Floral induction in *Brachypodium distachyon*

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Declaration

The research outlined in this thesis was carried by the author at the Cell & Developmental Biology Department, John Innes Centre, between April 2008 and April 2012. The work described herein is the original work of the author and includes nothing which is the outcome of work done in collaboration except when specifically indicated in the text. It has not been previously submitted to any institution for any qualification degree.

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Abstract

Brachypodium distachyon (L.) P. Beauv. is a recently fully-sequenced monocot plant, very closely related to wheat and barley, and an attractive model system to study the biology of temperate cereals.

The present work investigates the "mechanisms" of flowering time in Brachypodium (community standard line Bd21) by analyzing a group of core genes, mostly transcription factors, generally known to be functionally involved in the determination of the shoot apical meristem's reproductive fate. Its major aims were the identification of the related Bd21 homologues, and the examination of their functional conservation within the photoperiod inductive pathway, in order to determine the basic framework for flowering transition in temperate grasses. The experimental procedures included quantitative real time PCR, whole transcriptome microarray hybridization, constitutive gene expression, gene silencing via artificial microRNA expression, transcript immuno-localization, protein immuno-detection, and reporter studies. From these experiments, it was determined that while some genes remain functionally conserved among different plant systems, other signal integrators, thought to play an essential role as meristem identity genes, are not. From the characterization of the transitioning shoot apical meristem's transcriptome, it was possible to identify the network of biological processes and the underlying genetic mediators that, in Bd21, coordinate this highly complex developmental switch. Overall, the experimental work that is presented examines some of the rudiments of the floral induction in *Brachypodium distachyon* and, hopefully, will constitute a modest, yet relevant, contribution to the plant research community, on the reproductive development of temperate monocots.

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Abbreviations, symbols & units

% Number expression as a fraction of one hundred

(L.) Linnaeus (Carl Nilsson Linnæus)

< Less than

°C Degree Celsius

µ Micro ($x10^{-6}$)

AFO ABNORMAL FLORAL ORGANS

AG AGAMOUS

Agrot *Agrobacterium tumefaciens*

ALF ARABIDOPSIS LEAFY

amiRNA Artificial microRNA

Anti-DIG-AP Antibody against digoxigenin, conjugated with alkaline phosphatase

AP1 APETALA 1

AP2 APETALA 2

AP3 APETALA 3

APB Active PHYB binding

APE1 APURINIC/APYRIMIDIC ENDONUCLEASE 1

ARS Agricultural Research Service

At *Arabidopsis thaliana*

AtMYB33 *Arabidopsis thaliana* MYELOBLASTOSIS PROTEIN 33

BCIP 5-Bromo-4-chloro-3-indolyl phosphate

Bd *Brachypodium distachyon*

Bd21 *Brachypodium distachyon*, line 21

BdCO1 *Brachypodium distachyon* CO-like 1

BdCO2 *Brachypodium distachyon* CO-like 2

BdFD *Brachypodium distachyon* FD

BdFD1 *Brachypodium distachyon* FD-like 1

BdFD2 *Brachypodium distachyon* FD-like 2

BdFTL1 *Brachypodium distachyon* FT-like 1

BdFTL2 *Brachypodium distachyon* FT-like 2

BdFUL2 *Brachypodium distachyon*FUL2

BdGAI *Brachypodium distachyon* GAI

BdLFY *Brachypodium distachyon* LFY

BdPIF *Brachypodium distachyon* PIF

BdPIF1 *Brachypodium distachyon* PIF1

BdPIF7 *Brachypodium distachyon* PIF7

BdTFL1 *Brachypodium distachyon* TFL1

BdVRN1/FUL1 *Brachypodium distachyon* VRN1/FUL1

bHLH Basic helix loop helix

BLAST Basic local alignment search tool

bp Base pair

BSA Bovine serum albumin

bZIP Basic leucine zipper

c centi ($x10^{-2}$)

C3 First product of the Calvin-Benson cycle is a 3-carbon molecule (photosynthesis)

C4 First product of the Calvin-Benson cycle is a 4-carbon molecule (photosynthesis)

CAB CHLOROPHYLL A/B-BINDING PROTEIN

CCA1 CIRCADIAN AND CLOCK ASSOCIATES 1

CCT CO, CO-like, TOC1

cDNA Complementary deoxyribonucleic acid

CDPK CALCIUM-DEPENDENT PROTEIN KINASE

CDS Coding DNA sequence

CEC Compact and embryogenic callus

CEN CENTRORADIALIS

CHE CCA1 HIKING EXPEDITION

ChIP Chromatin immuno precipitation

CHV CHROMOSOMAL VIRULENCE

CO CONSTANS

co-2 CO mutant-2

co-9 CO mutant-9

Co-IP Co-immuno precipitation

Col-0 *Columbia-0*

CPS ENT-COPALYL DIPHOSPHATE SYNTHASE

Cq Quantification cycle

CRC CRABS CLAW

cRNA Complementary RNA

CRY1 CRYPTOCHROME 1

CRY2 CRYPTOCHROME 2

Ct Threshold cycle

CTAB Cetyltrimethylammonium bromide

$CuSO_4$ Copper sulphate

D8 DWARF-8

ddNTP Dideoxyribonucleotide triphosphate

DEPC Diethylpyrocarbonate

DIG Digoxigenin

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

DNAse Deoxyribonuclease

dNTP Deoxyribonucleotide triphosphate

DOE Department of Energy

DOT DOUBLE TOP

dsDNA Double-stranded DNA

dT Deoxy-thymine nucleotides

DTT Dithiothreitol

dUTP Deoxy-uracil triphosphate

E. coli *Escherichia coli*

E2 UBIQUITIN-CONJUGATING ENZYME (Step 2 of the ubiquitination process)

E3 UBIQUITIN LIGASE (Step 3 of the ubiquitination process)

EBS EARLY BOLTING IN SHORT DAYS

EDTA Ethylenediaminetetraacetic acid

EMS Ethylmethanesulfonate

EST Expressed sequence tag

EtOH Ethanol

F Farad

FAA Formaldehyde-acetic acid alcohol solution

FAO Food and Agriculture Organization of the United Nations

FLC FLOWERING LOCUS C

FRET Fluorescence resonance energy transfer

FRI FRIGIDA

FT FLOWERING LOCUS T

FUL1 FRUITFUL 1

g Gram

GA Gibberellic Acid

GA1 Gibberellic Acid 1

ga1-3 GIBBERELLIC ACID REQUIRING 1-3 mutant

GA13ox GIBBERELLIC ACID 13 OXIDASE

GA20ox GIBBERELLIC ACID 20 OXIDASE

GA2ox GIBBERELLIC ACID 2 OXIDASE

GA2ox4 GIBBERELLIC ACID 2 OXIDASE 4

GA2ox6 GIBBERELLIC ACID 2 OXIDASE 6

GA3 Gibberellic Acid 3

GA3ox GIBBERELLIC ACID 3 OXIDASE

GA4 Gibberellic Acid 4

GA5 Gibberellic Acid 5

GA6 Gibberellic Acid 6

GAI GIBBERELLIC ACID INSENSITIVE

GFP GREEN FLUORESCENT PROTEIN

GI GIGANTEA

GID1a-c GIBBERELLIC ACID-INSENSITIVE DWARF 1a-c

GID2 GA-INSENSITIVE DWARF 2

GRAS GAI, RGA, SCARECROW

GUS beta-GLUCURONIDASE

h Hour

Ha Hectare

Hd1 HEADING DATE 1

Hd3a HEADING DATE 3a

Heynh Heynhold (Gustav Heynhold)

Hg Hectogram

Hph HYGROMYCIN B PHOSPHOTRANSFERASE

HRP HORSE RADISH PEROXIDASE

HSL HORMONE-SENSITIVE LIPASE

HvCO1 *Hordeum vulgare* CO-like 1

HvFT1 *Hordeum vulgare* FT-like 1

HYG Hygromycin

IBI International Brachypodium Initiative

IBTC International Brachypodium Tagging Consortium

INRA Institut National de la Recherche Agronomique

IPTG Isopropyl β-D-1-thiogalactopyranoside

ISH *In situ* hybridization

IVT *In vitro* transcription

JGI Joint Genome Institute

JIC John Innes Centre

k Kilo ($x10^3$)

KAO ENT-KAURENOIC ACID OXIDASE

kb Kilo base pair

KNOX KNOTTED 1-LIKE HOMEOBOX

KO ENT-KAURENE OXIDASE

KS ENT-KAURENE SYNTHASE

l Litre

LB Left border

LB Lysogeny broth

LD Long day

LFY LEAFY

LHR Leucine heptad repeats

LHY LATE ELONGATED HYPOCOTYL

LiCl Lithium chloride

LpCO *Lolium perenne* CO

LpFT *Lolium perenne* FT

LtLFY *Lolium temulentum* LFY

m Metre

m Mili ($x10^{-3}$)

M Molar

MADS MINICHROMOSOME MAINTENANCE1; AGAMOUS; DEFICIENS; SERUM RESPONSE FACTOR

Mb Mega base

MeOH methanol

min Minute

miRNA micro RNA

MM Mismatch

mol Mole

mRNA Messenger RNA

MS Murashige and Skoog

Mya Million years ago

MYB Myeloblastosis

n Chromosome number

n Nano ($x10^{-9}$)

Na_2CO_3 Sodium carbonate

NaCl Sodium chloride

$NaHCO_3$ Sodium bicarbonate

NBT Nitro blue tetrazolium

NEB New England biolabs

NJ Neighbour-joining

NLS Nuclear localization signal

Noc Nopaline catabololism

NPGS National Plant Germplasm System

o/n Over-night

OD Optical Density

-OH Hydroxyl group

Onc Oncogenic

Ori Replication origin

OsAct1 *Oryza sativa* actin promoter and intron 1

p Pico ($x10^{-12}$)

P. BEAUV Palisot de Beauvois (Ambroise Marie François Joseph Palisot, Baron de Beauvois)

PBS Phosphate buffered saline

PCR Polymerase chain reaction

PEBP Phosphatidylethanolamine binding protein

Pfr Phytochrome, far-red light absorbing form

pH negative decimal logarithm of the molar concentration of dissolved hydronium ions (H_3O^+) in a solution

PHYA PHYTOCHROME A

PHYB PHYTOCHROME B

PHYC PHYTOCHROME C

PHYE PHYTOCHROME E

PI PISTILLATA

PIF PHYTOCHROME INTERACTING FACTOR

PIF1 PHYTOCHROME INTERACTING FACTOR 1

PIF3 PHYTOCHROME INTERACTING FACTOR 3

PIF4 PHYTOCHROME INTERACTING FACTOR 4

PIL5 PHYTOCHROME INTERACTING FACTOR 3-LIKE 5

PM Perfect match

PMSF Phenylmethylsulfonylfluoride

Poly(A) Poly adenosine monophosphate

Ppd1-D Photoperiod 1-D genome

Ppd-H1 Photoperiod-H1

Pr Phytochrome, red light absorbing form

PRR7 PSEUDO-RESPONSE REGULATOR 7

PRR9 PSEUDO-RESPONSE REGULATOR 9

qPCR Quantitative (reverse transcription) real time polymerase chain reaction

QTL Quantitative trait loci

RAF RAPIDLY ACCELERATED FIBROSARCOMA

RB Right border

RbCl Rubidium chloride

REL Relative expression levels

RFL RICE FLORICAULA LEAFY

RFLT1 RICE FLOWERING LOCUS T 1

RGA REPRESSOR ON THE GA1-3 MUTANT

RGL1 RGA-LIKE 1

RGL2 RGA-LIKE 2

RGL3 RGA-LIKE 3

RING REALLY INTERESTING NEW GENE

RNA Ribonucleic acid

RNAi Ribonucleic acid interference

RNAse Ribonuclease

RNAsin Ribonuclease inhibitor

Roc1/RBx1 REGULATOR OF CULLINS-1/RING-BOX PROTEIN 1

rpm Revolutions per minute

rRNA Ribosomal RNA

RT Room temperature

s Second

SAM Shoot apical meristem

SCF S-PHASE KINASE-ASSOCIATED PROTEIN 1, CULLIN, F-BOX PROTEIN

SCL Standard Community Line

SD Short day

SDS-PAGE Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SEM Scanning electron microscope

SEP SEPALLATA

Ser/Thr Serine/threonine

SH2 SARCOMA HOMOLOGY 2

SLN1 SLENDER 1

SLY1 Sleepy 1

SOC Super optimal broth with catabolite repression

SOC1 SUPPRESSOR OF OVEREXPRESSION OF CO 1

SSC Saline-sodium citrate buffer

ssDNA Single-stranded DNA

STM SHOOT MERISTEMLESS

SVP SHORT VEGETATIVE PHASE

T0 Obtained by the regeneration of transformed cells

T1 Obtained by the germination of T0 seed

TaCO1 *Triticum aestivum* CO 1

TAE Tris base; Acetic acid; EDTA

TaFDL2 *Triticum aestivum* FD-like 2

TaHd1 *Triticum aestivum* Hd1

Taq Pol *Thermus aquaticus* polymerase

TaVRN3 *Triticum aestivum* VERNALIZATION 3

TBE Tris base; Boric acid; EDTA

TBST Tris buffer saline; Tween-20

TCP TEOSINTE BRANCHED 1; CYCLOIDEA; PCFS

T-DNA Transfer Deoxyribonucleic acid

TdT TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE

TE Tris- EDTA

TFL1 TERMINAL FLOWER 1

Ti Tumor inducing

TNM Tris-HCl, NaCl, $MgCl_2$ buffer

TOC1 TIMING OF CAB EXPRESSION 1

tRNA Transfer RNA

TSF TWIN SISTER OF FT

U The amount of the enzyme that catalyzes the conversion of 1 micro mole of substrate per minute

U.V. Ultra violet

UBC18 UBIQUITIN CONJUGATING ENZYME 18

UDG URACIL DNA GLYCOSYLASE

US United States

USDA United States Department of Agriculture

V volt

v volume

Vir Virulence

VRN1 VERNALIZATION 1

VRN2 VERNALIZATION 2

VRN3 VERNALIZATION 3

w Weight

WFL WHEAT FLORICAULA/LEAFY

WMD3 Web micro designer 3

WRRC Western Regional Research Centre

WT Wild type

X-GAL Bromo-chloro-indolyl-galactopyranoside

X-Gluc 5-bromo-4-chloro-3-indolyl glucuronide

YAB3 YABBY 3

YEP Yeast extract; bacto-peptone

ZmUbi1 *Zea mays* ubiquitin promoter and intron 1

ZT Zeitgeber time

ZTL ZEITLUPE

Δ Minus (mutation)

λ_{max} Maximal wave length

Ω Ohm

Chapter 1

Introduction

The importance of monocot plants throughout the course of Human history has been outstanding, particularly the Poaceae (Gramineae) family of cereal grasses (Doebley, 2006). Historical and archaeological records report that from early ancient times, cereal grain species have been amongst the most significant edible plants to be used by the hunter-gatherer as part of his everyday diet, either by direct ingestion or by providing forage for hunted wild animals. Later, with a developed ability to cultivate wild grains and to domesticate these and other species, Man ceased to be a nomadic hunter-gatherer to become a sedentary farmer – the birth of the Neolithic revolution. With the development of better tools, the domestication of wild species, and the improvement of agricultural techniques, the intensive farming assured a steady high yield production that, at a amassed surplus, allowed densely populated settlements to thrive, freeing a significant proportion of the farming workforce for other activities like the arts and crafts, and the building up of armies. With the development of cities and social stratification arisen the first forms ideology, government and law, along with concepts of property and trade, all of which prompted the engenderment of the first inscriptions. Culturally, the use and importance of cereals strongly influenced human civilizations worldwide, remaining imprinted in the folklore of the nations as part of symbolism, linguistics (in idiomatic expressions such as «bread-winner» and «bread and water»), the performance of rituals and harvest festivals, and the worshiping of Gods. In fact the word "cereal" derives from Latin word "Ceres", which is the name of the Roman goddess of agriculture, grain crops, fertility and motherhood.

In our modern times cereal grains remain amongst the world's most important staple crops (Table 1.1) directly providing an elemental, yet essential, source of calories. For the past 50 years, according to FAO's records, the global wheat production levels have increased while the hectares harvested have remained more or less constant (Figure 1.1). This has happened as a result of increased production yields, probably due to the implementation of well-conceived crop-rotation plans, the use of seed from improved high-yield varieties and of mineral fertilizers, a robust phytosanitary control plan, the use of state of the art irrigation systems and of modern machinery. However, as the world population continues to rise, the challenge of feeding an ever growing number of people is a cause for concern, specially, with the mounting awareness of the urgency and imperativeness of the preservation of the ecosystems' sustainability – deforestation, soil mineral depletion or soil degradation (acidification and salinization), the eutrophication of aquifers and superficial waters, the toxic effects of antibiotic and pesticide use to consumers and wild life, and the climate change effects on global temperatures, and rainfall patterns. The question today is how to produce more food using less input? The answer may reside in the determined effort to continually develop better varieties with greater tolerance to drought and mineral toxicity, which are able to make a more efficient use of nutrients, withstand or avoid harsher ambient temperatures and are more resilient to pests and diseases. However, this is only possible if the scientific community is able to fully understand the physiology, the genetics, and the developmental biology of this group of plants, chiefly the flowering-associated processes; but despite their global relevance as staple crops, the biology of temperate cereals remains a relatively obscure field in plant science; as most of the plant knowledge that has been gathered over time relates to studies performed on the dicot model plant *Arabidopsis thaliana* L., the first plant genome to be fully sequenced (The Arabidopsis Genome Initiative, 2000), and a benchmark for scientific plant research worldwide.

Table 1.1: World's Top 20 highly produced commodities for the year 2010. Highlighted in black are staple crops (www.fao.org)

Rank	Commodity	Production (Tonnes)
1	Sugar cane	1711087173
2	**Maize**	**840308214**
3	**Rice, paddy**	**696324394**
4	**Wheat**	**653654525**
5	Cow milk, whole, fresh	600838992
6	**Potatoes**	**324420782**
7	**Soybeans**	**264991580**
8	Vegetables fresh nes	257065378
9	**Cassava**	**230265639**
10	Sugar beet	228453678
11	Tomatoes	151699405
12	**Barley**	**123544729**
13	Indigenous Pigmeat	109100198
14	**Sweet potatoes**	**107639494**
15	Bananas	102028171
16	Watermelons	99161274
17	Buffalo milk, whole, fresh	92473371
18	Indigenous Chicken Meat	85860953
19	Onions, dry	78534876
20	Apples	69511975

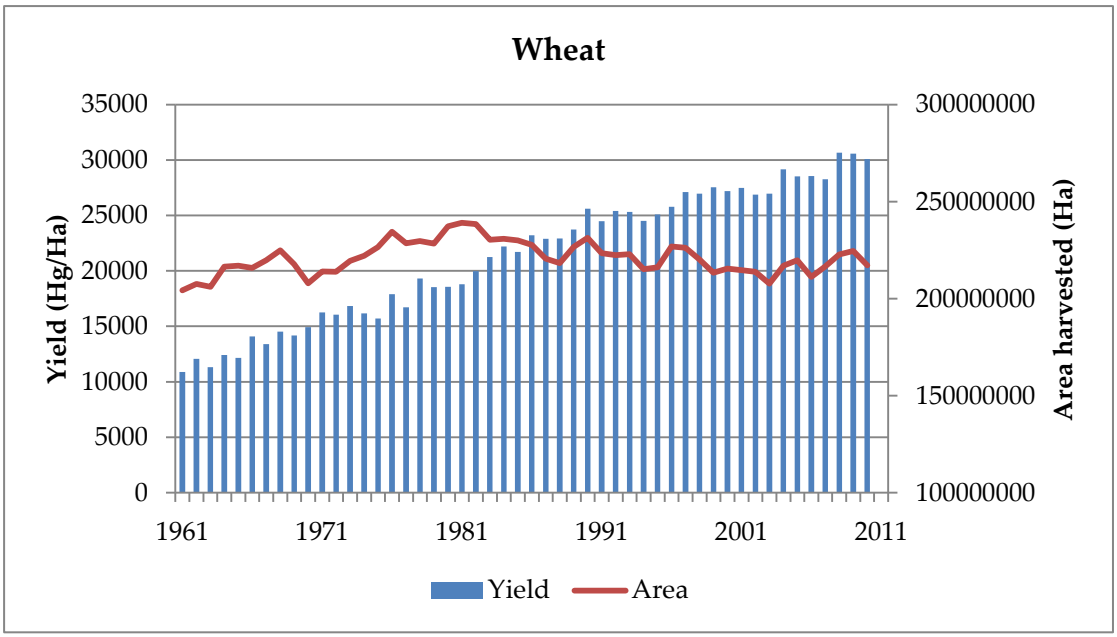


Figure 1.1: World's wheat production for the past 50 years. Yield (unit: hectogram per hectare) and area harvested (unit: hectare) (www.fao.org)

1.1 *Arabidopsis thaliana's* flowering regulatory network

The coordination of the always fluctuating external environmental signals with the internal physiological and developmental processes that lead to the reproductive fate in plants (reviewed in Bernier and Périlleux, 2005), have been the subject of intense study for the past several decades using mutants of *Arabidopsis thaliana* (L.) Heynh. (reviewed by Meyerowitz, 2001). From these studies, genes were identified and shown to be involved in an interconnected network of signalling pathways that converge towards the regulation of floral meristem identity genes (Mouradov *et al*, 2002), which are responsible for the triggering of the flowering response at the shoot apical meristem (Figure 1.2). In the SAM, LEAFY (LFY) and APETALA1 (AP1) up-regulate each other and are essentially expressed in the lateral primordia, where individual flowers form. LFY and AP1 also repress SUPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1), which promotes inflorescence fate rather than flower formation (Yu *et al*, 2004; Liu *et al*, 2007). In the centre of the SAM their activity is antagonized by TERMINAL FLOWER 1 (TFL1), whose expression maintains the indeterminacy of the SAM (Ratcliffe *et al*, 1999). Interestingly, TFL1 - a mobile signal, within the shoot meristem (Conti and Bradley, 2007) - has homology with the florigen FLOWERING LOCUS T (FT), and the modification of a single amino acid can convert TFL1 from a flowering repressor to an activator (Hanzawa *et al*, 2005; Ahn, 2006).

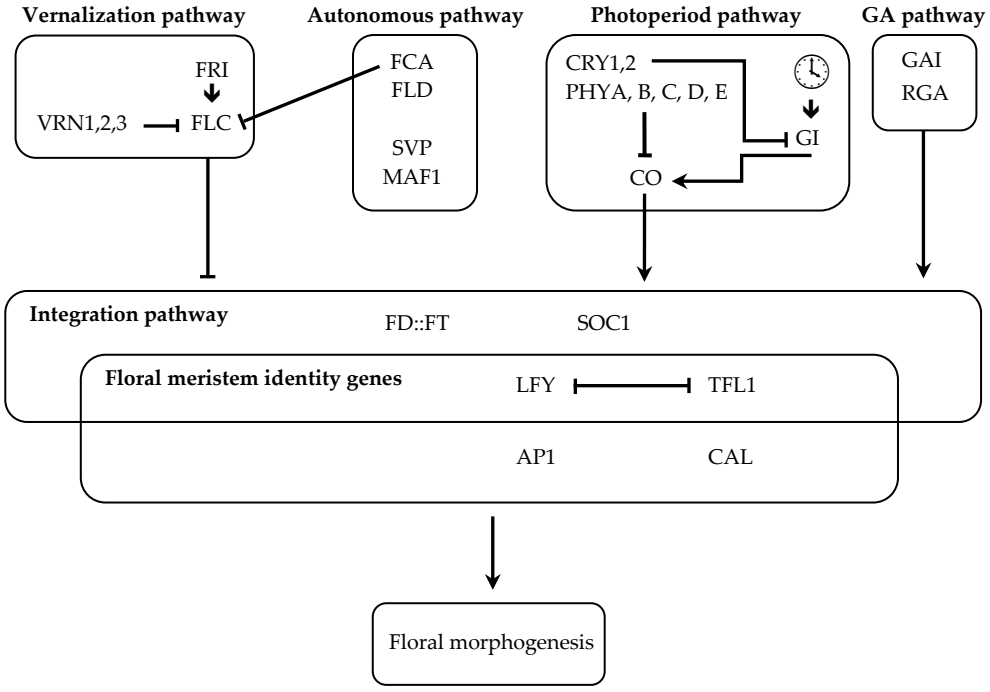


Figure 1.2: Schematic representation of the flowering inductive pathways. AP1 – APETALA1; CAL-CAULIFLOWER; CO – CONTANS; CRY1,2 – CRYPTOCHROME 1,2; FCA – FLOWERING TIME CONTROL LOCUS A; FLC – FLOWERING LOCUS C; FLD – FLOWERING LOCUS D; FRI – FRIGIDA; FT – FLOWERING LOCUS T; GAI – GA INSENSITIVE; GI - GIGANTEA; LFY - LEAFY; MAF1 – MADS AFFECTING FLOWERING 1; PHYA-E – PHYTOCHROME A-E; RGA – REPRESSOR OF GA1-3; SOC1 – SUPRESSOR OF OVEREXPRESSION OF CONSTANS 1; SVP – SHORT VEGETATIVE PHASE; TFL1 – TERMINAL FLOWER 1; VRN1-3 – VERNALIZATION 1-3 (Adapted from Komeda, 2004)

1.1.1 The vernalization pathway

Through the vernalization signalling pathway (Figure 1.2), flowering is promoted after a prolonged exposure to cold temperatures between 0° and 10°C. This phenomenon, commonly known as vernalization, occurs naturally during the winter months and is a frost avoidance strategy characteristic of accessions from higher latitudes and, therefore, colder climates. Cultivars that require vernalization to undergo floral transition ("winter" accessions), express an active floral repressor gene called *FRIGIDA* (*FRI*) that strongly represses floral transition by positively regulating the MADS box gene *FLOWERING LOCUS C* (*FLC*), a key integrator of the floral repression response (Choi *et al*, 2011).

Following the exposure to a cold period, the *VERNALIZATION* (*VRN*) genes are up-regulated to stably repress *FLC* in increasingly warmer temperatures, and throughout the following mitotic development, allowing floral transition to occur (Michaels and Amasino, 1999). Additionally, the expression of *VERNALIZATION INSENSITIVE 3* (*VIN3*) and *MADS AFFECTING FLOWERING 2* (*MAF2*) genes, ensure that short cold episodes occurring during Autumn will not be enough to trigger the floral transition which would, otherwise, compromise the seed production (Ratcliffe *et al*, 2003; Sung and Amasino, 2004). FLC epigenetic repression ceases later, during meiosis, when the vernalization response is due to be reset for the next generation (Reviewed by Schmitz and Amasino, 2007).

1.1.2 The autonomous pathway

The autonomous signalling pathway (Figure 1.2) was found to promote flowering independently of environmental factors (Boss *et al*, 2004), but no clear input signal has yet been identified as the trigger of the several subsets of independent genes identified so far; Blázquez *et al*, in 2003, suggested that ambient temperature perception may act through the autonomous pathway, but this may also be involved in fitness assessment (plant age, water/nutrients content and health conditions). Members of this pathway are *FLOWERING TIME CONTROL LOCUS A* (*FCA*), which is an RNA-binding protein that interacts with FY protein, a RNA 3'-end processing/poly-adenilation factor (Quesada *et al*, 2003, Simpson *et al*, 2003), and *FVE* that, together with *FLOWERING LOCUS D* (*FLD*), is involved in chromatin regulation (He *et al*, 2003). Not much is known about these genes, apart from being positive regulators of flowering through their common repressive function on *FLC* (Reviewed by Schmitz and Amasino, 2007). In contrast, *SHORT VEGETATIVE PHASE* (*SVP*) and *FLOWERING LOCUS M* (*FLM*) or *MADS AFFECTING FLOWERING 1* (*MAF1*) genes exert a repressive effect on flowering independently of *FLC*, and are known to be involved in photoperiod perception (Hartmann *et al*, 2000; Scortecci *et al*, 2003).

1.1.3 The photoperiod pathway

In Arabidopsis, light promotes flowering through the photoperiod signalling pathway (Figure 1.2) on exposure to a single 16 hour long day (LD) (Turck *et al*, 2008), either at high intensity from red light-fluorescent lamps (100 $\mu mol.m^{-2}.s^{-1}$) or at low intensity from far-red

light-incandescent lamps (10 μmol.m^{-2}.s^{-1}) (King *et al*, 2008). Light intensity and quality are major inputs perceived by photoreceptors like *PHYTOCROME A* (*PHYA*) and *CRYPTOCHROME 2* (*CRY2*), which mediate the positive effects on flowering of far-red and blue light, respectively; and *PHYTOCHROME B* (*PHYB*) - a red light photo-detector - that acts as a repressor of flowering and is involved in the plant's shade avoidance response (Lorrain *et al*, 2008). These photoreceptors, or phytochromes, are synthesised in an inactive red-light absorbing (Pr) form and undergo photo-conversion to a biologically active far-red light absorbing form (Pfr); a reaction which is optimized at red wavelengths. Conversion of Pfr back to the inactive Pr is optimized by far-red wavelengths, resulting in a dynamic photo-equilibrium of Pr and Pfr in almost all irradiation conditions (Franklin, 2008). After the exposure to red-light and conversion to the Pfr, the phytochrome undergoes nuclear translocation and binds to the PHYTOCHROME INTERACTING FACTORS (PIFs), a family of basic helix-loop-helix (bHLH) transcription regulators. This interaction promotes the phosphorylation, ubiquitination and degradation of PIFs (Castillon *et al*, 2007), thereby preventing these PIFs from binding to their target gene promoters and activating gene expression. The external light signals detected by the phytochromes are immediately transduced to the circadian clock components that permit the plant to sense the duration of day-night cycle, in the leaf: *GIGANTEA* (*GI*) (Park *et al*, 1999; Mizoguchi *et al*, 2005) and *CONSTANS* (*CO*) (Yanovsky and Kay, 2002). *GI* is not similar to any protein with known function (Fowler *et al*, 1999), but *CO* encodes a nuclear zinc-finger protein, which mRNA expression occurs both in SD and LD (Suárez-López *et al*, 2001), but accumulation of protein is strongly dependent on the presence of light sensed by *PHYA* and *CRY2* (Valverde *et al*, 2004). Extended day length stabilises CO protein levels, allowing it to activate its target gene: *FLOWERING LOCUS T* (*FT*), encoding a phosphatidylethanolamine binding protein (PEBP)/RAF-kinase-inhibitor-like protein (Kardailsky *et al*, 1999); and a mobile signal that travels from the leaves, through the phloem, to the SAM (Zeevaart, 2006, 2007; Jaeger and Wigge, 2007; Corbesier *et al*, 2007) where it forms a complex with the basic domain/leucine zipper protein FD. This FT/FD heterodimer then activates the downstream floral meristem identity genes, like *APETALA1* (*AP1*), triggering floral transition (Abe *et al*, 2005; Wigge *et al*, 2005). *FT* may also be directly up-regulated by the interconnected autonomous and vernalization pathways (Blázquez *et al*, 2003; Levy *et al*, 2002), producing, also by this way, the mobile transition signal.

1.1.4 The gibberellin pathway

The gibberellic acid signalling pathway (Figure 1.2) is extremely complex as it coordinates endogenous metabolic information, signal perception, and interconnected transduction cascades, into a precise and synchronized physiological response. Gibberellins (GAs) are plant hormones that control growth and development throughout the life cycle of a plant; but not all GA molecules are bioactive, the majority are either biosynthetic intermediates or a catabolite of a bioactive GA (Figure 1.3) (Olszewski *et al*, 2002). The molecules with biological activity are known to be implicated in seed germination, hypocotyl elongation, leaf expansion, pollen tube growth, flowering initiation, flower organ development and

fruit development (reviewed by Ueguchi-Tanaka *et al*, 2007; Olszewski *et al*, 2002; Sun and Gubler, 2004; Fleet and Sun, 2005; Yamaguchi, 2008).

1.1.4.1 GA metabolism

The metabolic pathway involves the sub-cellular compartmentalization of the biosynthetic enzymes, like CPS and GA3ox, whose proteins catalyse the first and final steps, respectively in the formation of bioactive GAs (GA_1 and GA_4), and the catabolic GA2ox enzyme, that determines their inactivation (Figure 1.3).

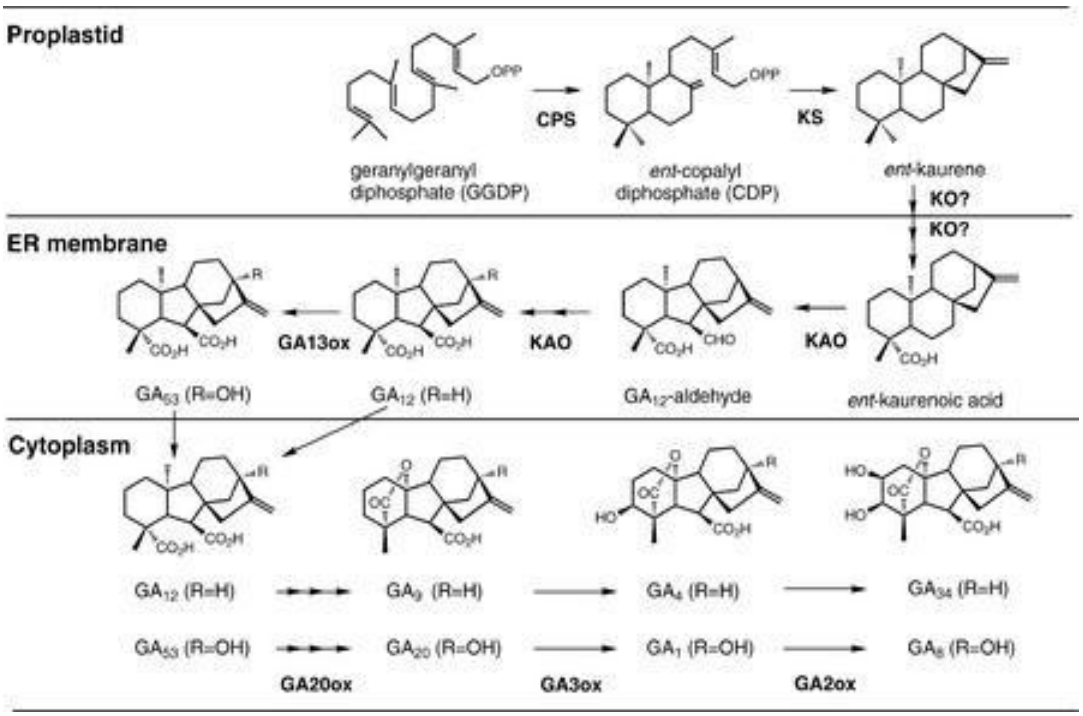


Figure 1.3: GA metabolic pathways in higher plants. The enzyme names are in bold below each arrow. GA_1 and GA_4 are bioactive GAs, and GA_8 and GA_{34} are their inactive catabolites. CPS (ent-copalyl diphosphate synthase), KS (ent-kaurene synthase), KO (ent-kaurene oxidase), KAO (ent-kaurenoic acid oxidase), GA20ox (GA20 oxidase), GA3ox (GA3 oxidase, and GA2ox (GA2 oxidase) (Adapted from Olszewski. *et al*, 2002)

In Arabidopsis, the enzymes responsible for the first GA biosynthetic steps, CPS (ent-copalyl diphosphate synthase), KS (ent-kaurene synthase) and KO (ent-kaurene oxidase appear to be encoded by single-copy genes, expressed in vascular elements of expanded leaves and in rapidly growing tissues (Silverstone *et al*, 1997; reviewed by Hedden and Phillips, 2000). Loss of function mutations at these loci, such as ga1-3 (GA REQUIRING 1 (GA1) or ent-COPALYL DIPHOSPHATE SYNTHASE 1 (cps)) impair flowering in SD (Wilson *et al*, 1992), when the photoperiod pathway is inactive, and severely compromise the plant's morphology - displayed by dark-green leaves and a dwarf phenotype (Sun and Gubler, 2004). By contrast, null mutations in the dioxygenases that catalyse the final reactions of the GA pathway only exhibit intermediate semi-dwarf phenotypes. This can be explained by the fact that these steps are encoded by small multigene families, each exhibiting functional redundancy within: GA20ox and GA3ox are encoded by at least four genes each and GA2ox by at least six genes (Hedden and Phillips, 2000). Exogenous applications of physiologically active GAs, however, are known to restore the phenotype of

mutants impaired in GA biosynthesis and are also known to promote stem elongation and floral transition in wild type Arabidopsis, especially in SD (Xu *et al*, 1997). GA overdosed plants or recessive lost-of-function mutants with increased GAs levels or enhanced GA signalling, on the other hand, show pale-green leaves and a slender/tall phenotype (Sun and Gubler, 2004). The feedback and feedforward regulations that provide a mechanism for GA homeostasis and assure plant's normal development, are restricted to the some dioxygenase genes involved in GAs biosynthesis and do not occur earlier in the pathway (Hedden and Phillips, 2000). Therefore, over-expression of CPS has no dramatic effects on growth and development in Arabidopsis but, in contrast, manipulation of the GA20ox, GA3ox and GA2ox protein levels may shift the balance between inactive/bioactive GAs inside the plant with concomitant repercussions in its physiological development (Hedden and Phillips, 2000).

During the floral induction in *Arabidopsis*, the expression level of *AtMYB33*, a GA-induced MYB transcription factor, increases in the shoot apex (Gocal *et al*, 2001). The GA-induced *AtGAMYB33* protein binds to a specific 8-bp cis-element (MYB transcription factor-binding site) on the promoter of the floral meristem identity gene *LFY,* activating its expression (Blázquez and Weigel, 2000; Gocal *et al*, 2001), and directly instigating the floral transition. *SOC1* expression is also up-regulated in the presence of bioactive GA prior to floral transition and reinforces LFY activity; however, gibberellins have not been shown to activate flowering directly by up-regulating *FT* expression (Moon and Suh *et al*, 2003). In fact, mutations at the *EARLY BOLTING IN SHORT DAYS* (*EBS*) locus, a repressor of *FT* (Piñeiro *et al*, 2003), cause an early flowering phenotype in SD, distinctively due to GA biosynthesis (Gómez-Mena *et al*, 2001). The homeotic gene *AGAMOUS* (*AG*), expressed after floral induction signal from LFY, terminates meristem activity and promotes development of floral organs. Its expression directly elevates the expression of *GA3ox1*, which may cause an increase in GA levels in the floral meristem and promote the shift from meristem identity to differentiation (Gómez-Mena *et al*, 2005). The KNOX protein *SHOOT MERISTEMLESS* (*STM*), on the other hand, up-regulates the expression of the GA catabolic genes *GA2ox4* and *GA2ox6* at the base of the meristem (Hay *et al*, 2002) to control the level of active GA in the SAM and maintain the indeterminate state of the corpus cells.

1.1.4.2 GA signalling

Three GA-*INSENSITIVE DWARF 1a-c* (*GID1a-c*) genes were identified as encoding soluble nuclear-enriched GA receptors; enzymatically inactive relatives of the hormone-sensitive lipases (HSLs) which bind directly to bioactive GAs forming GID1-GA complexes with DELLA proteins (Murase and Hirano *et al*, 2008), and inducing DELLA proteins' degradation via the SCF-ubiquitination complex/26S proteasome pathway. The DELLA proteins' degradation elicits, as a consequence, changes in downstream gene expression cascades that effect GA-induced growth and development responses (Figure 1.5) (Ueguchi-Tanaka *et al*, 2005; Nakajima *et al*, 2006). The DELLA proteins constitute a subfamily of the GRAS (GAI, RGA, and SCARECROW) family of putative transcription factors. They are nuclear-localized, growth restraining, GA-signalling repressors that block GA-induced development and are defined by their unique DELLA domain near the N-terminal region, essential to perceive the GA signal; a conserved GRAS domain at the C-terminus with both

functional and regulatory activities; polymeric Ser/Thr motifs (poly S/T), which could be targets of phosphorylation or glycosylation; Leu heptad repeats (LHR), which may mediate protein-protein interactions; putative nuclear localization signals (NLS); and a putative SH2 phosphotyrosine binding domain (Pysh *et al*, 1999; Peng *et al*, 1999) (Figure 1.4).

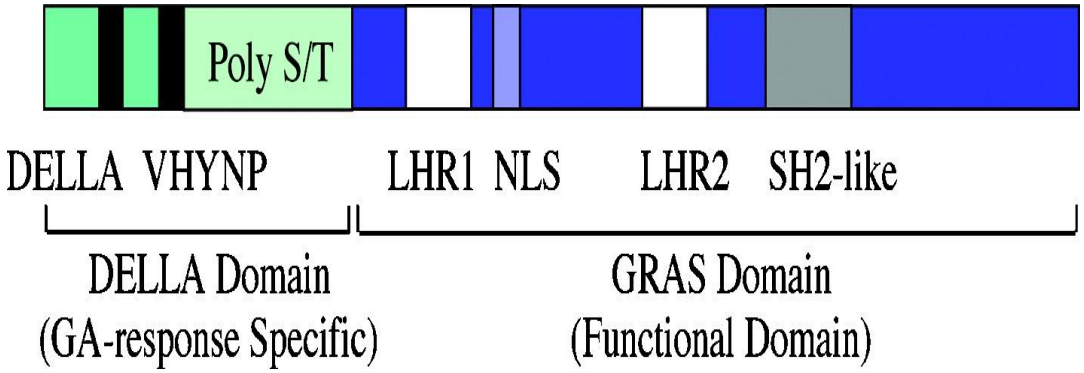


Figure 1.4: The DELLA subfamily of GRAS proteins. The unique N-terminal "DELLA domain" of the DELLA proteins contains two highly conserved motifs (named DELLA and VHYNP) and a Poly S/T region. The C-terminal region is conserved among all GRAS family members. Poly S/T, polymeric Ser and Thr; LHR, Leu heptad repeat; NLS, nuclear localization signal. (Adapted from Sun and Gubler, 2004)

DELLA proteins are highly conserved among different plant species, including lycophytes and bryophytes (Gao *et al*, 2008; Yasumura *et al*, 2007); but unlike the five DELLA proteins in *Arabidopsis* [*GIBBERELLIC ACID INSENSITIVE* (*GAI*), *REPRESSOR OF GA1-3* (*RGA*), *RGA-LIKE 1* (*RGL1*), *RGA-LIKE 2* (*RGL2*), and *RGA-LIKE 3* (*RGL3*)] only one functional ortholog has been identified in rice (*SLENDER RICE 1*, *SLR1*), wheat (*REDUCED HIGHT 1*, *Rht1*), barley (*SLENDER 1*, *SLN1*), maize (*DWARF*-8, *D8*) (Ikeda *et al*, 2001; Andersen *et al*, 2005), grape (*VvGAI*) and tomato (*LeGAI*) (Boss and Thomas, 2002; Vandenbussche *et al*, 2007; Bassel *et al*, 2008). In Arabidopsis, *GAI* and *RGA* are the major repressors of GA responses during vegetative growth and floral induction. *RGA* and *RGL2* jointly repress petal, stamen and anther development and *RGL1* activity enhances this effect (Lee *et al*, 2002; Cheng *et al*, 2004; Tyler *et al*, 2004; Yu *et al*, 2004b; Achard *et al*, 2006). The importance of DELLA proteins as repressors of GA signalling is illustrated by observations that DELLA loss-of-function mutations suppress the *ga1-3* phenotype (Thomas and Sun, 2004). A single DELLA protein gene knockout in *Arabidopsis* only produces subtle effects on GA responses, due to functional redundancy among the five DELLA family members. Knockout of all five DELLAs, results in constitutively active GA response and a dramatically taller phenotype (reviewed by Thomas and Sun, 2004; Kumar and Lucyshyn *et al*, 2012).

The *GA-INSENSITIVE DWARF2* (*GID2*) and *SLEEPY1* (*SLY1*) genes (Sasaki *et al*, 2003) McGinnis *et al*, 2003; Gomi *et al*, 2004) of rice and *Arabidopsis*, respectively, encode homologous F-box proteins, that modulate the GA responses by controlling the stability of DELLAs, as part of the SCF complex. GA, on the other hand, represses the transcription of the GA receptor and SLY1 F-box protein genes, which serve to fine tune the system at the level of the individual cell (Tyler *et al*, 2004). It is now understood that deletions in the DELLA domain impair its interaction with the GID1-GA complex, producing constitutively active repressors that are not inactivated by GA, nor are destroyed by proteolysis via SCF complex (Dill *et al*, 2001). The SCF complex (Skp1, Cullin, F-box and the RING finger binding protein Roc1/RBx1) acts as a multi-protein E3 ligase aggregate that catalyses the

transfer of a poly-ubiquitin chain from the E2 ubiquitin conjugating enzyme to proteins destined for 26S proteasomal degradation (Figure 1.5).

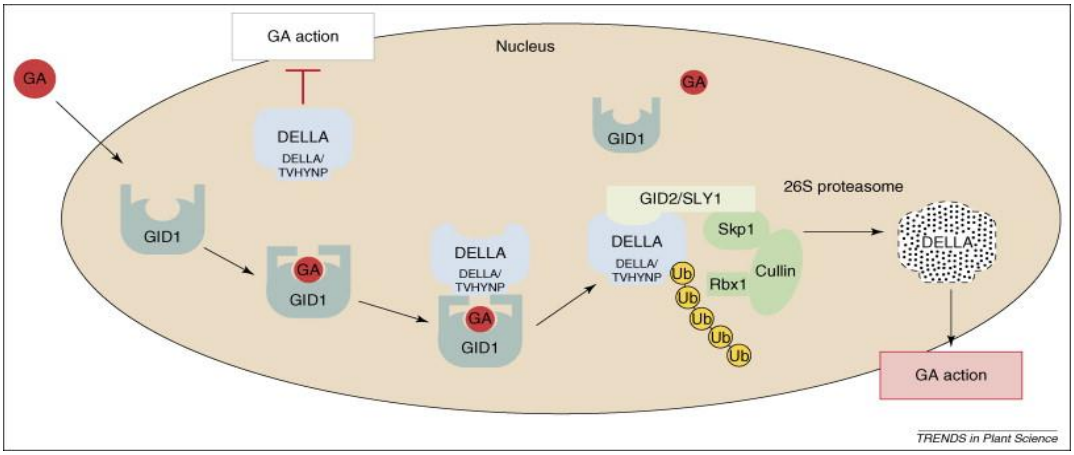


Figure 1.5: GA-induced developmental responses via DELLA protein degradation. Under low GA concentrations, DELLA proteins repress the GA response. Under high GA concentrations, a soluble receptor, GID1, binds to GA; forming a GID1-GA complex that stabilizes when interacts with DELLA proteins at the DELLA and TVHYNP domains. This GID1-GA-DELLA protein complex is targeted by the $SCF^{GID2/SLY1}$ complex for DELLA protein proteasomal degradation, releasing GA response. (Adapted from Hirano *et al*, 2008)

As with the Autonomous pathway, the key signal that drives the GA endogenous pathway into floral transition is unknown (Simpson and Dean, 2002). There is much evidence that environmental stimuli including light and temperature, interfere with this pathway by either changing the GA concentrations and/or altering the responsiveness of the target tissues to GA (Kamiya and Garcia-Martínez, 1999). In the dark, the bHLH protein PIL5 (PHYTOCHROME INTERACTING FACTOR 3-LIKE 5) also known as PIF1, PHYTOCHROME INTERACTING FACTOR 1, indirectly represses the expression of GA biosynthetic genes and conversely activates the gene encoding GA2ox2 (Oh *et al*, 2006). PIL5 also interacts with the promoter regions of DELLA genes and activates their expression (Oh *et al*, 2007). DELLA proteins, in their turn, interact directly with PIF3 and PIF4, thus preventing these PIFs from binding to their cognate promoters and thereby antagonizing PIF-dependent transcriptional activation (Oh *et al*, 2007; de Lucas *et al*, 2008; Davière *et al*, 2008; Feng *et al*, 2008). Upon exposure to light PIL5 is degraded by light-activated phytochromes, which affect a large diversity of early photo-morphogenic responses by accelerating GA biosynthesis and GA response through DELLA protein degradation, and by allowing PIF3 and PIF4 to retain their transcriptional activity (Yamaguchi *et al*, 1998; Oh *et al*, 2006). Recent studies have revealed that DELLA proteins have broad interactions with other endogenous phytohormones with important roles in many aspects of plant growth, development and adaptation to environmental stresses (Fu and Harberd 2003; Achard *et al*, 2006, 2007a, 2007b).

Chapter 2

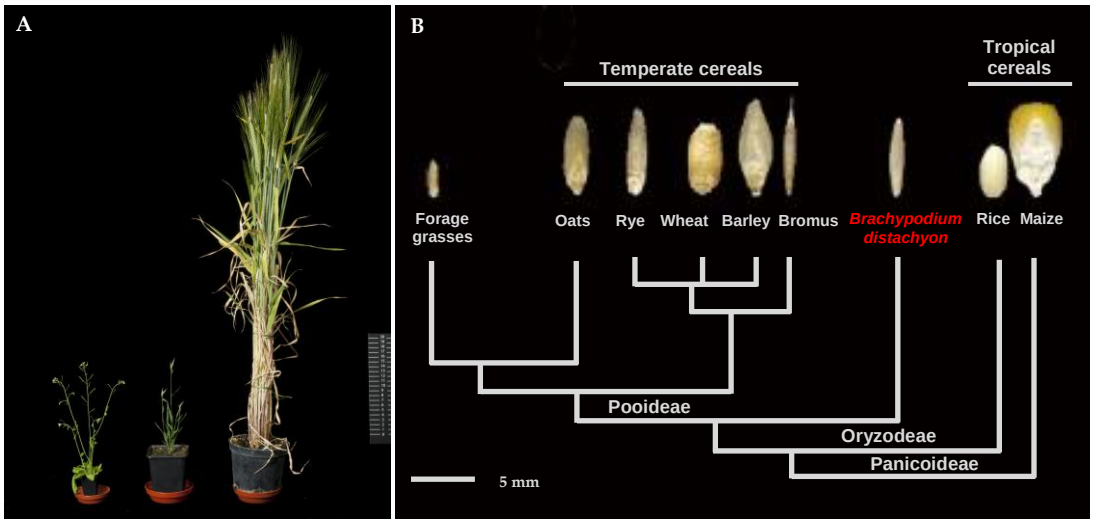
Brachypodium distachyon (L.) P. Beauv. - A new model for temperate grasses

2.1 Introduction

As monocots, cereal plants diverge significantly from the standard research model, *Arabidopsis thaliana*, routinely used in plant science labs worldwide. These differences, mostly concerning the size of the genomes, the elementary anatomy of the plants, their architecture, physiology, geographic centre of origin, and artificial selection through domestication, make Arabidopsis an inadequate system for monocot research. From all cereal crops, rice (*Oryza sativa* L.) is an attractive system for grass genomics due to its small genome size and available genome sequence (Garvin *et al*, 2008); however, in temperate climates, growing rice plants in large numbers is expensive, difficult and can only comprise one or two life cycles a year. In addition, it is not particularly well-suited as a robust model system for all grasses, as the biology of temperate grasses is sufficiently divergent from rice, which evolved as a semi-aquatic plant, in the tropics. Alternatively, plant biologists have turned to the economically important cereals like wheat, and maize; however, research has been frequently hindered by the large genome sizes, by polyploidy, by long life cycles, and by the high stature of the plants (Garvin *et al*, 2008). The challenge to study the biology of temperate grasses with the level of depth imposed on Arabidopsis, requires a monocot model plant that will facilitate the development of community resources, is easy to manipulate with relative low maintenance costs, and that possesses the physiology of the temperate cereals. The research of temperate grasses' biology, its regulatory strategies and environmental sensitivity developed in the course of genetic evolution, by natural selection and/or artificial domestication, will not only enrich our knowledge of the fundamental biology of plants, but it can be transposed into increased economic potential through agricultural or technological use.

2.2 *Brachypodium distachyon* (L.) P. Beauv.

Brachypodium (from the Greek brachys "short" and podium "foot", referring to its subsessile spikelets) is a genus representing some temperate wild grass species. In particular, *Brachypodium distachyon* (L.) P. Beauv., also described as "purple false brome", is a grass species native to southern Europe, northern Africa and south-western Asia, closely related to the four major cool season grass tribes of greatest economic importance (Figure 2.1): Triticeae, Aveneae, Poeae, and Bromeae and, therefore, more genome closely related to these crops than rice (Garvin *et al*, 2008). In fact, using available genomic and expressed sequence tag sequences, Bossolini *et al* estimated, in 2007, that Brachypodium is 10-15 Mya closer to wheat, than rice. Early studies also indicated that gene order (synteny) is largely conserved between Brachypodium and the small grain cereals, (Reviewed by Opanowicz *et al* 2008).



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Parameter	Arabidopsis	Rice	Barley	Wheat	Brachypodium
Height (cm)	15-20	100	50-120	50-100	15-20
Density (plants/ m²) [a]	2000	36	80-120	50	1000
Growth requirements (controlled conditions) [b]	Simple	Demanding	Intermediate	Intermediate	Simple
Efficiently crossed?	Yes	Yes	Yes	Yes	Yes
Reproduction	Selfing	Selfing	Selfing	Selfing	Selfing
Typical generation time (weeks)	8-12	12-24	10-20	10-20	8-12
Seed per plant	>1000	>1000	150-200	50-150	100-1000
Transformation	Extremely easy	Highly efficient	Efficient, but labour intensive	Inefficient	Highly efficient
Genome size (Mb)	119 [c]	382 [c]	5500	16000	272 [c]
Assembled genome sequence	Finished	Finished	In progress	In progress	Finished
T-DNA resources	Extensive	Extensive [d]	None	None	10 000
Cell wall type	Type 1	Type 2	Type 2	Type 2	Type 2
Photosynthesis	C3	C3	C3	C3	C3

[a] High-density planting under laboratory conditions. [b] The difficulty of growing plants is dependent upon their size and the range of environmental conditions tolerated. Thus, small plants that tolerate varied conditions have simple growth requirements and large plants that need carefully controlled environmental conditions have demanding growth requirements. [c] Assembled genome size. [d] The availability of the resources is constrained by quarantine restrictions and intellectual property concerns.

Figure 2.1: *Brachypodium distachyon* (L.) P. Beauv. as a model system for temperate grasses. A) *Arabidopsis thaliana* (L.) Heyhn, Columbia-0, Left; *Brachypodium distachyon* (L.) P. Beauv, Bd21, Middle; *Hordeum vulgare* L., Golden promise, Right. B) Phylogenetic tree of cereal grasses (Adapted from Opanowicz *et al*, 2008). C) Comparative table of traits between model systems and crops (Adapted from Brkljacic *et al*, 2011).

Brachypodium distachyon is an annual grass, with a basal chromosome number of n=5 (Reviewed by Opanowicz *et al* 2008), that possesses the suite of biological traits desired in a model plant, quite similar to those found in the dominant dicot model *Arabidopsis thaliana* (L.) Heyhn. These desirable features include a small genome (272 Mb) somewhere between rice and *Arabidopsis*, availability of diploid ecotypes, self-fertility promoting inbreeding, efficient transformation protocol (Alves *et al*, 2009), small stature (20 cm at maturity), a moderately rapid life cycle (< four months), high seed set, lack of seed head shatter, extensive natural variation in agronomic traits, and simple growth requirements - adapted to temperate environments. Such characteristics, evidenced by Draper *et al* in 2001, excited a range of research groups and led to the emergence of an international Brachypodium research community, and the formation of the International Brachypodium Initiative (IBI) in 2005 (Garvin *et al*, 2008). As a consequence, a set of inbred Brachypodium lines, from the United States Department of Agriculture - National Plant Germplasm System (NPGS) collection was developed from a single seed descent (Garvin *et al*, 2008). The community standard line Bd21, source of DNA and RNA for both the whole genome and EST sequencing projects, is a single seed descent inbred line derived from accession PI 254867 (Garvin *et al*, 2008).

As a new model plant, Brachypodium's potential as a suitable system to study the biology of cereal crops has been under scrutiny (Li *et al*, 2012; Yordem *et al*, 2011) and, interestingly, with a photosynthetic pathway belonging to the C3 cluster – not the most efficient under hot and dry conditions – *Brachypodium distachyon* opens the possibility to investigate the viability of converting other C3 cereals like the highly economically important wheat and rice, into the C4 pathway – a project already under way with the support of the Bill & Melinda Gates Foundation (http://c4rice.irri.org/).

2.2.1 Botanical description and morphology

Brachypodium distachyon is a cleistogamous[1] caespitose[2] bright green or glaucous[3] annual monocot pooid. It possess ascending or geniculate[4] culms (Figure 2.2-A); with glabrous[5], hollow and terete[6] internodes; and nodes noticeably pubescent[7] (Figure 2.2-A2). The leaves are cauline, not distinctly distichous[8]; with sheaths usually glabrous, with open margins (Figure 2.2-A1); the auricles are absent; the ligules are membranous and pubescent (Figure 2.2-A3); the blades are flat, glaucous, sparsely hairy, with veins unequally prominent, and thickened margins (Figure 2.2-A3). The glumes are persistent, dissimilar and shorter than the spikelet (Figure 2.2-A6). The inflorescence is a bilateral spike or raceme, with a persistent rachis (Figure 2.2-A4). The spikelets, which disarticulate above the 2 opposite, unequal, shorter than first floret, and awnless glumes, are solitary, laterally compressed, shortly pedicellate, awned, and ascending. They are composed of 7 to 15 florets that are reduced at the apex; the rachilla does not extend beyond upper floret (Figure 2.2-A6). Each floret possesses a chartaceous[9], 7-veined, glabrous, and acuminate[10] lemma, with an apical and straight awn; the palea is 2-veined, awnless, and ciliate (Figure 2.2-A5 and 2.2-C); the 2 lodicules are oblong, acute[11], sparsely hairy and ciliate. There are 2 stamens with yellow anthers. The ovary is pubescent on apex (Figure 2.2-B and 2.2-C). The fruit is a dorsiventrally compressed and ellipsoid caryopse, adnated[12] to the lemma (Figure 2.2-A7). (http://botany.csdl.tamu.edu/FLORA/taes/tracy/610/brachypodium.html; http://herbarium.usu.edu/webmanual/info2.asp?name=Brachypodium_distachyon&type=treatment)

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[1] From the Greek *kleistós* (closed) and *gamos* (reproductive organs). Self-pollinating and fertilization occurring in unopened flowers. (http://dictionary.reference.com/)

[2] From the New Latin *Caespitōsus* (growing in dense tufts) (http://dictionary.reference.com/)

[3] From the Latin *Glaucus* or Greek *Glaukós* (silvery, grey, bluish-green or greenish-blue) (http://dictionary.reference.com/)

[4] From the Latin *Geniculātus* (having knee-like joints or bends) (http://dictionary.reference.com/)

[5] From the Latin *Glaber* (hairless) (http://dictionary.reference.com/)

[6] From the Latin *Teres* (smooth and usually cylindrical and tapering) (http://dictionary.reference.com/)

[7] From the Latin *Pūbēscere* (covered with a layer of fine short hairs) (http://dictionary.reference.com/)

[8] From the Latin *Distichus* or Greek *Dístichos* (arranged alternately in two vertical rows on opposite sides of an axis) (http://dictionary.reference.com/)

[9] From the Latin *Charta* (paper) and *Aceous* (like) (http://dictionary.reference.com/)

[10] From the Latin *Acūmināt*us (tapering to a point) (http://dictionary.reference.com/)

[11] From the Latin *Acūtus* (having a sharp end or point) (http://dictionary.reference.com/)

[12] From the Latin *Adnātus* (growing closely attached to an adjacent part or organ) (http://dictionary.reference.com/)

Figure 2.2: *Brachypodium distachyon* (Bd21) wild-type's morphological traits. A) Tiller, scale bar: 1 cm; A1) Leaf, scale bar: 0.5 cm; A2) Node, scale bar: 0.1 cm; A3) Ligule, scale bar: 0.1 cm; A4) Spike, scale bar: 0.5 cm; A5) Floret, scale bar: 0.3 cm; A6) Spikelet, scale bar: 0.5 cm; A7) Caryopsis (seed), scale bar: 0.2 cm. B) SEM microscopy photograph of a dissected of *Brachypodium distachyon* (Bd21) wild-type floret: I) Palea; II) Lemma [removed]; III) Lodicules [x2 – right one removed]; IV) Stamens [x2], composed by (a) filament, and (b) anther; V) Carpel, composed by (a) ovary, (b) style [x2], and (c) stigma [x2]. C) Schematic representation of a *Brachypodium distachyon*'s floret. Symbols in grey represent aborted organs.

2.2.2 Growth requirements

The environments in which Brachypodium is most commonly found often experience hot, dry summers and mild, wet winters, such as the climate found around the Mediterranean and Californian chaparral communities (Figure 2.3). In fact, Bd21 has its geographic origin on a road to Mosul, Iraq (Vogel, 2009 *in* http://brachypodium.pw.usda.gov/files/Bdmodel2009.pdf).

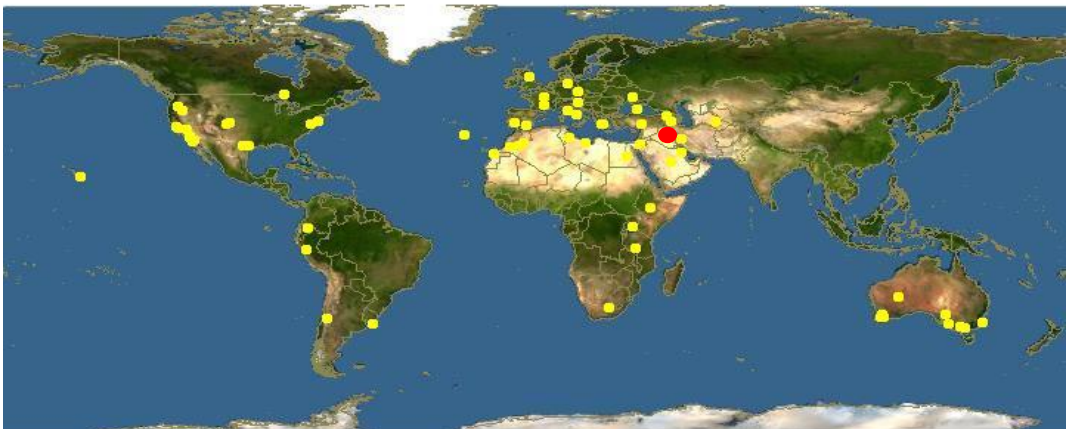

Figure 2.3: Geographical distribution of Brachypodium accessions. Bd21's geographic origin highlighted in red. (Adapted from http://www.discoverlife.org/mp/20q?search=Brachypodium+distachyon)

Brachypodium exhibits very simple growth requirements, developing well in both glasshouses and controlled environmental rooms. Because it has a small stature and does not outcross, Brachypodium can be sowed at a density of 1 000 plants/m^2 (http://brachypodium.pw.usda.gov/files/Bdmodel2009.pdf). It grows well in a number of different compost mixtures; however, it does not tolerate excessive watering. A generic setting of 20 °C constant temperature, and long photoperiods (with a mix of fluorescent and incandescent lights) bypasses the need for vernalization. Bd21 will flower readily with 20 h of light, and the first seeds will be ready to harvest in 2 months. Flowering can be further accelerated with more extreme light regimes: in conditions of 24 h of light, inflorescences of Bd21 plants have been observed to begin emerging in as few as 17 days. Conversely, a 14 h photoperiod will suppress the floral transition of the community standard line, which will result in plants with a large amount of vegetative biomass (Figure 2.4) (Reviewed by Garvin *et al*, 2008).

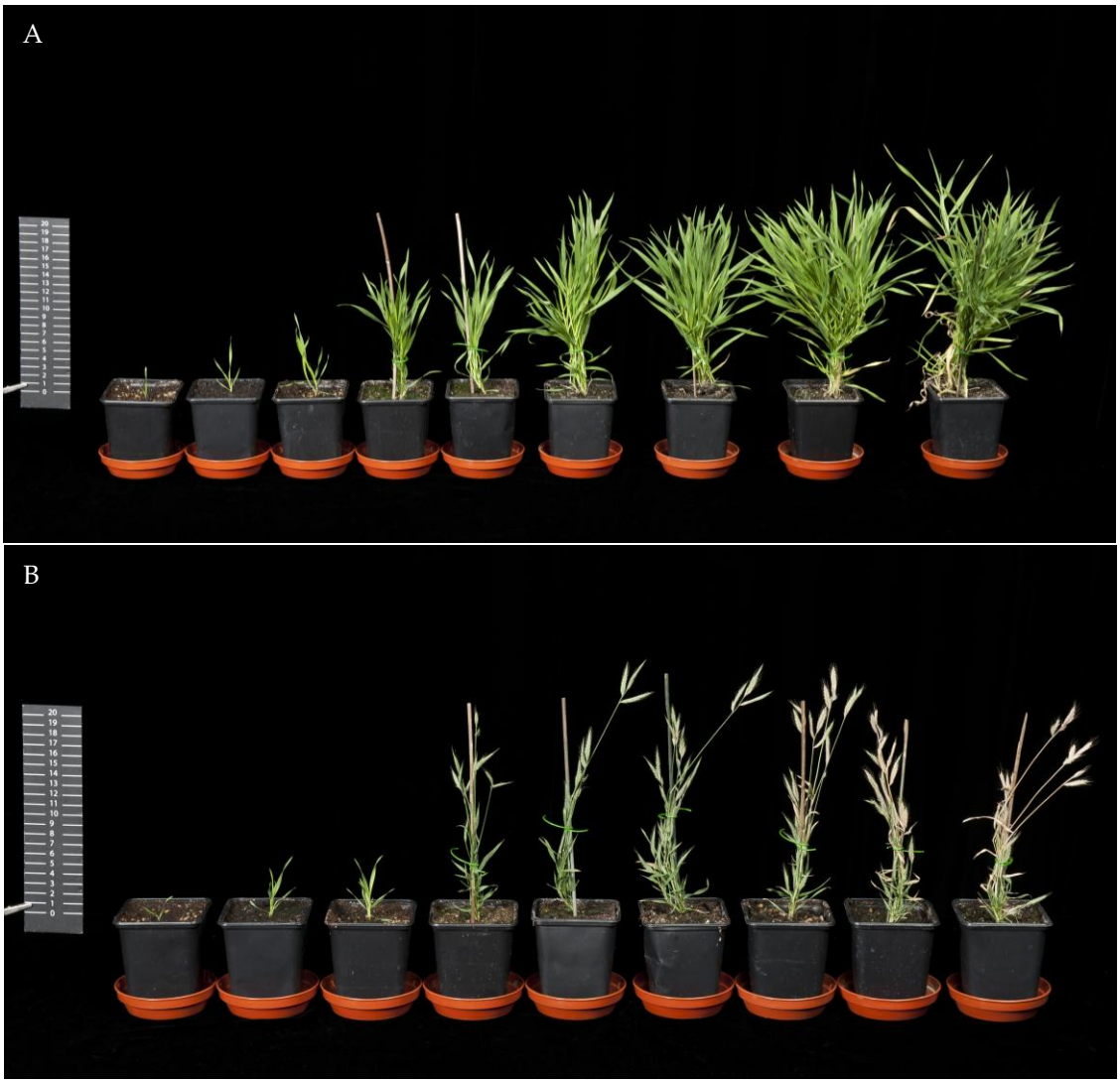

Figure 2.4: *Brachypodium distachyon*'s (Bd21) developmental series over a 9 week period. A) Non-flowering inductive photoperiod of 14 h light, 10 h dark. Plants develop high levels of vegetative biomass. B) Inductive photoperiod of 20 h light, 4 h dark. Plants develop lower levels of vegetative biomass, heading date occurs after the 3rd week of growth, and fully dried seeds are ready for harvesting by the 10th week.

2.2.3 Crossing

Brachypodium exhibits an inbreeding reproductive strategy, with anthers and stigmas enclosed tightly within the palea and lemma. This makes crossing different plants slightly more demanding technically. When conducting crossing experiments, floral spikelet emergence proved to be the best stage to remove the anthers and apply pollen to the stigmas (Draper, 2001) (Figure 2.5-A). The emasculation of the floret (♀) can be performed using a needle to, carefully, separate the lemma from the palea (Figure 2.5-B) and remove both immature anthers (Figure 2.5-C* and 2.5-D). The pollen harvest from the donor plant (♂) can be achieved by cutting the floret with sharp scissors and by gently pushing the anthers out (Figure 2.5-E – 2.5-H). Once collected, the anthers must be placed inside the previously emasculated floret (♀) immediately and as near as possible to the stigmas (○); following which the floret should be closed with a string to prevent the desiccation of the reproductive organs and of the pollen grains (Figure 2.5-I – 2.5-K). All florets that were successfully crossed will experience the subsequent filling of the grain (Figure 2.5-L).

Figure 2.5: Crossing Brachypodium. A) Developmental stage of the floret to be pollinated (♀) - the anthers must be immature; B – D) Emasculation of the floret to be pollinated – excision of the anthers; E – H) Pollen harvest for pollination of the emasculated floret (♂); I - K) Pollination of the emasculated floret: to prevent desiccation the anthers must be quickly inserted in the new floret, as near as possible to the stigmas, after which the palea and lemma need to be tightly closed with a string; L) F1's immature seed. (l) lemma, (p) palea, (a, *) immature anthers, (s) pubescent stigmas, (o) ovary, (○) cross. (Adapted from Garvin 2009 *in* **http://www.ars.usda.gov/SP2Userfiles/person /1931/BrachypodiumCrossing.pdf**, and Steinwand and Vogel 2010 *in* **http://wheat.pw.usda.gov./bBEST/file/xingBrachy100203pdf**)

2.3 Brachypodium community resources

2.3.1 Genome sequence

The US Department of Energy Joint Genome Institute (JGI) has completed a draft assembly of *Brachypodium distachyon*'s whole genome sequence (community standard line Bd21); accessible through the http://www.brachypodium.org/ or http://mips.helmholtz-muenchen.de/plant/brachypodium/ webpages (Table 2.1). The draft 8x genome assembly is thought to be of «unprecedented quality», with gaps corresponding to no more than 0.4% of the whole sequence. These gaps should be soon resolved, as DOE/JGI has initiated a new project targeting to improve the quality of uncertain sequences, and to finalize its completion (Brkljacic *et al*, 2011). In parallel with the genome sequencing project, expression sequence tag (EST) libraries have been generated and are partially sequenced (Vogel *et al*, 2006). In addition to the 20 000 EST already deposited into Genbank, back in 2005, an additional 180 000 ESTs are expected to be released as part of the genome sequencing project of the DOE/JGI. (http://www.brachypodium.org).

2.3.2 Microarray chips

Taking advantage of the release of the 8x genome sequence assembly, Todd Mockler from the Oregon State University has created a Brachypodium Affymetrix microarray platform (Table 2.1), which contains about 2.55 million expression probes, covering both exon and intron sequences; and roughly 3.95 million intergenic probes. These arrays can be ordered individually from the Nottingham Arabidopsis Stock Centre, or directly from Affymetrix's website (www.orau.gov/gtl2011/abstracts/mockler_todd.pdf).

2.3.3 Genetic transformation

The availability of an efficient transformation method is essential for a model plant system. Agrobacterium-based transformation technologies are generally better suited to generate transgenic plants containing a low number of transgene copies with limited rearrangements, which can integrate at one or multiple unlinked Mendelian loci into the nuclear genome (Travella *et al*, 2005). Due to the specific anatomy of its florets, the floral dipping method usually used to transform Arabidopsis is not suitable to transform *Brachypodium* (Alves, unpublished). The published transformation method of *Brachypodium distachyon* standard community line (SCL) Bd21 (Alves *et al*, 2009), is more laborious and involves the production of compact and embryogenic *callus* (CEC) derived from immature embryos (Figure 2.6); the desiccation of CEC after inoculation with Agrobacterium; and the use of hygromycin as a selectable marker for the identification of transgenic *calli* and plants; with the optional use of the green fluorescent protein (GFP) as a reporter. Approximately eight independent fully developed transgenic plants can be successfully regenerated from each immature embryo of Bd21 (Alves *et al*, 2009), with transgenic seed (T0 generation) ready to be harvested ~12 weeks after transformation.

Figure 2.6: Genetic transformation of Bd21 via tissue culture. Each Petri dish contains several independently transformed lines of *callus,* derived from excised immature embryos of Bd21 plants, regenerating transformed shoots under Hygromycin selection.

2.3.4 Mutant collections

Brachypodium is an emerging model system, and for that reason the mutant collections available are still limited to a few labs (Table 2.1). There is a collection of fast-neutron mutants belonging to Dr Debbie Laudencia Chingcuanco (USDA) and Dr Mary Byrne (JIC); another collection of ethylmethanesulphonate (EMS) mutants from the United States Department of Agriculture (http://brachypodium.pw.usda.gov); two collections of targeted induced local lesions in genomes (Tilling) mutants, one from the French Institut National de la Recherche Agronomique (INRA) (http://urgv.evry.inra.fr/UTILLdb), and another from the Boyce Thompson Institute (http://bti.cornell.edu/brachybio); and 2 T-DNA insertion mutant collections, one from the United States Department of Agriculture-ARS Western Regional Research Centre (WRRC) (http://brachypodium.pw.usda.gov/TDNA/), and another from the BrachyTAG group at the John Innes Centre, England (http://www.brachytag.org/), from where the first Brachypodium mutant has been recently identified (Vain *et al*; 2011). The International Brachypodium Tagging Consortium (IBTC), composed by 8 laboratories from 5 countries, has been recently created with the mission to significantly expand the production and characterization of T-DNA mutants. It is the intention of the IBTC to reach their objectives of 60 000 T-DNA lines by 2013, and seed requests will be made available to the scientific community via online databases (more information on http://www.brachypodium.org/).

2.3.5 Artificial micro RNA

While mutant collections are still being generated and few have been characterized, it is possible to generate Brachypodium lines in which specific transcripts (single or multiple) have been selectively silenced through the constitutive expression of particular 21 mer artificial microRNAs. Prof. Dr. Detlef Weigel and his team, in Tübingen, have expanded their pre-existing artificial microRNAs web designer tool (Figure 2.7 and Table 2.1) to include Brachypodium's genome (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi). Basically, the amiRNA designer will, given the genome sequence and the parameters imposed by the user, define oligos to be engineered with the rice endogenous miRNA precursor, to target the gene of interest via the expression of a small RNA (Warthmann *et al*, 2008).

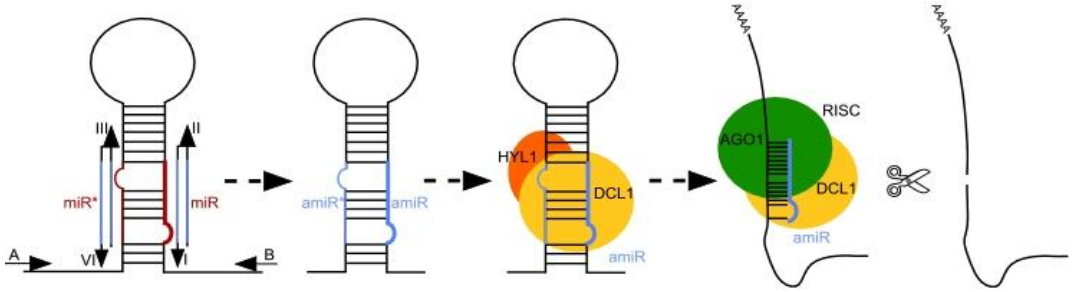

Figure 2.7: Schematic representation of the gene silencing technology mediated by the production of artificial microRNAs (adapted from **http://wmd3.weigelworld.org/cgi-bin/webapp.cgi**).

Table 2.1: Summary table of internet resources for Brachypodium research (adapted from Brkljacic *et al*, 2011).

Resource	Institute	URL	Description
Arizona Genomics Institute	Arizona State University	http://www.genome.arizona.edu	BAC libraries
BrachyBase	Oregon State University	http://brachybase.org/	Genome sequence
BrachyBio	Boyce Thompson Institute for Plant Research	http://bti.cornell.edu/brachybio	TILLING population, resources for teachers
Brachypodium distachyon Information Resource	Oregon State University	http://www.brachypodium.org/	Central location for information
Brachypodium Genome Information	Munich information Center for Protein Sequences	http://mips.helmholtz-muenchen.de/plant/brachypodium/	Genome sequence
Brachypodium Resources	USDA-ARS, Western Regional Research Center	http://brachypodium.pw.usda.gov/	T-DNA lines, methods (crossing, mutagenesis, transformation), germplasm
BrachyTAG	John Innes Centre	http://www.brachytag.org	T-DNA lines, protocols, transformation vectors, and service
CoGe	University of California, Berkeley	http://syntheny.cnr.berkeley.edu/CoGe	Comparative genomic tools
ELEMENT	Oregon State University	http://element.cgrb.oregonstate.edu/	Promoter searching tool
Garvin Laboratory	USDA-ARS Plant Science Research Unit	http://www.ars.usda.gov/pandp/docs.htm?docid=18531	Germplasm, crossing method
GrainGenes	USDA-ARS, Western Regional Research Center	http://wheat.pw.usda.gov	Comparative genomics tools
Gramene	Cold Spring Harbor Laboratory	http://www.gramene.org	Comparative genomics tools
Iowa State University Plant Transformation Facility	Iowa State University	http://www.agron.iastate.edu/ptf/index.aspx	Transformation Service
ModelCrop	John Innes Centre	http://www.modelcrop.org/	Genome sequence
NASC's International Affymetrix Service	University of Nottingham	http://affymetrix.arabidopsis.info/	Microarray service
Phytozone	JGI and Center for Integrative Genomics	http://www.phytozome.net/	Comparative genomics tools
PlantGDB	Iowa State University	http://www.plantgdb.org/	Comparative genomics tools
PlexDB	Iowa State University	http://www.plexdb.org/	Expression data
QuantPrime	University of Potsdam	http://www.quantprime.de	Design oligonucleotides for qPCR
TILLING database	Unité de Recherche en Génomique Végétale	http://urgv.evry.inra.fr/UTILLdb	TILLING collection
USDA NPGS	USDA	http://www.ars-grin.gov/npgs	Germplasm
WMD3 web microRNAs Designer	Max Planck Institute for Developmental Biology	http://wmd3.weigelworld.org/	Tools for designing artificial microRNAs

2.4 Aims and Outline of the Thesis

In spite of all the major scientific advances achieved in plant research in the last decade, the genetics behind flowering in monocot plants is still poorly understood. Using *Brachypodium distachyon*, the emergent model system for temperate grasses, the present work aimed to characterize the morphological evolution of the SAM following exposure to inductive conditions and determine if key elements of the photoperiod inductive pathway mediating flowering transition in Arabidopsis, a dicot plant, remain functionally conserved in Bd21.

The thesis is organized as follows: Chapter 1 is an introduction to the most comprehensive flowering regulatory network, *Arabidopsis thaliana*'s, and its known triggering cues. Chapter 2 introduces *Brachypodium distachyon* as a model system for temperate monocots and summarizes the international resources made available to the community standard line Bd21. Chapter 3 explores Bd21's photoperiod inductive pathway by focusing on the search for "florigen" and the potentially conserved up-stream genetic elements that in coordination with an entrained circadian clock gate the sunlight cue. Chapters 4 and 5 are dedicated to the transformation occurring at the shoot apical meristem after flowering evocation; but while Chapter 4 examines the expression of Bd21's homologues to well described meristem identity genes and other central elements coordinating flowering commitment, Chapter 5 is focused to the whole transcriptome profile of the transitioning meristem during the inflorescence and floral development. Chapter 6 summarizes the experiments performed and highlights the main conclusions. Chapter 7 describes the experimental procedures.

Chapter 3

Photoperiod perception and signal transmission

3.1 Introduction

Plants are particularly sensitive to the environmental changes occurring in the region where they established themselves. Due to their motionless nature, stress avoidance strategies are limited; stress anticipation and stress management are the keywords to plant fitness, so much so that their environmental plasticity has been built on the continuous monitoring of its surroundings, and on the ability to respond accordingly.

With the arrival of springtime, plants enter the reproductive phase and begin to differentiate extraordinary structures, intentionally developed for the production of seeds. But why do plants recurrently synchronize their developmental decisions according to a particular time of the year? What is so special about this particular season that signals flowering plants that it's time to make that ultimate decision to arrest vegetative growth, and re-orientate its energy towards the establishment of a progeny? And more intriguingly, how does that happen - how can springtime stimulate floral transition? What goes on inside the plant while major environmental changes are taking place in its surroundings?

3.1.1 The annual seasons and circadian rhythms

Spring is one of the four seasons, conventionally defined by meteorologists, for the temperate regions of the globe. As it follows the end of the cold winter, and precedes the beginning of the warm summer, springtime can be defined as the season that commences on the vernal equinox - the day of the year when day-time and night-time have equal duration, and terminates on the summer solstice - the longest day of the year (Figure 3.1-A). In other words, it is the temperate season of increasingly long photoperiods. But how can plants sense this?

Circadian[1] rhythms have been described for many different kinds of organisms (Reviewed by Devlin and Kay, 2001) and, overall, they provide living beings with the ability to synchronize key physiological and metabolic processes with cyclic environmental cues, called zeitgebers[2], in a regular and dependable way, so to assure optimal growth in a particular environment. One of the most important zeitgebers is, of course, sunlight (Figure 3.1-B), and it has been known, mainly from work done on *Arabidopsis thaliana* that plants possess, in their leaves, light sensitive proteins, photoreceptors, that perceive not only the duration of the day/night cycle (photoperiod), but also the intensity and the quality of the radiation available.

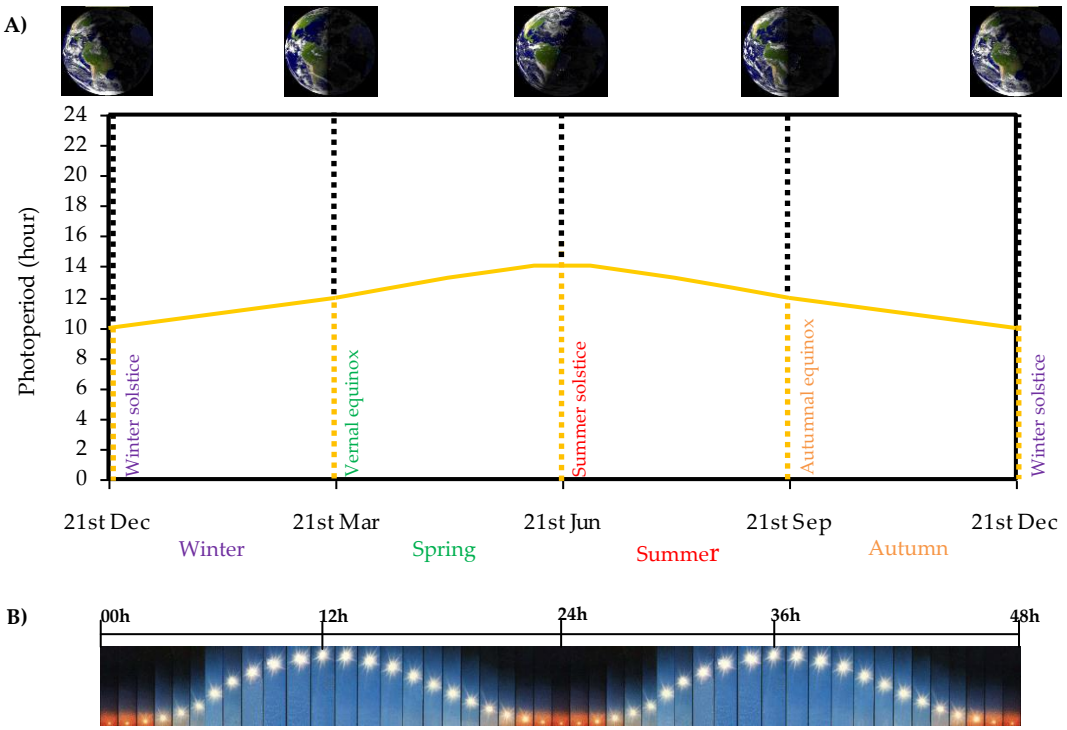

Figure 3.1: Sunlight's oscillatory patterns. A) Photoperiod across the seasons in Iraq, the geographical origin of Bd21 ecotype – latitude 30° 00′ N, as a result from the Earth's orbit around the Sun . Day time hours in yellow; night time hours in black (Earth's photographs adapted from http://en.wikipedia.org/wiki/Sunlight). B) Earth's solar day - A 48h sequence of photographs depicting the apparent movement of the Sun in the sky as a result from the Earth's rotation around its own axis (modified from http://www.unifr.ch/biochem/index.php?id=99)

[1] From the Latin *Circa* (around) and *Diem* (day) (http://dictionary.reference.com/,) (Halberg *et al*, 2013)

[2] From the German, zeit (time) and geber (giver) (http://dictionary.reference.com/), (Aschoff, 1960).

3.1.1.1 Light sensing photoreceptors

So far, the blue light responsive CRYPTOCHROMES[3] (CRY1 and CRY2), the phototropins, and the ZEITLUPE[4]'s (ZTL) family of F-box proteins; along with the red/far-red light (R/FR) detecting PHYTOCHROMES[5] (PHYA-PHYE) have been identified as vital components of the light perception complex (Fankhauser and Staiger, 2002; Nagy and Schafer, 2002; Kim *et al*, 2007; Imaizumi *et al*, 2003; Baudry *et al*, 2010; Somers *et al*; 1998; Devlin and Kay, 2000; Yanovsky *et al*, 2000; Martínez-García *et al*, 2000). From all these light sensitive molecules, PHYTOCHROME B (PHYB) has been shown to be a central regulator of plant development under high light intensities, such as those found in the open field. PHYB, is the red light responsive mediator of the "shade avoidance syndrome" (Lorrain *et al*, 2008) in Arabidopsis, and apart from regulating flowering time (Reed *et al*, 1993), is probably involved in the perception and entrainment of the dawn and dusk cues into the clock's central oscillator. The remaining members of the phytochrome family, PHYA, and PHYC to PHYE, are assigned to mediate other developmental processes, like seed germination in the absence of light (Botto *et al*, 1996; Shinomura *et al*, 1996; Furuya and Schafer, 1996; Marrocco *et al*, 2006), or to assist PHYB in the competitive response for sunlight under the canopy (Furuya and Schafer, 1996; Somers *et al*, 1998; Devlin and Kay, 2000; Yanovsky *et al*, 2000). With their expression circadianly regulated (Devlin and Kay, 2001), phytochromes are synthesised in an inactive form, and undergo photo-conversion upon absorbance of the appropriate radiation, becoming functionally active (reviewed by McClung, 2006) (Figure 3.2-A). Once activated, these proteins are translocated into the nucleus where they supply the light input to the circadian clock through the physical interaction with the PHYTOCHROME INTERACTING FACTORs (PIFs), a family of basic helix-loop-helix (bHLH) transcription regulators (Figure 3.2-B). This interaction promotes the phosphorylation, ubiquitination and degradation of PIFs (Castillon *et al*, 2007), thereby preventing these PIFs from binding to their target gene promoters and activating gene expression. Far-red enriched light, for example is known to induce flowering in Arabidopsis through the up-regulation of FT (Cerdán and Chory, 2003; Halliday *et al*, 2003; Valverde *et al*, 2004; Reed *et al*, 1994; Bagnall and King, 2001). Conversely the opposite happens on exposure to red light.

In summary, the complexity of this highly sophisticated arrangement allows plants to maintain a entrained circadian rhythmn under all light conditions (Figure 3.2), and this is only achievable because in its core, the system is sustained by a combination of highly sensitive photoreceptors, which are degraded under higher light intensities, with more stable but less sensitive photoreceptors, which act at higher light intensities without overbearing the circadian phototransduction pathway (Devlin and Kay 2001).

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[3] From the Greek *kruptós* (hidden) and *khrôma* (color) (http://dictionary.reference.com/)
[4] From the German *zeitlupe* (slow motion) (http://dictionary.reverso.net)
[5] From the Greek *phutón* (plant) and *khrôma* (color) (http://dictionary.reference.com/)

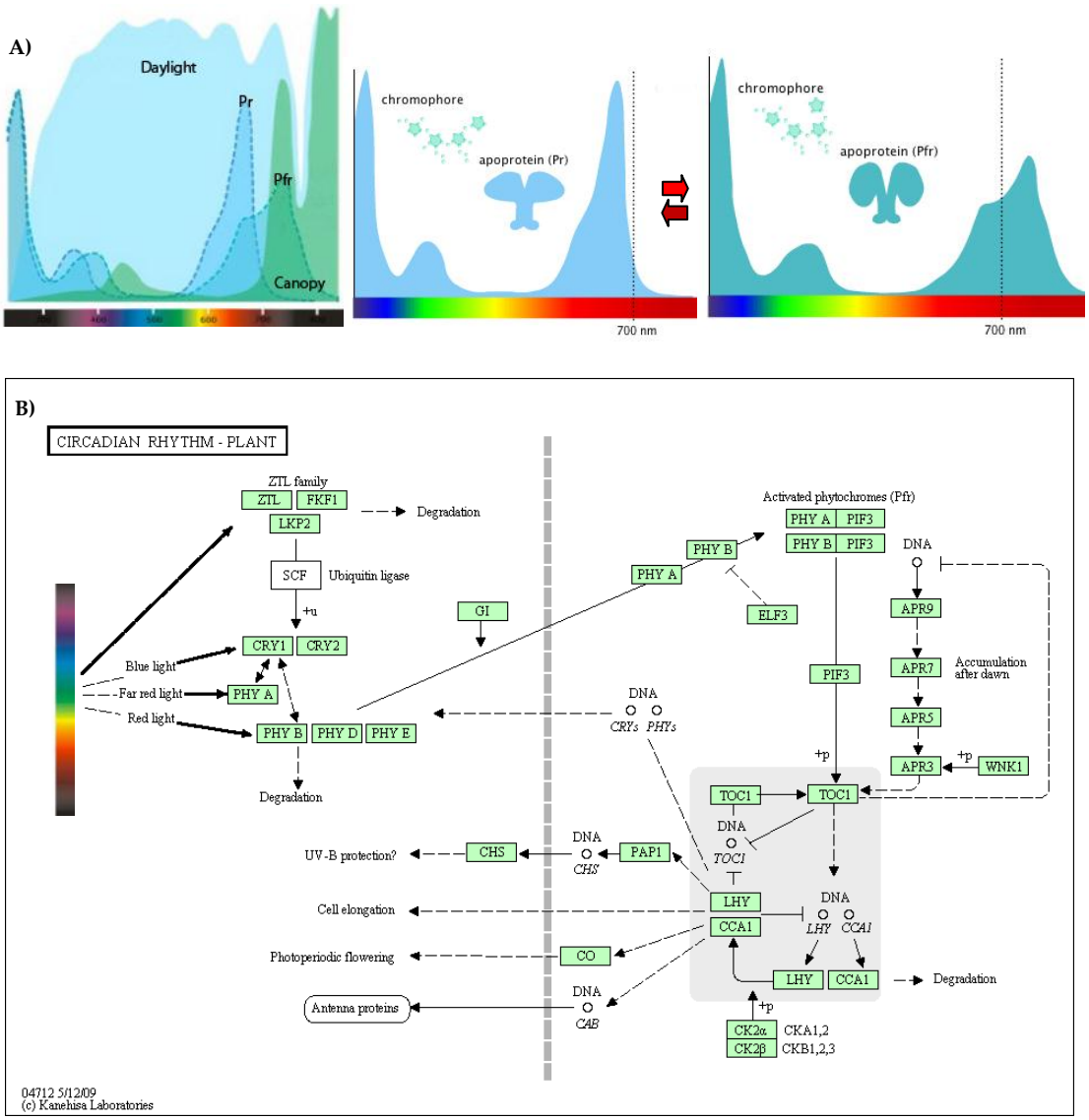


Figure 3.2: Plant phytochromes and the circadian rhythm. A) Pr and Pfr absorption maxima are shown in relation to daylight spectra above and below a canopy. The conversion of the chromophore by red or far-red radiation incites a corresponding adjustment to the phytochrome tertiary structure, significantly changing its chape, color, absortion spectra and biological activity (modified from http://www.huh.harvard.edu/research/mathews-lab/LabWebsite/phyintro.html); B) Schematic representation of the biological complexity of gene regulation mediated by light and the circadian rythm - *Arabidopsis thaliana* (Adapted from http://www.genome.jp/kegg/pathway/ath/ath04712.html).

3.1.1.2 The circadian clock

The circadian clock, as a whole, is a highly complex arrangement of tightly regulated genes that, in order to function properly and offer the plant an adaptive advantage must be precisely synchronized (entrained) with the daily environmental changes imposed by the day/night cycle, and the advancing seasons (Johnson *et al*, 2003; Hotta *et al*, 2007). Supplied with the light input perceived by the leaves, through a blend of intertwined and highly specialized photoreceptors, the core oscillator consists of, at least, three interconnected feedback loops (the morning, the central, and the evening loops) that generates a self-sustained rhythm (Locke *et al*, 2005; Locke, 2006; Zeilinger *et al*, 2006; Lu and Tobin, 2011) in tune with the apparent (all) light conditions. These loops, responsible for keeping the pace of the clock, are thought to be generated by the regulation of the expression of the Myb transcription factors CIRCADIAN AND CLOCK ASSOCIATES 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY); and the pseudo-response regulator TIMING OF CAB EXPRESSION 1 (TOC1) (Strayer *et al*, 2000; Alabadí *et al*, 2001). The current working model describes the occurrence of a central negative feedback loop in which CCA1 and LHY, primarily expressed in the morning, repress both the evening abundant TOC1 which, in itself, acts as a positive regulator of CCA1 and LHY (McClung, 2006); and a TCP transcription factor CCA1 HIKING EXPEDITION (CHE), which again represses CCA1 directly. In the morning, however, CCA1 and LHY act as positive regulators of other PSEUDO-RESPONSE REGULATOR proteins, PRR7 and PRR9, only to serve as negative regulators of CCA1 and LHY, themselves (Farré *et al*, 2005; Salomé and McClung, 2005; Locke *et al*, 2006; Pokhilko *et al*, 2010). The composition of the evening loop, on the other hand, remains elusive. It has been suggested that TOC1 is positively regulated via an unknown Y element, possibly (but not exclusively) GIGANTEA (GI), that once activated would, again, feedback negatively on itself, enclosing in this way this regulatory circle (Figure 3.3).

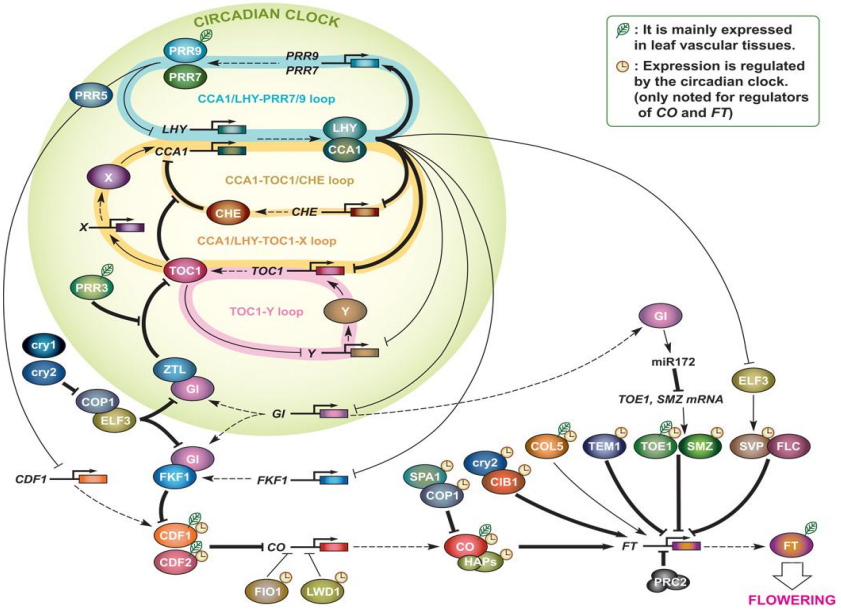


Figure 3.3: Schematic representation of Arabidopsis core oscillator model, and its output. The morning loop is shaded in blue, the central loop is shaded in yellow, the evening loop is shaded in pink (adapted from Imaizumi, 2010)

3.1.2 Flowering time

3.1.2.1 CONSTANS – the photoperiod gauge

One of the known outputs of the photoperiod inductive pathway via the circadian clock is GI, and its downstream target CONTANTS (CO) (Figure 3.3). *CO* encodes a nuclear B-box type zinc-finger transcription factor (Putterill *et al*, 1995) with a CCT (CO, CO-like, TOC1) domain (Strayer *et al*, 2000; Robson *et al*, 2001), whose stability is strongly dependent on the presence of the far-red and blue light sensed by *PHYA* and *CRY2* (Valverde *et al*, 2004), in spite its circadian regulated expression occurring independently of the photoperiod (Suárez-López *et al*, 2001). Extended day length stabilises CO protein levels (Suárez-López *et al*, 2001) (Imaizumi *et al*, 2005) (Turck *et al*, 2008), allowing it to activate its target gene, *FLOWERING LOCUS T* (*FT*), encoding a phosphatidylethanolamine binding protein (PEBP)/RAF-kinase-inhibitor-like protein (Kardailsky *et al*, 1999); and a mobile signal (florigen) that travels from the leaves, through the phloem, to the SAM (Jaeger and Wigge, 2007; Corbesier *et al*, 2007) where it triggers the development of flowers (Figure 3.4). Loss of function mutations of CO lead to late flowering phenotype under LD but not on SD (Putterill *et al*, 1995), while the constitutive expression promotes early flowering in both SD and LD (Onouchi *et al*, 2000).

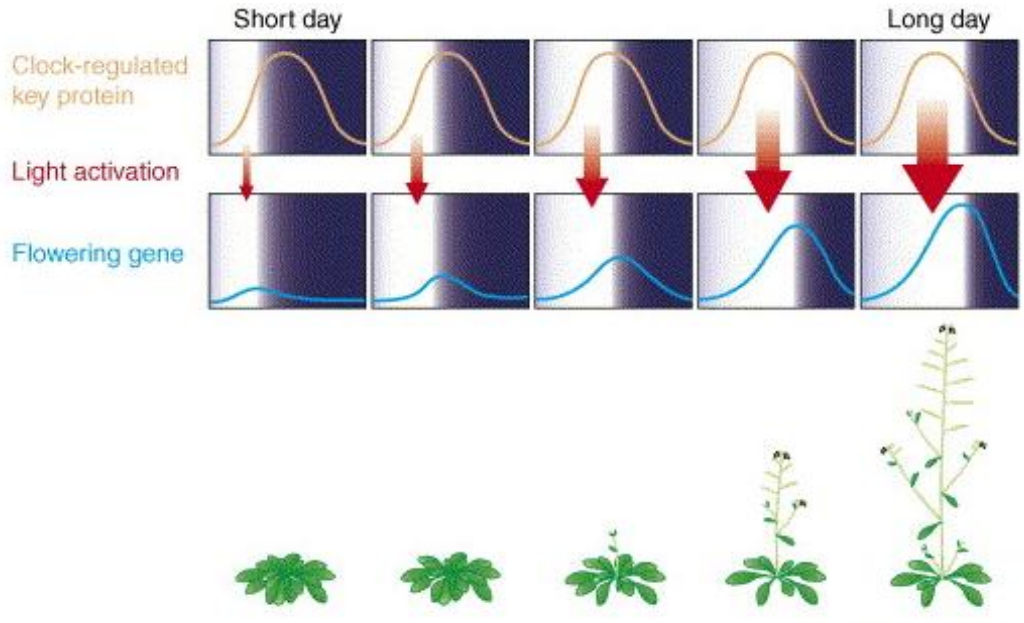


Figure 3.4: Schematic representation of the external coincidence model in Arabidopsis. Clock-regulated CO responds to extended photoperiods through the upregulation of florigen (adapted from Imaizumi and Kay, 2006).

It is thought that monocots and dicots share the layout of photoperiodic inductive pathway, in spite of the few differences that have already been reported: contrary to Arabidopsis' CO, rice's Heading date 1 (Hd1) promotes flowering under SD conditions instead of LD, through the induction of Heading date 3a (Hd3a), an FT-like protein (Yano *et al*, 2000;

Kojima *et al*, 2002; Hayama *et al*, 2003; Tamaki *et al*, 2007). Sequence homology and comparative mapping revealed that barley's HvCO1 is collinear with Hd1 (Griffiths *et al*, 2003) and that both peptides are predicted to form a coiled region in the centre of the protein; a feature not predicted for CO. HvCO1 and Hd1 also exhibit aminoacid variations that are thought to interfere with the proper function of the second B-box, indicating that cereals probably do not require two B-boxes to be functional. This could explain why HvCO1 is not able to complement Arabidopsis' *co-2* mutant, nor induce FT expression (Campoli *et al*, 2012). However, LpCO, the CO orthologue in *Lolium perenne*, was able to complement the *co-2* mutant and promote early flowering in Arabidopsis wt, even though it also possesses a non-conserved second B-box (Martin *et al*, 2004). Curiously, it has been reported that none of the CO orthologues in barley or wheat can be associated with any known photoperiod responsive quantitative trait loci (QTL) (Griffiths *et al*, 2003), and that, for barley, the pseudo-response regulator Ppd-H1 (similar to PRR7 from Arabidopsis) is the determinant gene for the photoperiod response (Turner *et al*, 2005), acting independently of HvCO1 to up-regulate HvFT1 (barley FT orthologue) (Campoli *et al*, 2012). However, HvCO1 was able to induce early flowering in both LD and SD, when constitutively expressed in Golden Promise, a spring barley cultivar; even though HvFT1 mRNA was only up-regulated in over-expressing lines grown in LD (Campoli *et al*, 2012). In Arabidopsis, on the other hand, the Ppd-H1 equivalent, PRR7, is not a major determinant of flowering time among natural populations (Ehrenreich *et al*, 2009). These significant differences between monocots and dicots, along with the inconsistencies among cereal species denote that generalizations regarding the genetic elements regulating the photoperiodic induction of flowering are reductive, and that the understanding of flowering transition in temperate monocots requires dedicated attention.

3.2 Rational

Being recognized as a central integrator of all inductive cues, and thought to be the long sought "florigen", FT is the epicentre of the flowering transition. In order to explore the photoperiodic induction of flowering in a new plant system it is important, to first of all, identify its functional orthologue, as flowering time research has to be placed in the context of FT expression.

As a signature element of the photoperiodic pathway, in Arabidopsis, CO is known to induce flowering through the up-regulation of FT under long photoperiods. With Brachypodium (Bd21) being highly responsive to daylength and known to flower early in days with extended hours of sunlight, the photoperiodic pathway seems to have prevalence among the other known flowering inductive cues, such as vernalization, and ambient temperature (Dr. Scott Boden, personal communication). The Bd21 CO orthologue was determined, its expression analysed as well as its ability to functionally contribute to the light-mediated induction of flowering in both Bd21 and Arabidopsis.

It has been recently demonstrated that PIF4 regulates Arabidopsis' architecture via an ambient thermo-sensory response (Franklin *et al*, 2011) and the induction of flowering in non-inductive photoperiods (Kumar and Lucyshyn *et al*, 2012). Kumar and Lucyshyn *et al*, 2012 also demonstrated that this thermo-induction of flowering in SD, by PIF4, results from the direct binding of the bHLH transcription factor to FT promoter, inducing its expression independently of CO. This study also correlated the delay in flowering at lower temperatures with the PIF4 protein level activity, and gibberellins. It is known that DELLA proteins are able to interact with PIF proteins, resulting in the PIF confiscation and degradation. In a quintuple *della* mutant, the authors reported early flowering when compared to wt. Since this "mechanism" bypasses the photoperiod induction pathway in Arabidopsis, epitomized by CO, it was analysed if in Brachypodium the correspondent PIF4 orthologue would have a predominant role in the induction of flowering in the same way it was demonstrated in Arabidopsis.

3.3 Results

3.3.1 BdFTL2 is *Brachypodium distachyon*'s functional orthologue of Arabidopsis' FLOWERING LOCUS T

The protein sequence of Arabidopsis FLOWERING LOCUS T (FT), was blasted against the Bd21 genome (modelcrop.org, v1.0 genome assembly), in order to identify, *in silico*, candidates for the correspondent functional orthologue in *Brachypodium distachyon* This analysis yielded several PEBP-like proteins with higher amino-acid similarity (highest score, smallest sum probability) which were aligned with the protein sequences of FT (Arabidopsis), Hd3a (Rice), and other cereal orthologues to confirm the presence of conserved domains (Figure 3.6). With a difference of only seven amino acids from TaVRN3, HvFT1 and LpFT, Bradi1g48830.1 stood out as the most likely candidate to be the functional orthologue of FT in Brachypodium (Figure 3.5). A supposition supported by Higgins *et al*, 2010, as BdFTL2 (Figure 3.7).

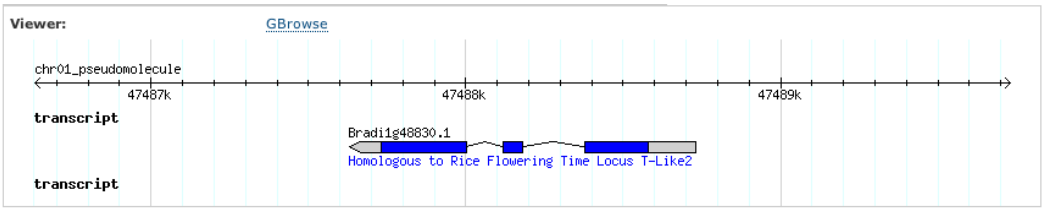


Figure 3.5: Schematic representatition of Bradi1g48830 locus (Adapted from http://mips.helmholtz-muenchen.de/plant/brachypodium/).

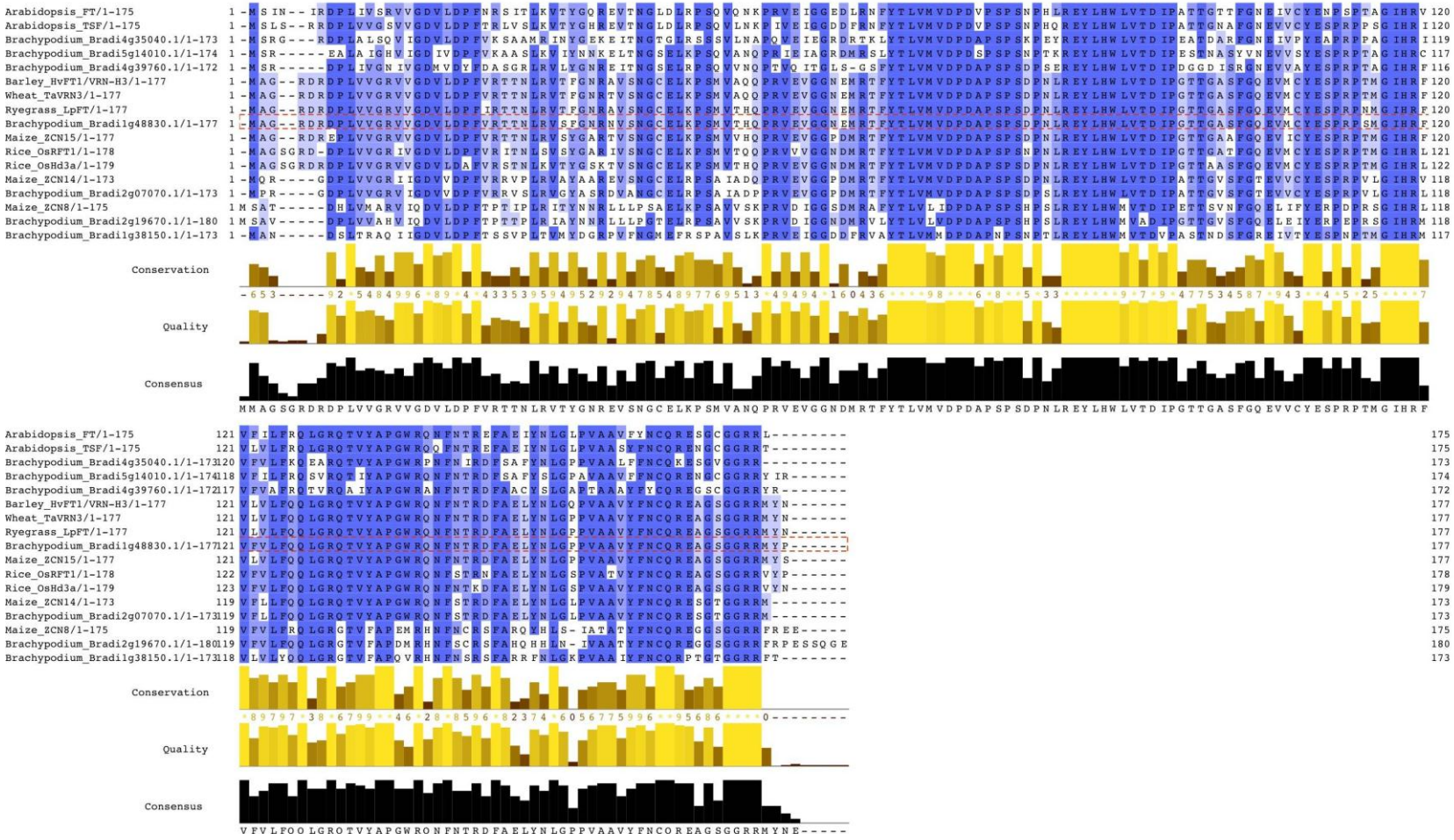


Figure 3.6: ClustalW alignment of FT and the correspondent orthologues in different cereal species. Bradi1g48830.1 identified by the dashed-red box. Blue highlights represent aminoacid residue conservation amongst the aligned proteins: highly conserved aminoacid residues in dark blue and conserved aminoacid in light blue. Dashes represent residue insertions or deletions amongst orthologue peptide sequences. Yellow bars represent higher levels of aminoacid conservation between FT orthologues, while brown bars represent regions with lower levels of aminoacid conservation between the aligned sequences. Black bars represent the conservation frequency of the aminoacid residues that make up the consensus sequence.

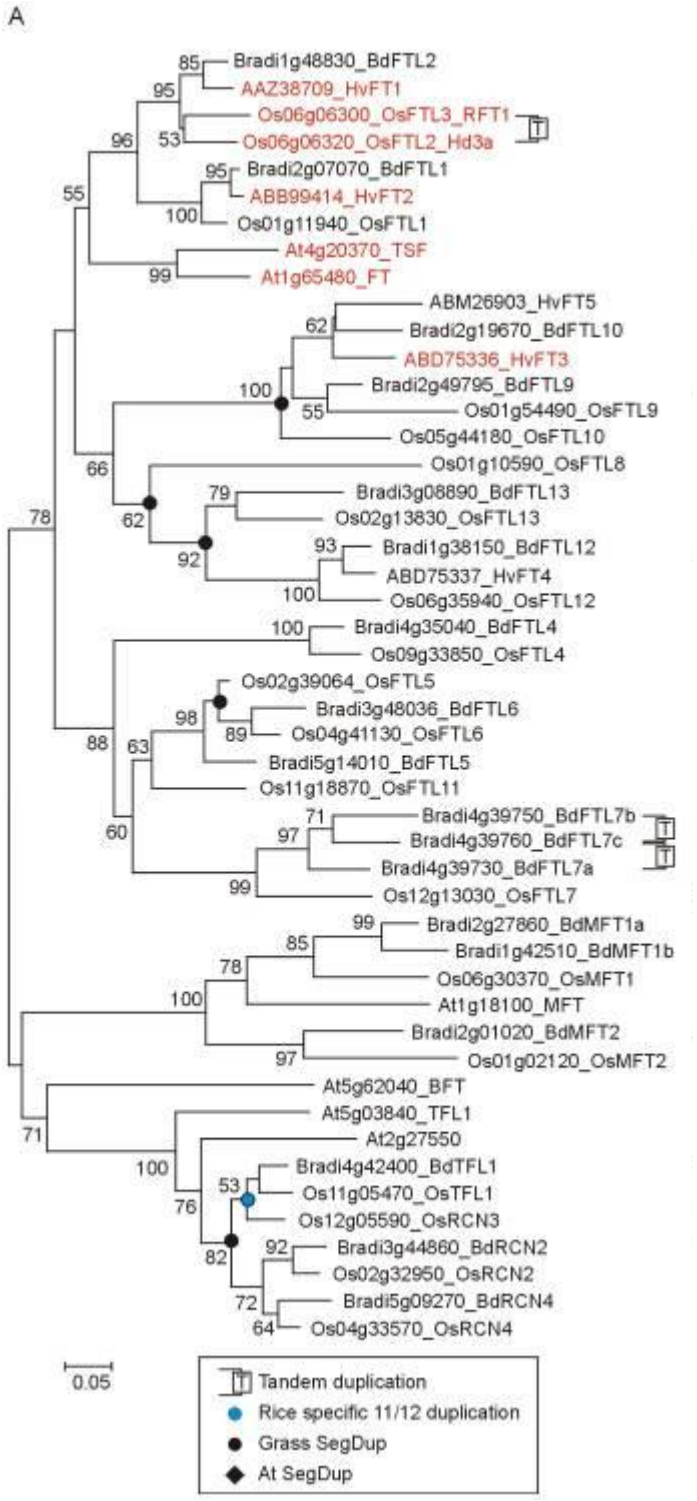


Figure 3.7: Phylogenetic relationship of FT family proteins. A) NJ tree of FLOWERING LOCUS T and related proteins (PEBP (PF01161) domain alignment) (Adapted from Higgins *et al,* 2010).

3.3.1.1 BdFTL2 expression is induced in the leaf's vasculature upon shift to LD

Although Bradi1g48830 locus (BdFTL2 thereafter, according to Higgins *et al*, 2010) was identified as the strongest candidate to be the FT orthologue in Bd21, this had to be confirmed *in vivo*. As Arabidopsis FT is known to be up-regulated in the leaves exposed to LD, travelling through the vasculature up to the shoot apical meristem to deliver the flowering signal (Zeevaart 2006, 2007; Jaeger and Wigge, 2007; Corbesier *et al*, 2007); it was necessary to determine if other BdFT-like genes would share the same expression domain. Therefore, the expression levels of all BdFT-like genes identified in this study were assessed, by qPCR analysis, in leaves of LD grown plants. This analysis clearly confirmed that BdFTL2 is the most up-regulated BdFT-like gene to be expressed in leaves of flowering Brachypodium (Figure 3.8), reinforcing the hypothesis that BdFTL2 is the true FT orthologue. This result was also confirmed through BdFTL2 transcript immuno-localization in leaves of flowering Bd21 plants (Figure 3.9), where the mRNA of BdFTL2 could be visualized around the vasculature, consistent with the hypothesis of FT being "florigen".

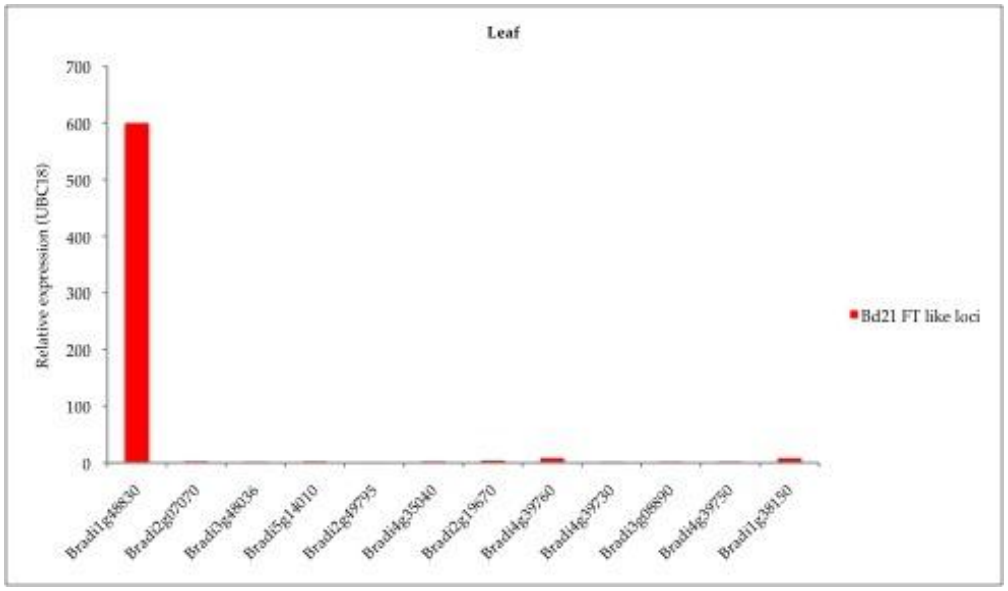


Figure 3.8: Relative expression of Bd21 FT-like transcripts in leaf samples of *Brachypodium distachyon* grown in LD. The most significantly FT-like transcript being up-regulated is Bradi1g48830, the expression levels of all the other transcrips analysed remained low. Reference gene UBC18_Bradi4g00660 (Schawrtz *et al*, 2010).

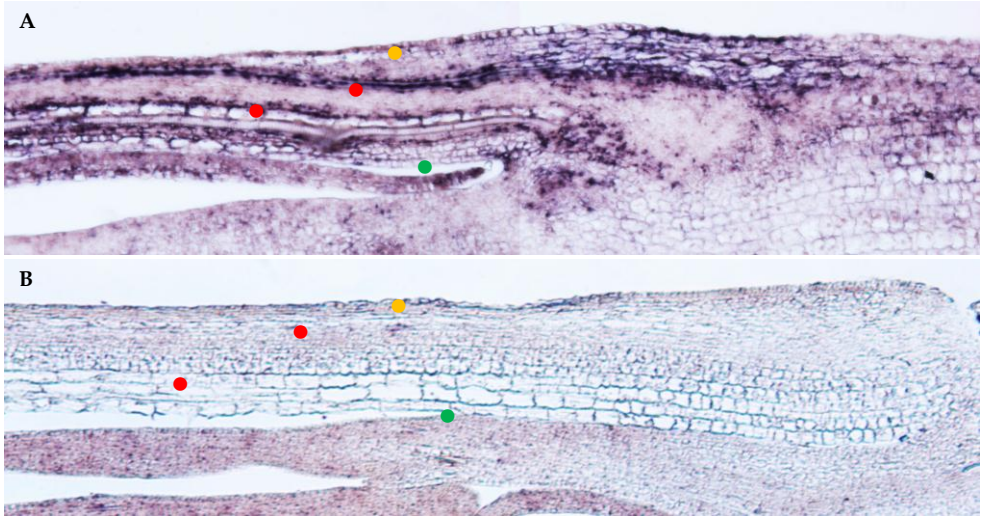


Figure 3.9: BdFTL2 full transcript immunolocalization in the vasculature of leaves of flowering Bd21 plants (longitudinal view). BdFTL2 [A], Negative control (Bradi1g59250) [B]. Orange dots indicate the abaxial epidermis, green dots indicate the adaxial epidermis, and red dots indicate the vasculature.

The daily expression dynamic of BdFTL2 was also analysed, by qPCR, in leaf samples of Bd21 plants grown in both SD and LD. Once again, the results were consistent with previously published results that demonstrated that FT is not expressed in SD, but is up-regulated upon shift into LD (Suárez-López *et al*, 2001; Turner *et al*, 2005). The BdFTL2's expression pattern displayed by flowering induced plants is characterized by a very sharp morning peak, followed by another mRNA accumulation during the night period (Figure 3.10).

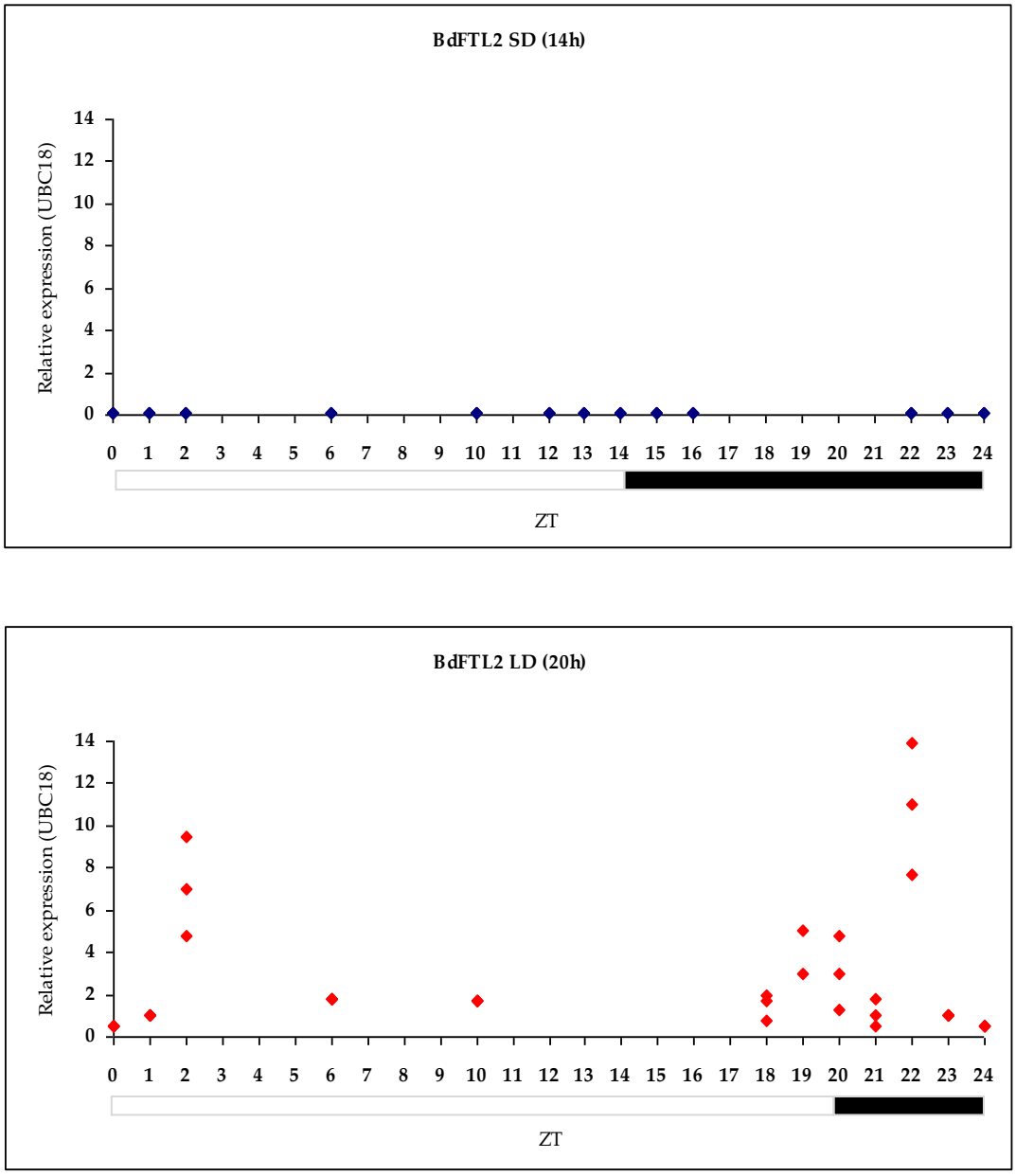


Figure 3.10: Relative expression levels of BdFTL2 in a time course series across a SD and LD (Top and Bottom, respectively), reference gene UBC18_Bradi4g00660 (Schawrtz *et al*, 2010). Data courtesy of Dr. Scott Boden, JIC.

3.3.1.2 The over-expression of BdFTL2 induces flowering in regenerating callus

BdFTL2 was a good candidate to be Brachypodium's FT orthologue, but it was still necessary to determine its ability to induce early flowering when constitutively expressed, as it does its Arabidopsis counterpart (Kardailsky *et al*, 1999). To assess this, the CDS of Bradi1g48830.1 was cloned into a constitutive expressing Gateway vector driven by the ZmUbi1 promoter, (courtesy of Dr. Ben Trevaskis, Hemming *et al*, 2008) and introduced into Bd21 according to Alves *et al*, 2009. Throughout the regeneration period, the *callus'* cells over-expressing the T-DNA entered the reproductive phase immediately without differentiating any vegetative structure, regenerating only spikes and spikelets (Figure 3.11). All the reproductive organs within the florets seemed to be in place, however, without leaves, stems, or roots they senesced without filling the endosperm. This result demonstrates that in a tissue composed exclusively by meristematic cells, BdFTL2 has the ability to suppress the formation of all vegetative organs, and induce flowering directly without further need of other *stimuli*.

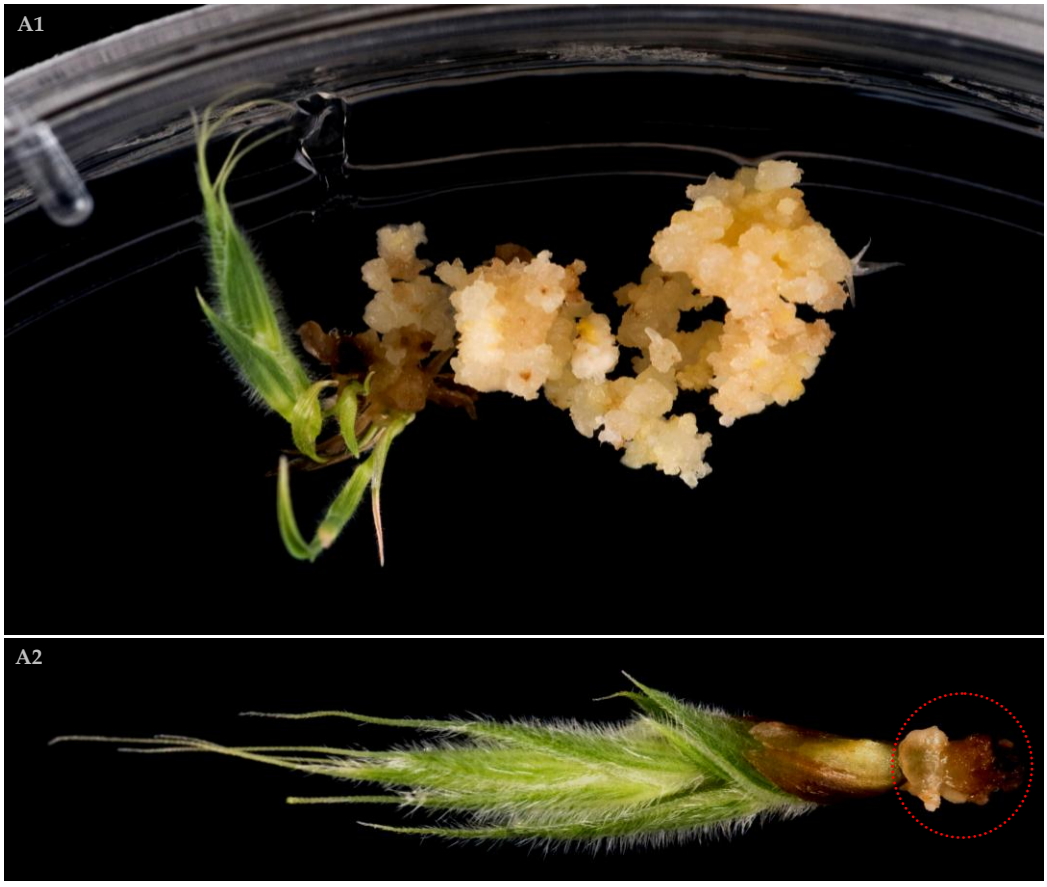


Figure 3.11: The regeneration of transgenic calli over-expressing BdFTL2 (Bradi1g48830.1) [A1, A2]. Highlighted in red is the leftover callus from where the regeneration of the spike occured.

3.3.1.3 Silencing BdFTL2 through the over-expression of a amiRNA may compromise inflorescence development

Given the current lack of characterized Bd21 mutants in the scientific community, a strategy of targeted gene silencing was devised in order to study the effects of the mis-expression of this gene in the overall developmental process of the plant and, in particular, its impact on flowering induction. As a known key element of the photoperiodic inductive pathway, it would be expected that plants with reduced levels of FT would flower later in LD, compared to wt. In order to achieve this objective, an artificial microRNA (amiRNA) was created, through the use of the amiRNA WMD3 web designer tool (Figure 3.12)(Ossowski, Joffrey, Schwab, Riester and Weigel personal communication) and a rice endogenous miRNA precursor (Warthmann *et al*, 2008). This amiRNA was, then, cloned into a constitutive expression plasmid, under the rice Actin promoter and its leading intron (Himmelbach *et al*, 2007), and introduced into Bd21 according to Alves *et al*, 2009.

Although the data gathered corresponds to the T0 generation, and it still requires further confirmation, it was observed at this point an enhanced sterility rate among the population of independent transgenic lines. In the most extreme cases, the inflorescence development was compromised by the cessation of floret differentiation potentially due to the inflorescence's precocious heading, leading to the desiccation of the meristem. The few florets developed exhibited cell collapse throughout the whole reproductive structure, including the internal organs (lodicules, carpel, and stamens) that, although distinctively observable, remained incipiently developed (Figure 3.13). This phenotype was not carried through the next generation, and no difference in flowering time was observed between the amiRNA lines tested and the control plants (data not shown); a possible explanation is the fact that only lines with very low levels of expressed amiRNA were able to produce fully matured fertile seed.

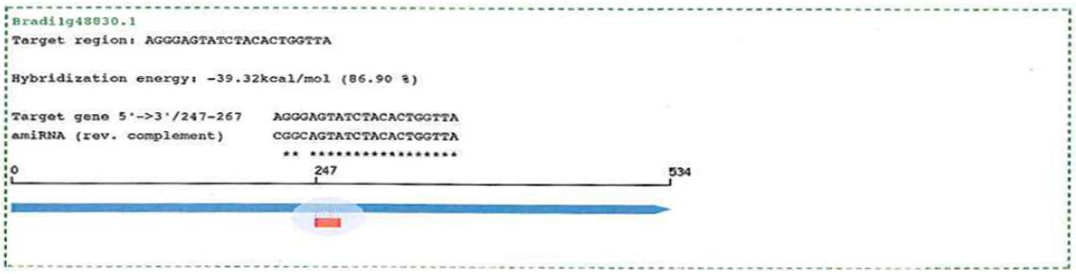


Figure 3.12: Schematic representation of the 21mer designed by the WMD3, and the correspondent target sequence in Bradi1g48830.1. Designed 21mer (red), targeted sequence (light blue) in Bradi1g48830.1's ORF of 534 bp (dark blue) (Ossowski, Joffrey, Schwab, Riester and Weigel personal communication).

Figure 3.13: Transgenic plants expressing an artificial microRNA sequence that specifically targets BdFTL2 mRNA for degradation (pOsAct1::amiRNA-BdFTL2) [A]; Spike development detail [B1, pOsAct1::amiRNA-BdFTL2 (scale bar: 0,4 cm)- B2, wt control (scale bar: 0,2 cm)]; Spikelet development detail [C1, pOsAct1::amiRNA-BdFTL2 (scale bar: 1 mm) - C2, wt control (scale bar: 2 mm)]; Dissected floret exhibiting atrophied reproductive organs (carpel and stamens) [D1, pOsAct1::amiRNA-BdFTL2 (scale bar: 0,2 mm) – D2, wt control (scale bar: 0,2 mm)]; SEM microscopy of a dissected floret: lod, lodicule; car, carpel; sta, stamen [E1 pOsAct1::amiRNA-BdFTL2 (scale bar: 100 µm) - E2 wt control (scale bar: 500 µm)].

3.3.2 CONSTANS orthologue, BdCO1, is not decisive for the photoperiodic induction of flowering in Bd21

3.3.2.1 BdCO1 is the phylogenetic orthologue of Arabidopsis CONTANS

The protein sequence of the *Arabidopsis thaliana* CONSTANS (CO) was blasted against the Bd21 genome (http://www.modelcrop.org, v1.0 genome assembly), and the results with higher amino-acid similarity (highest score, smallest sum probability) were used to identify, *in silico*, candidates for the correspondent functional orthologue in *Brachypodium distachyon*. Through a protein similarity analysis Bradi1g43670.1 was identified as the most likely candidate to be the functional orthologue of CO in Brachypodium (Figure 3.15). The same locus of Bradi1g43670.1 was also identified by Higgins and colleagues in 2010, through a more comprehensive study, which included the physical maps of the BdCO1 and BdCO2 gene regions (Figure 3.16). The same locus is identified in the MIPS genome browser as homologous to HvCO1-like (Figure 3.14).

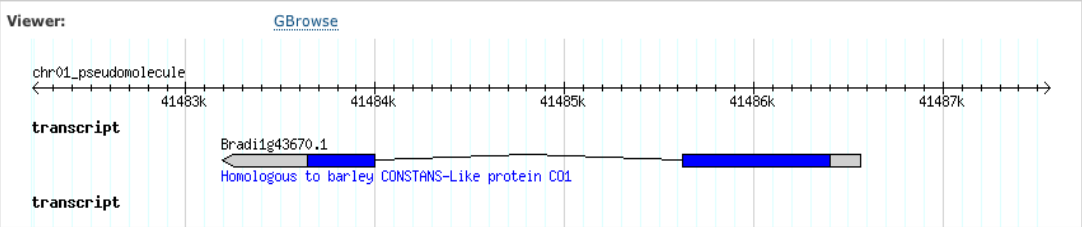


Figure 3.14: Schematic representation of Bradi1g43670 locus (adapted from http://mips.helmholtz-muenchen.de/)

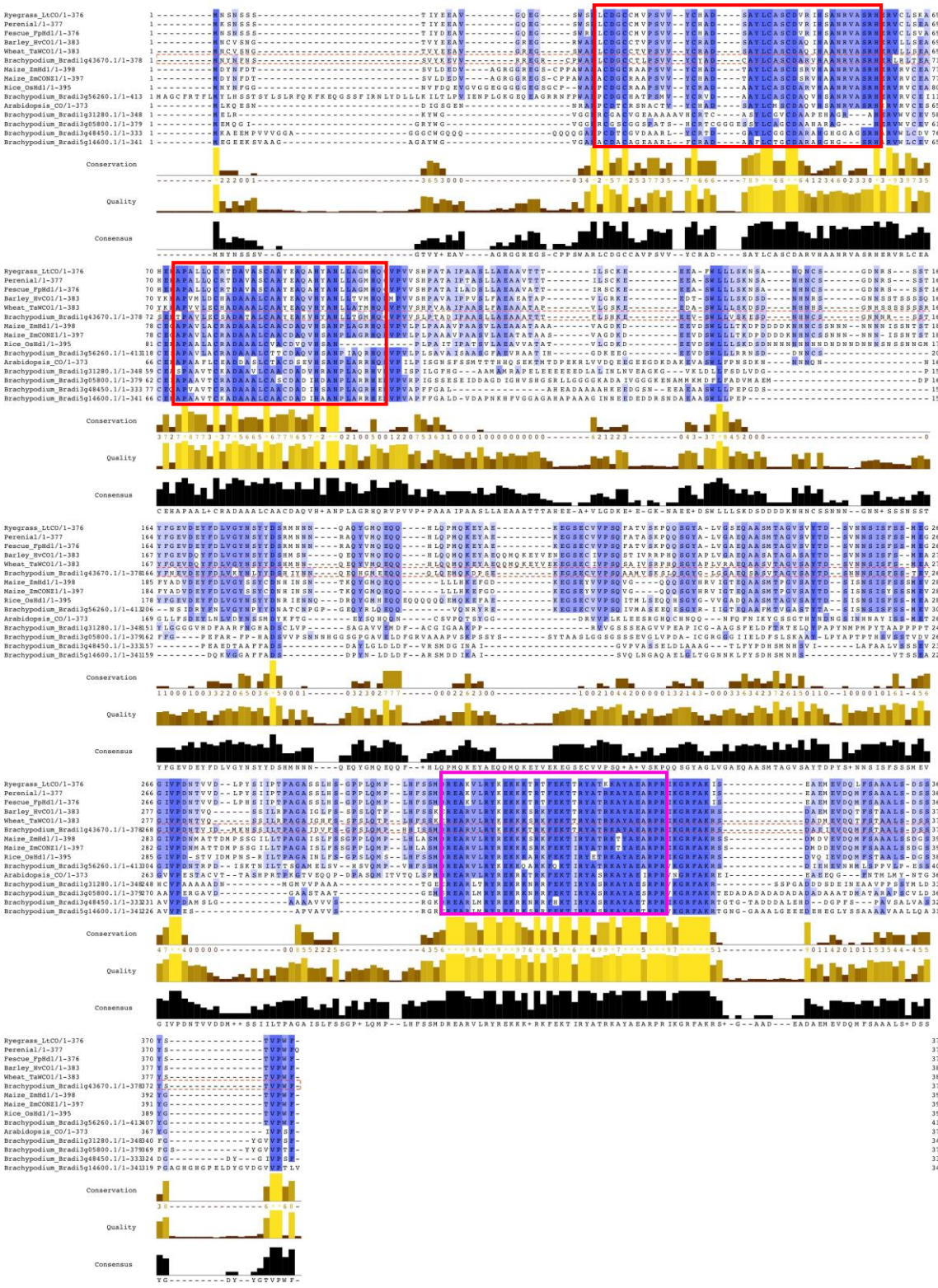


Figure 3.15: ClustalW alignment of CO and the correspondent orthologues in different cereal species. Bradi1g43670.1 identified by the dashed-red box Red: B-Box; Pink: CCT domain (Robson *et al*, 2001). Blue highlights represent aminoacid residue conservation amongst the aligned proteins: highly conserved aminoacid residues in dark blue and conserved aminoacid in light blue. Dashes represent residue insertions or deletions amongst orthologue peptide sequences. Yellow bars represent higher levels of aminoacid conservation between CO orthologues, while brown bars represent regions with lower levels of aminoacid conservation between the aligned sequences. Black bars represent the conservation frequency of the aminoacid residues that make up the consensus sequence.

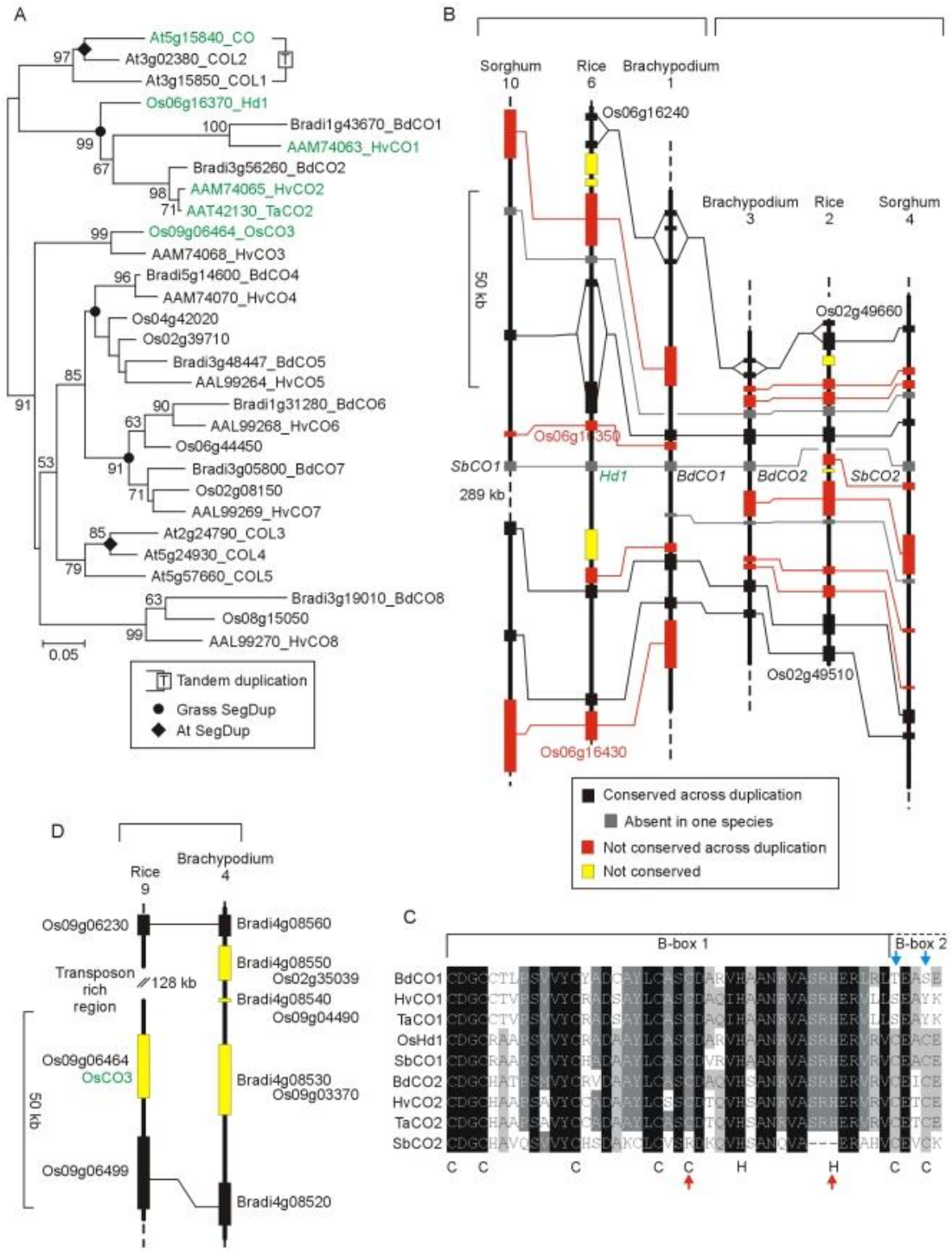


Figure 3.16: Phylogenetic relationship of CO proteins and physical maps of the BdCO1 and BdCO2 gene regions (Adapted from Higgins *et al* 2010)**.** NJ tree of CONSTANS and related proteins (whole protein alignment). B) Structure of the segmental duplication containing the CO1 and CO2 genes of the grasses. C) Amino-acid sequences of B-box1 and the start of B-box2 from rice, barley, Brachypodium and sorghum proteins. Blue arrows – conserved C residues not present in B-box 2 of the temperate species. Red arrows – conserved C and H residues missing from B-box 1 of the sorghum CO2 protein. Conserved amino acids defining B-boxes are shown below. D) Colinear segments of rice chromosome 9 and Brachypodium chromosome 4 showing the absence of the CO3 gene from Brachypodium.

3.3.2.2 The over-expression of BdCO1 delays flowering in Bd21 in LD, but promotes flowering in SD

According to previously published studies, the constitutive expression of CO induces flowering by up-regulating the expression of FT (Suárez-López *et al*, 2001). In order to assess if BdCO1 was able to anticipate flowering time by inducing BdFTL2 expression, the CDS of Bradi1g43670.1 was cloned, via Gateway technology, downstream of the ZmUbi1 promoter (courtesy of Dr. Ben Trevaskis, Hemming *et al*, 2008), and introduced into Bd21 according to Alves *et al*, 2009. Ten random independent transgenic lines were selected for analysis. The flowering time results for both SD and LD conditions are summarised in the Figure 3.18, as the average of five plants per line and per treatment. The expression levels of BdCO1, BdFTL2, and Hygromycin were also measured by qPCR for each and every independent line (3 biological replicates and 2 technical replicates) confirming the presence of the T-DNA and characterizing the lines according to the transgenes' expression levels (Figure 3.19). Although all transgenic lines showed very high levels of BdCO1 and flowered in SD this was not correlated with increased levels of BdFTL2 mRNA (Figure 3.19). In fact, the over-expression of BdCO1 in Brachypodium resulted in a consistent late flowering phenotype in LD (Figure 3.17), in spite of the assessed high levels of mRNA (Figure 3.19) present in the transgenic plants.

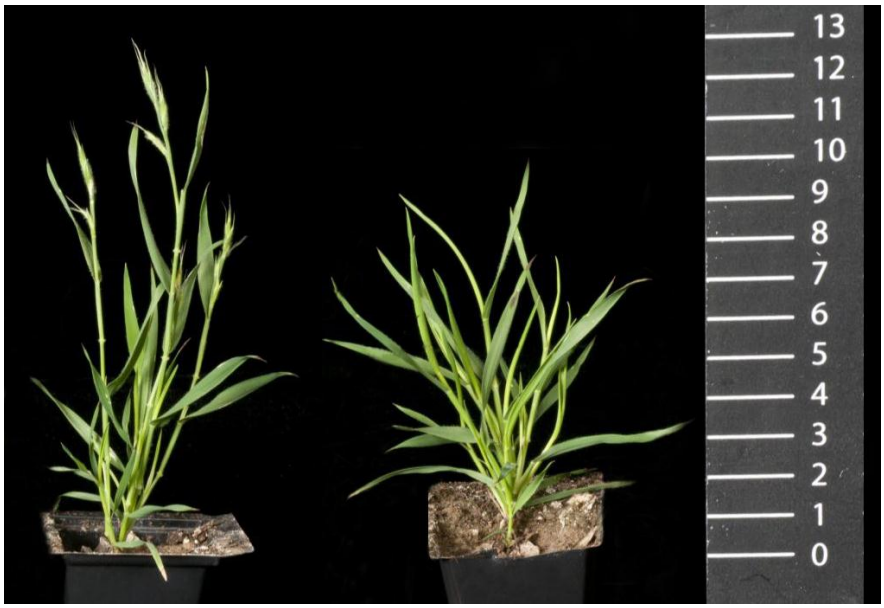


Figure 3.17: Flowering phenotype of BdCO1 over-expressing transgenic grown in LD.
Left, Bd21 wild type; Right, pUBI::BdCO1

This particular observation is not consistent with the published behaviour of CO over-expressing transgenic lines in Arabidopsis (Putterill *et al*, 1995; Suárez-López *et al*, 2001), and yet the contradictory Hd1 of rice is known to delay flowering when over-expressed under inductive conditions - coupled with the suppression of Hd3a expression - (Ishikawa *et al*, 2011), while being a fundamental floral inducer in SD, and repressor in LD via up/down-regulation of Hd3a (Ishikawa *et al*, 2005).

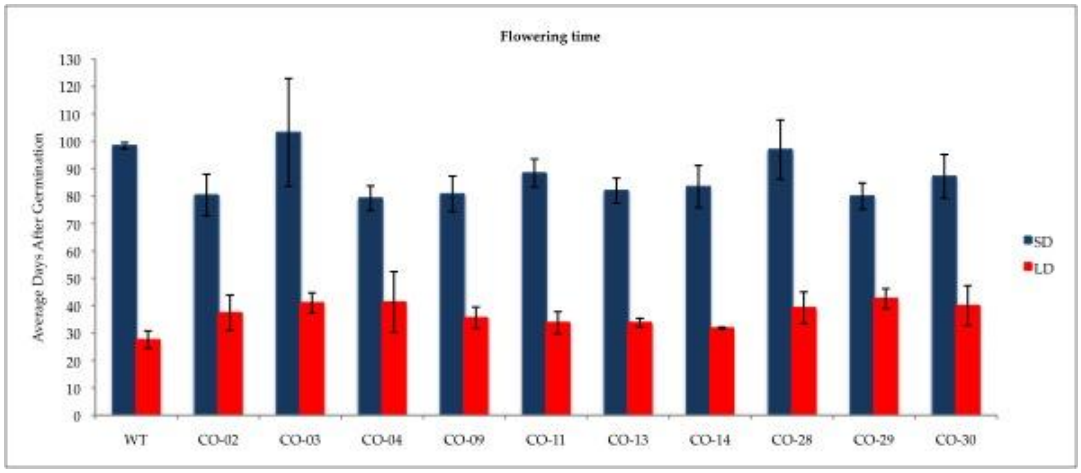


Figure 3.18: Flowering time of independent transgenic lines of Brachypodium over-expressing tagged BdCO1 (Bradi1g43670.1) protein.

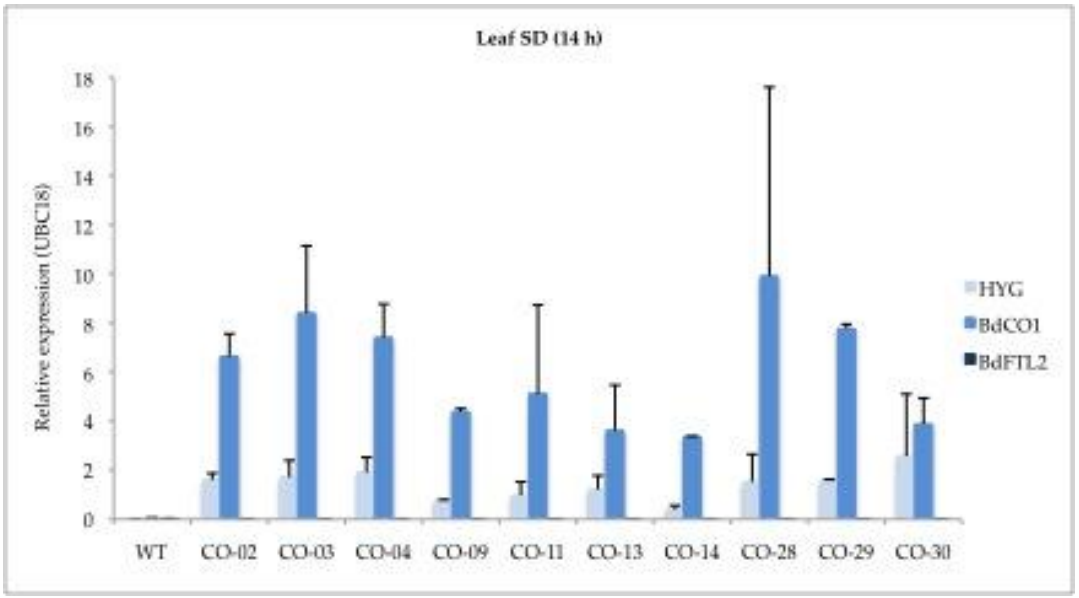


Figure 3.19: Relative expression levels of BdCO1 (Bradi1g43670.1), BdFTL2 (Bradi1g48830.1), and HYG (hygromycin resistance) in leaf samples of Brachypodium independent transgenic lines over-expressing BdCO1 (Bradi1g43670.1), grown in SD. Reference gene UBC18_Bradi4g00660 (Schawrtz *et al*, 2010).

3.3.2.3 Constitutively expressed BdCO1 is regulated at the protein level across the day, independently of the light conditions

Since the transgenic plants constitutively expressing BdCO1 were able to flower in SD but no FT mRNA levels were detected, it was important to first of all to correlate this phenotype with the presence of high levels of exogenous BdCO1 protein. In order confirm the presence of the transgenic protein in leaves of transformed Bd21, a western blot was performed by harvesting leaf samples in a daily time series that spanned light and dark periods of a SD. The immuno-detection was performed by using a commercial HRP-conjugated antibody against the 3xFlag tag. The results, summarized in Figure 3.20 revealed a few interesting points. The first is that, in spite of this being a constitutively highly expressed protein, there was, still, a very strong and efficient regulation happening at the leaf level, in terms of protein stability across the whole 24 h period of analysis. It has been reported previously that CO is stabilized by light, and destabilized in darkness (Jang *et al*, 2008), a feature that accounts for the coincidence model, of CO mediating the photoperiodic response in increasing LD (spring), because it's the only time of the year when the high levels of translated protein coincide with an extended light period that prevents the protein from being degraded. However, in the protein assay performed on BdCO1 it was clear that light itself is not sufficient to stabilize this protein, given the sharp protein decay between ZT=1 and ZT=2. In addition, high levels of protein were detected during the night period at time point ZT=22, and especially at ZT=23, indicating that the current model of CO being unstable in the dark (Jang *et al*, 2008) cannot be applied to BdCO1. Ishikawa *et al*, 2011, also, have reported that Hd1, rice CO orthologue, is equally detectable in leaf samples collected during the light and dark periods of SD grown 35S::Hd1-10xmyc over-expressing rice lines, ruling out the possibility that the late flowering phenotype is a direct consequence of Hd1 light-mediated degradation. Consistent with previous accounts is the fact that the protein can be detected during the final hours of daylight, only to be degraded in the first hours of darkness. Although not all 24 time points were employed in this analysis, it seemed that the protein levels oscillate during the day/night cycle and that its stability may be regulated by the circadian clock. At this time, since CO is thought to be itself an output of the circadian clock, it was necessary to determine the endogenous pattern of expression of BdCO1 mRNA and compare if the degradation of the protein has parallel with the regulation of the mRNA transcript. The assessment was performed by a qPCR run on leaf samples from plants grown in the same light conditions, and harvested at the exact time points used for the protein immuno-detection assay and, in fact, there is a resemblance between the control of transcription (Figure 3.21) and the protein abundance (Figure 3.20). This would imply that, overall, BdCO1 is under a very tight control during the day/night cycle, and that the protein levels are required to drop at particular times of the day (irrespectively of light conditions) to a point below immunological detection, which is accomplished by degrading the protein, and reducing transcription.

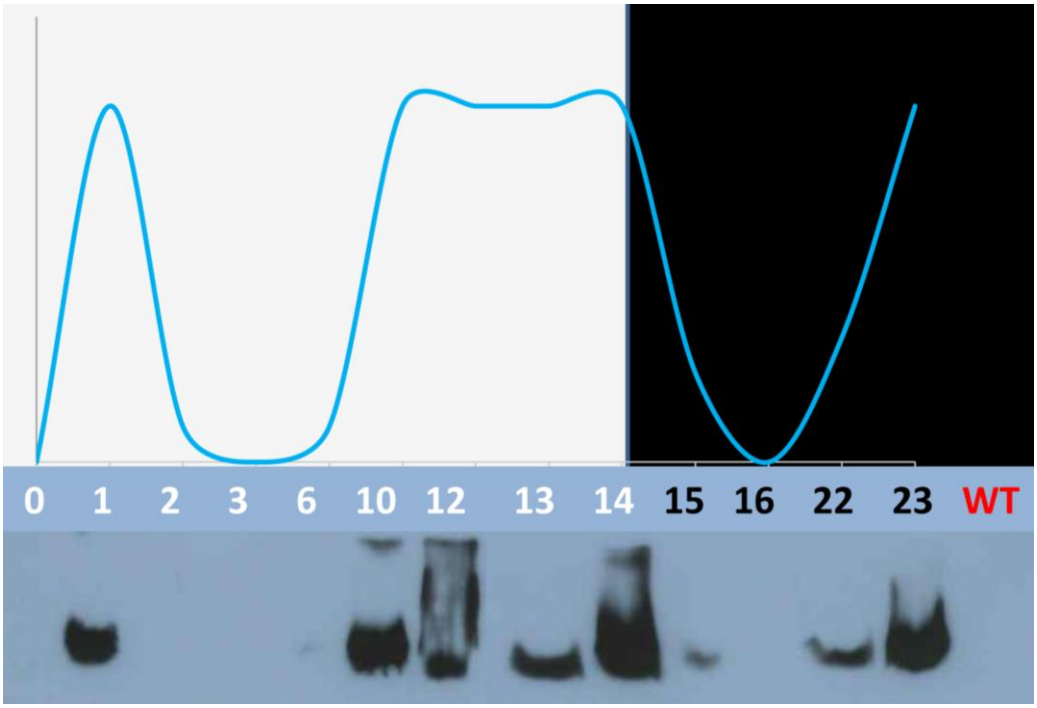


Figure 3.20: Western blot of pUBI:6xHis-CO-3xflag in a time-course series (SD), ZT=0 to ZT=23, WT – wild type negative control. Antibody against flag-tag.

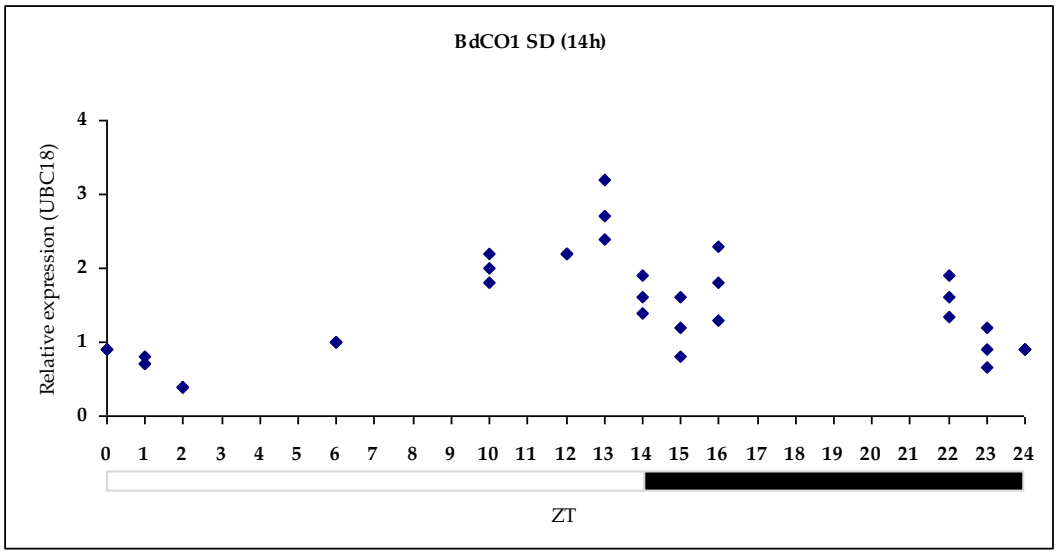


Figure 3.21: Relative expression levels of BdCO1 (wt) in a time course series across a SD, reference gene UBC18_Bradi4g00660 (Schawrtz *et al*, 2010). Data courtesy of Dr. Scott Boden, JIC.

The confirmation that the transgene has been actively transcribed and translated into a protein combined with a late flowering phenotype displayed by the over-expressing lines grown in LD, contradict available data in Arabidopsis and barley (Putterill *et al*, 1995; Suárez-López *et al*, 2001; Campoli *et al*, 2012); and yet it is supported by the report from Ishikawa *et al*, 2011, where the over-expression of Hd1 in rice is coupled with the suppression of Hd3a expression and flowering delay under inductive conditions. This either implies that BdCO1 is not the functional orthologue of CO in Brachypodium, and therefore it does not mediate the photoperiodic induction of flowering, as hypothesised; the double tag on the protein imposes an artificial dominant-negative effect over the endogenous gene that masquerades its true behaviour; or in spite of being the functional orthologue, BdCO1 role in the flowering transition of Brachypodium is more closely related to the one of Hd1. In order to investigate these possibilities, three separate experiments were designed and carried out in parallel: (1) the transformation of Brachypodium with the constitutively expressed native version of BdCO1, (2) the creation BdCO1 single tagged over-expression transgenic lines in Bd21, and (3) the confirmation of the functionality of the tagged protein through the complementation of a *co* mutant. Unfortunately, the cloning of the native BdCO1 CDS proved to be unsuccessful after many attempts and, therefore, the transformation of Brachypodium was not performed. The cloning of the CDS of Bradi1g43670.1, C-terminally tagged with a 3x flag tag, via Gateway recombination, into the pIPKb003 over-expression vector, driven by the OsAct1 promoter (Himmelbach *et al*, 2007), was performed and introduced into Bd21 according to Alves *et al*, 2009. But the flowering time assessment of the constitutively expressing BdCO1-3xflag lines was not completed due to time restrictions. Concerning the functionality of the tagged protein, ideally, this should have been tested by complementing a Bd21 *co1* mutant and analysing the resulting phenotype; however, given the current lack of characterized Brachypodium mutants, this experiment had to be performed in *Arabidopsis thaliana* through the transformation of the *co-9* mutant.

3.3.2.4 Constitutive expression of C-terminally tagged BdCO1 partially rescues Arabidopsis *co-9* mutant's late flowering phenotype

The C-terminally 3xFlag tagged CDS of BdCO1 (Bradi1g43670 locus) used in the transformation of Brachypodium *calli* was transferred into a modified pJHA212K (Yoo *et al* 2005) Gateway compatible plasmid, under the 35S promoter. The T-DNA from this construct was introduced into Arabidopsis wild type, ecotype Columbia-0, and *co-9* mutant through the floral dip method developed by Clough and Bent in 1998. Both transgenic and control seeds were germinated at the same time in MS-kanamycin and MS, respectively, and grown at 22 °C under 16 h of light (LD). The flowering phenotypes of 35S::BdCO1-3xFlag in both backgrounds is summarized in Figure 3.22. The over-expression of BdCO1-3xFlag tagged in the wt background slightly delayed flowering (days after germination) compared with Col-0 (Figure 3.22), across all the 15 independent lines analysed. On the other hand, the over-expression of BdCO1-3xFlag in the *co-9* background rescued the extremely late flowering phenotype, characteristic of *co-9* mutant, for 10 out of 15 independent lines analysed (Figure 3.22). This, however, was still later than Col-0 and the 35S::BdCO1-3xFlag (Col-0), which indicates that although the protein is functional it can only perform a partial complementation of the mutation (Figure 3.22).

Figure 3.22: *Arabidopsis thaliana* transgenics (WT and *co* mutant) constitutively expressing BdCO1, grown in LD. Left, Columbia-0 wild type; Center-Left, 35S:BdCO1 in Columbia-0 wt background; Center-Right, *constans co-9* mutant; Right, 35S:BdCO1 in *constans co-9* mutant background.

With the confirmation that the protein is able to accelerate flowering in the *co-9* background, and cause later flowering phenotype on Col-0 in LD, it became clear that perhaps the tags do not necessarily interfere with the protein function, and that the phenotype of the over-expressing Bd21 lines reflect its functional role, in Brachypodium. A fact that is maybe correlated with the findings reported by Nemoto *et al*, 2003, in which the wheat orthologue of Hd1 (TaHd1) was able to complement rice *hd1* mutant, by restoring the early flowering phenotype under SD, and delaying flowering in LD. In addition, Shaw, Turner, and Laurie (poster communication), have observed that early flowering cultivars of wheat carrying the photoperiod insensitive allele Ppd1-D (PRR7) display increased levels of FT mRNA in the morning, coincident with Ppd1-D morning peak of expression, when compared with their counterpart late flowering cultivar, Paragon. Also, TaCO1 activity in these Ppd1-D cultivars doesn't seem to be positively correlated with their early flowering phenotype; in fact the expression pattern of TaCO1 is antagonist to the one of Ppd1-D and FT, being very low in the morning and increasing towards the end of the day. Paragon's later flowering phenotype, on the other hand, was characterized by lower levels of FT (and Ppd1-D) mRNA, and higher levels of TaCO1 (Shaw *et al*, poster communication).

3.3.3 BdPIF-like proteins mediate the photoperiodic induction of flowering

Since BdCO1 is not likely to mediate the photoperiodic induction of flowering, as it has been described for other systems, it's possible that in Brachypodium other genetic element is overriding this process. This was already acknowledge by Ishikawa *et al*, 2011, when they described a correlation between the low levels of Hd3a in the Hd1 over-expressing lines with active PHYTOCHROME B. At this point attention was turned to a group of proteins from the bHLH group called PHYTOCHROME INTERACTING FACTORs (PIFs), that are known to be involved in the perception of light as mediators of the "shade avoidance syndrome" in Arabidopsis, and in the CO-independent induction of flowering, via PIF4, a direct inducer of FT (Kumar and Lucyshyn *et al*, 2012).

3.3.3.1 The over-expression of BdPIF1 and BdPIF7 induces flowering in both SD and LD

In order to determine if a Bd21 orthologue of PIF4 could mediate light perception leading to the induction of flowering, a protein BLAST search was performed, using PIF4 protein as query. bHLH proteins display a very low level of aminoacid conservation outside the APB and bHLH domains, which makes it very difficult to pinpoint a trustworthy potential candidate (Dr Monika Kavanová personal communication). A phylogenetic analysis was performed using the sequences of the conserved domains, in an attempt to narrow the search for a suitable candidate (Figure 3.23).

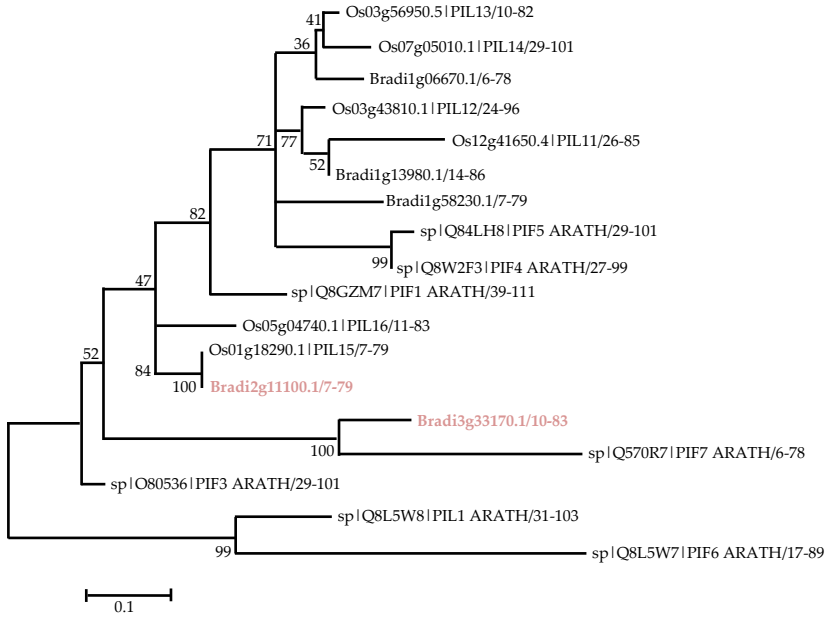


Figure 3.23: Phylogenetic tree of the bHLH family of proteins. The aminoacid sequence of the APB and bHLH domains were used to calculate the phylogeny using Maximum Likelihood Method. Bootstrap support values from 1000 bootstrap replicates are shown for each node as percentages. Highlighted in pink are Bradi2g11100.1 (BdPIF1) and Bradi3g33170.1 (BdPIF7). (Data courtesy of Dr. Monika Kavanová, JIC)

From this analysis five Bd21 loci were cloned into a constitutive expressing Gateway vector driven by the ZmUbi1 promoter, (courtesy of Dr. Ben Trevaskis, Hemming *et al*, 2008). Bd21 transgenics were obtained through the Agrobacterium-mediated transformation method of Alves *et al*, 2009. As a result of the ectopic expression of the different candidates, two the BdPIF-like genetic sequences, highlighted in pink in Figure 3.23 (Figure 3.24), did display early flowering in both LD and SD. The loci Bradi2g11100.1 (BdPIF1) and Bradi3g33170.1 (BdPIF7) flowered 1 week earlier than Bd21 wt, in LD (quantification data not shown); while in SD, Bradi2g11100.1 flowered 5 weeks after germination (Figure 3.25, Figure 3.26), and Bradi3g33170.1 flowered 8 weeks after germination, while wt plants never flowered (Figure 3.25)(Data courtesy of Dr Monika Kavanová).

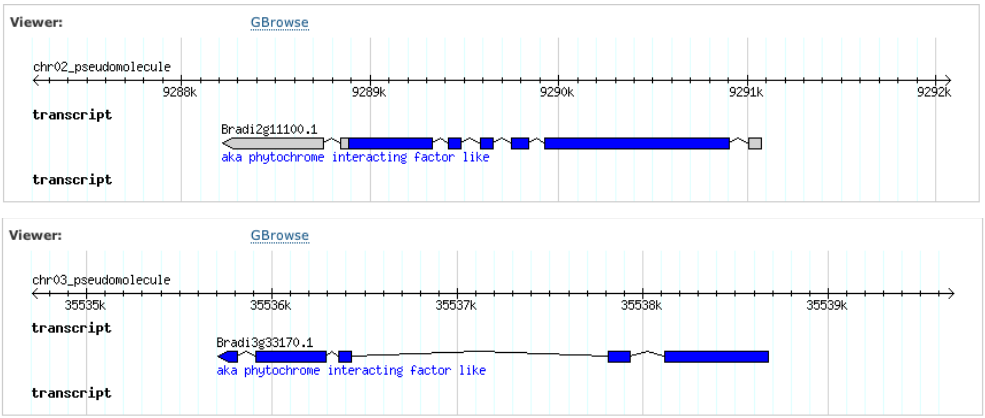


Figure 3.24: Schematic representation of Bradi2g11100 and Bradi3g33170 loci. (adapted from http://mips.helmholtz-muenchen.de/)

As shown in Figure 3.26, the over-expression of BdPIF1 is not only able to induce flowering under non-inductive conditions, by-passing the need for long photoperiod; but in fact the overall architecture of the transgenic plant resembles that of a Bd21 WT plant flowering in LD. In other words, pUBI::BdPIF1 in SD restores the wt flowering time phenotype characteristic of LD grown wt.

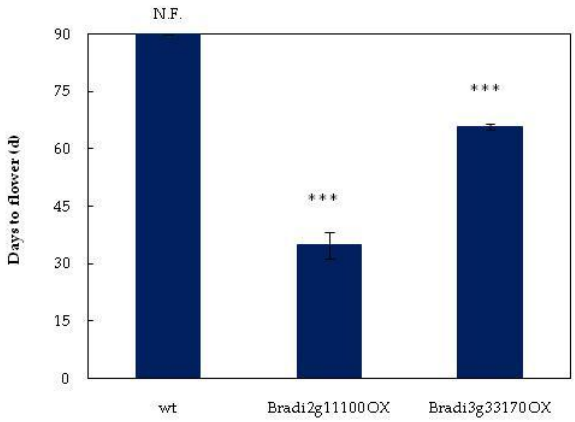


Figure 3.25: Over-expression of BdPIF1 and BdPIF7 accelerates flowering transition under non-inductive photoperiods. Brachypodium plants were grown in SD (14h light/10h dark), 22°C. Wt plants did not flower in these conditions for at least 3 months. Average of 3-4 independent lines The statistical significance was assessed by a t-test. ***, P<0.001 (Data courtesy of Dr Monika Kavanová, JIC).

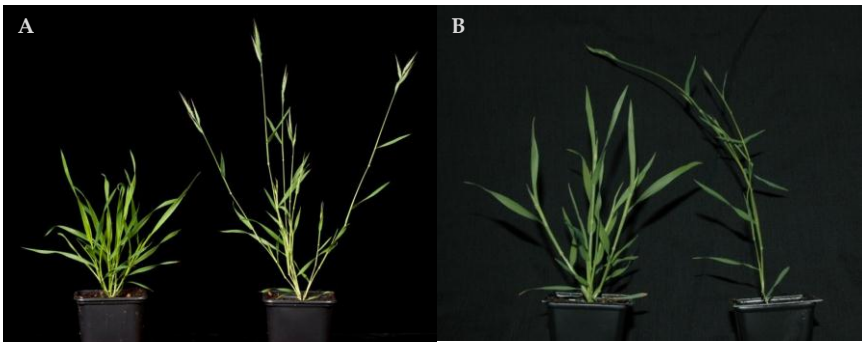


Figure 3.26: Overexpression of PIF1 (Bradi2g11100.1) accelerates flowering both in SD and LD conditions. A) SD (14h light/10h dark), 22°C; B) LD (20h light/4h dark), 22°C. BdPIF1ox (right), and Bd21 wild type (left)(Data courtesy of Dr Monika Kavanová, JIC).

3.3.3.2 BdPIFs' activity may be regulated by BdDELLA protein via bioactive GA

Kumar and Lucyshyn in 2012 correlated the delay in flowering of Arabidopsis at lower temperatures with the DELLA-mediated repression of PIF4 activity, demonstrating that the quintuple *della* mutant, flowers early when compared to wt, and that applications of GA, in a GA deficient background, lead to increased FT expression levels. Other reports in Arabidopsis, had also documented that flowering induction upon exposure to LD is accompanied by the inactive-PHYB up-regulation of gibberellin biosynthesis (Hisamatsu *et al*, 2005; Hisamatsu and King, 2008; King and Hisamatsu *et al*, 2008). This direct relation between light perception, gibberellin bio-synthesis, GA-mediated DELLA degradation, PIF4 stabilization, and FT up-regulation leading to floral transition is very plausible in the context of flowering regulation in cereals; particularly after the published studies of King *et al*, 2006 on the LD grass *Lolium temulentum*. In this publication, the authors reported that GA (mainly GA_5 and GA_6) is highly florigenic without causing excessive stem elongation (Evans *et al*, 1990; King *et al*, 2003), suggesting even that GA has a predominant role in the induction of flowering, parallel to LtFT. However, direct regulation of FT expression through GA signaling is not implausible, in fact, King and Hisamatsu *et al*, 2008 pointed out that Arabidopsis FT promoter contains three GA response elements and a pyrimidine box that could account for GA-mediated regulation of FT. In order to test if GA would be florigenic in Brachypodium, through the stabilization of PIF proteins, by targeting the degradation of Bd21's only GA INSENSITIVE (GAI-DELLA) protein, several experiments were set up. The first was the confirmation that Bd21 does have a functional BdGAI protein - identified in a BLAST search using, as query, Arabidopsis GAI protein sequence (Figure 3.27, Figure 3.28). This was achieved by over-expressing the native genomic sequence of Bradi1g11090 (Figure 3.27) under the maize ubiquitin promoter and leading intron, plus a stable BdGAI version where the DELLA domain had been removed. By removing the DELLA domain it was expected that the GAI protein would become insensitive to the GA levels inside the plant, and so it would display a stable dwarf phenotype, consistent with reduced height mutants from wheat (Peng *et al*, 1999). This was, in fact, confirmed as the native BdGAI still retains susceptibility to regulation by endogenous GA levels, with the constitutive expresser exhibiting a normal plant architecture despite the ectopic expression, and the stable BdGAI version with a deleted DELLA domain being completely dwarfed with defective internode elongation (Figure 3.29). The flowering time of these lines appeared to be delayed when compared to wt plants, as one would expect, however, due to the lack of internode elongation the determination of the date of head emergence wasn't considered the most accurate way of ascertain the flowering time phenotype of these lines. A more precise way to determine this would be the dissection of the SAMs under a binocular microscope and the comparison of their morphology for both the control and the transgenic plants. Due to time restrictions it was not possible to perform this assessment and include it in this chapter, but it would be pertinent to carry out this measurement later on as a continuation of this study.

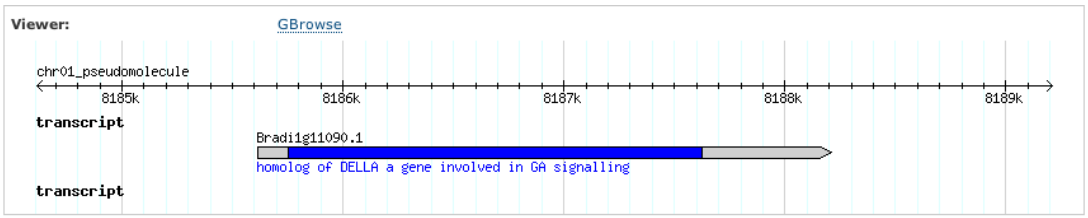


Figure 3.27: Schematic representation of Bradi1g11090 locus (adapted from http://mips.helmholtz-muenchen.de/)

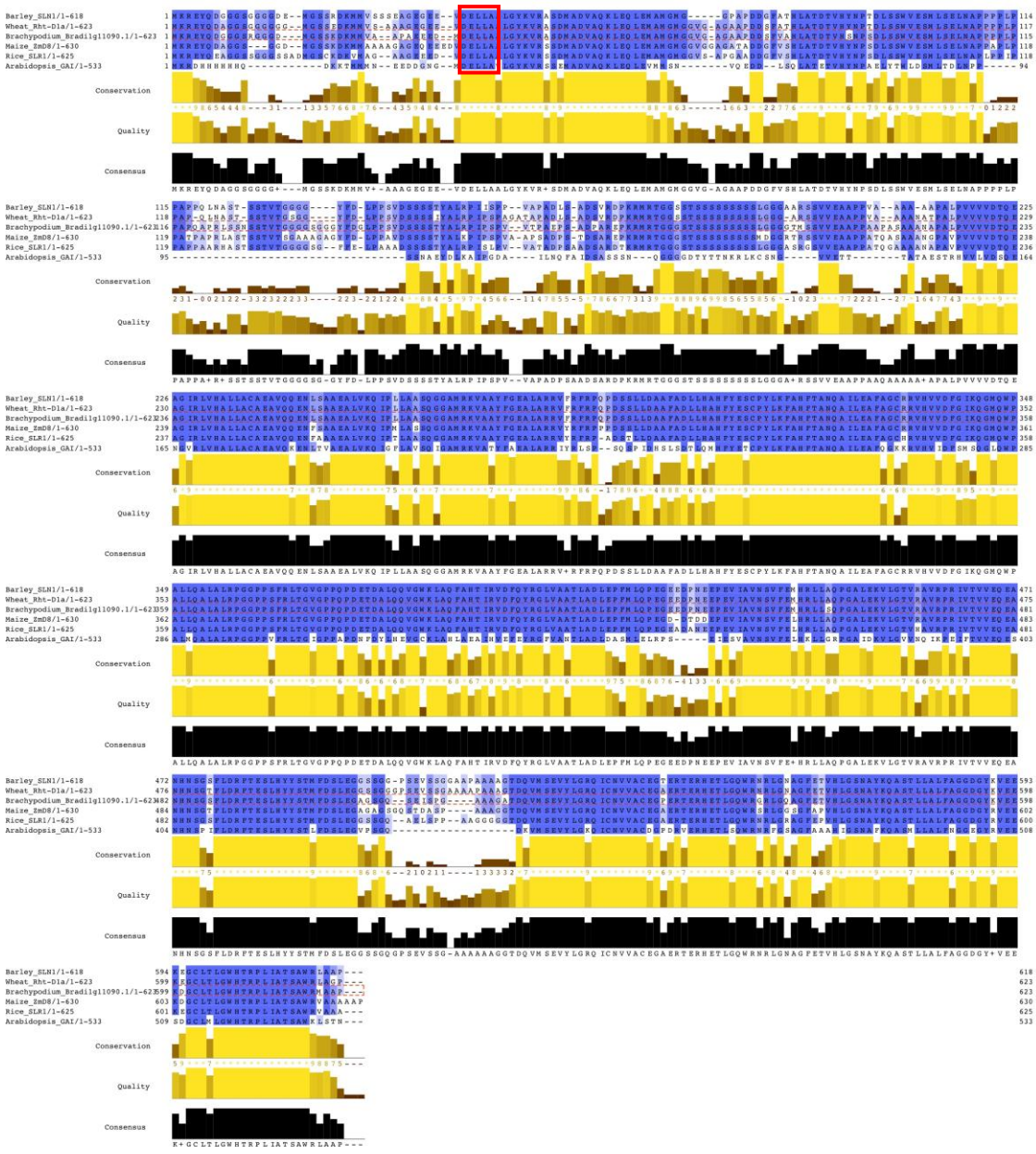


Figure 3.28: ClustalW alignment of BdGAI and the correspondent orthologues in different plant species. Bradi1g11090.1 identified by the dashed-red box. Highlighted in red are the conserved amino acids characteristic of the DELLA domain, essential for gibberellin signalling (Peng *et al*, 1997; Gubler *et al*, 2002; Itoh *et al*, 2002). Blue highlights represent aminoacid residue conservation amongst the aligned proteins: highly conserved aminoacid residues in dark blue and conserved aminoacid in light blue. Dashes represent residue insertions or deletions amongst orthologue peptide sequences. Yellow bars represent higher levels of aminoacid conservation between GAI orthologues, while brown bars represent regions with lower levels of aminoacid conservation between the aligned sequences. Black bars represent the conservation frequency of the aminoacid residues that make up the consensus sequence.

Following the generation of the transgenic lines, a qPCR analysis was performed to determine the BdFTL2 levels of the pUBI::ΔDELLA-GAI plants grown in LD compared to wt. The leaf samples were harvested from synchronized and florally induced plants grown for 7 LD of 20 h, at 22 °C, at ZT=2 – coincident with BdFTL2 peak of expression in LD of Figure 3.10 (the analysis included two technical and three biological replicates). It was anticipated that a stable BdGAI protein would sequester BdPIF proteins, preventing them to induce flowering via the up-regulation of BdFTL2. This prediction was confirmed, as the results in Figure 3.30 demonstrate the levels of BdFTL2 were significantly lower in the

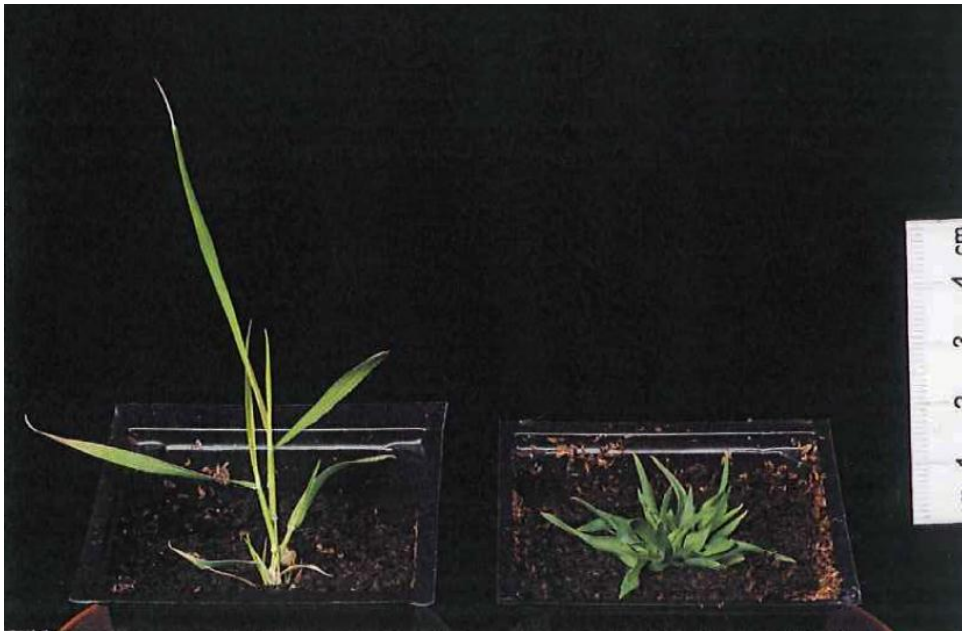


Figure 3.29: Bd21 transgenic plants constitutively expressing BdGAI (Bradi1g11090): Native protein - pUBI::GAI [left]; protein without the DELLA domain - pUBI::ΔDELLA_GAI [right]. Data courtesy of Dr Monika Kavanová.

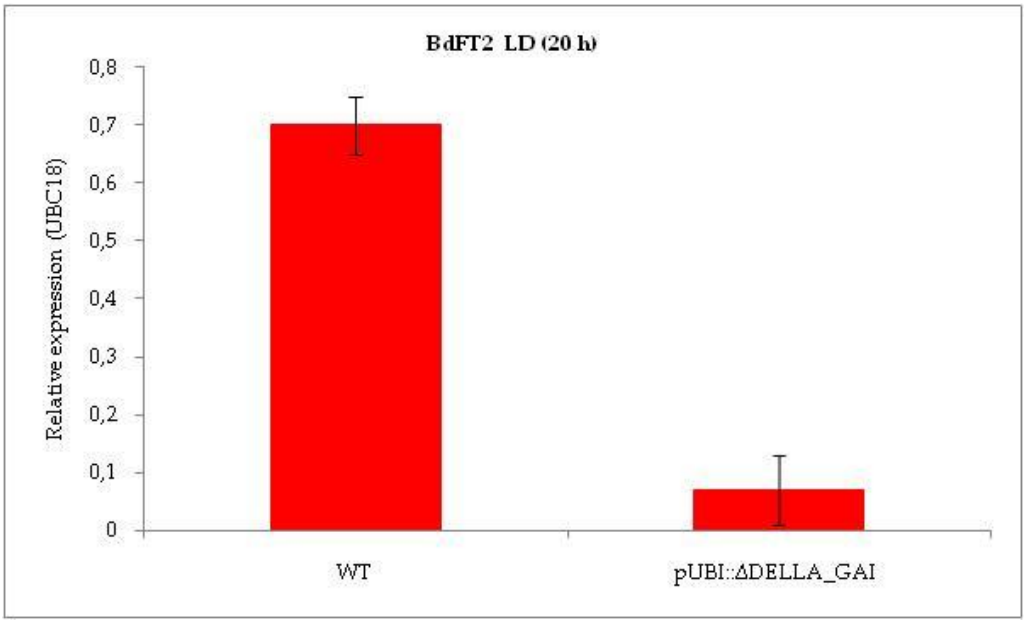
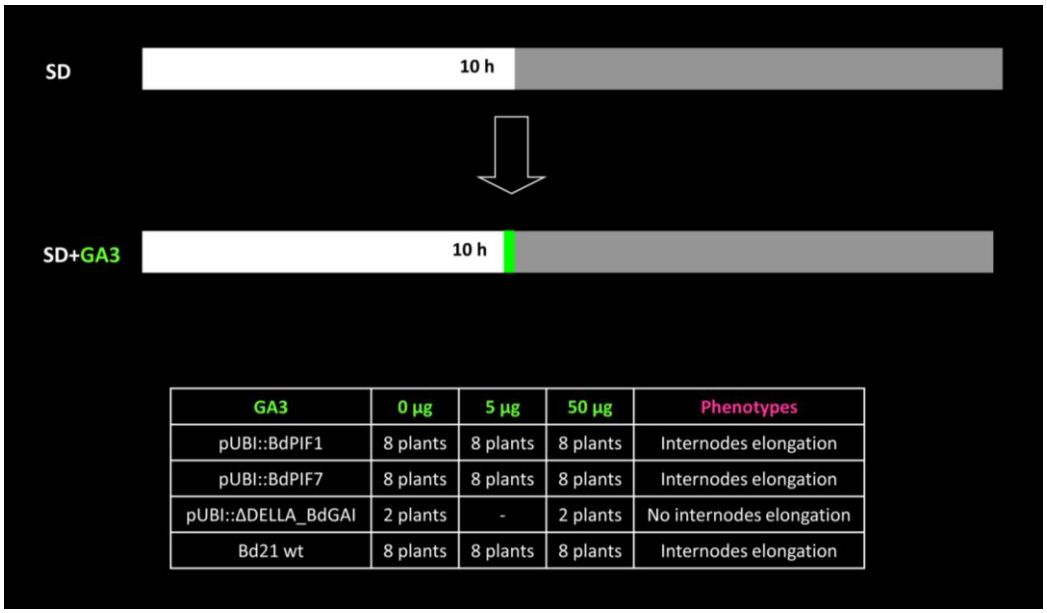


Figure 3.30: Relative expression levels of BdFTL2 in the pUBI::ΔDELLA_GAI and Bd21 wt, 7 days after shift into LD, at ZT=2. Reference gene UBC18_Bradi4g00660 (Schawrtz *et al*, 2010). Data courtesy of Dr Scott Boden, JIC.

stable BdGAI transgenics, as opposed to wt plants. This result corroborates the evidences already presented in other studies of a flowering response mediated through BdFTL2, and gauged by tightly controlled endogenous GA levels (Hisamatsu and King, 2008; Kumar and Lucyshyn, 2012).

In accordance with these results and given that the expression levels of two GA biosynthetic enzymes, from the GA20ox group, increase upon transition from SD to LD (Dr Scott Boden personal communication; King *et al*, 2006) it was hypothesized that in SD the levels of endogenous GA stay low, allowing BdGAI to remain stable and to sequester BdPIFs proteins, preventing them from inducing flowering; while in LD this scenario would become inverted. If true, it was predicted that by applying GA exogenously to wt plants, the BdGAI levels would drop significantly to allow lingering levels of BdPIF to accelerate flowering transition. In the case of applying GA to the BdPIFs over-expressers, immediate flowering transition would be expected. In order to test these hypotheses, four consecutive applications (every 2 days) of GA3 were directly applied to the upper leaves of SD grown plants. Given the unprecedented experiment in Bd21, different amounts were employed for testing: 0 μg (control), 5 μg and 50 μg, applied to the center of the leaf blade, at dusk, according to Evans *et al*, 1990. The analysis was performed using Bd21 wt, the constitutive expressers of BdPIFs and of the stable BdGAI protein (control).



GA3	0 μg	5 μg	50 μg	Phenotypes
pUBI::BdPIF1	8 plants	8 plants	8 plants	Internodes elongation
pUBI::BdPIF7	8 plants	8 plants	8 plants	Internodes elongation
pUBI::ΔDELLA_BdGAI	2 plants	-	2 plants	No internodes elongation
Bd21 wt	8 plants	8 plants	8 plants	Internodes elongation

Figure 3.31: Schematic representation of the experimental set up of the GA3 application to SD grown Bd21, and constitutive expressing BdPIFs and stable BdGAI lines. The summary of the phenotypes observed is presented in the table.

The results summarized in Figure 3.31 show that GA3 was absorbed by the plant tissue, translocated into the lower parts of the plants where it, eventually, stimulated the internode elongation, through the removal of the growth repression installed by BdGAI. This phenotype was equally evident in both wt and BdPIFs over-expressing plants treated with either 5 μg or 50 μg of GA3. None of the plants treated showed a flowering phenotype, demonstrating that although biologically active, and able to target BdGAI for degradation,

GA3 is not florigenic. This observation implies that the sole removal of BdGAI through GA3 application is not sufficient to allow the BdPIFs, already in excess amounts, to anticipate, even further, the flowering transition in SD; this might be because BdGAI levels in the pUBI::PIFs background are so low compared to the BdPIFs that they are not restricting their activity in what flowering induction is concerned. As expected, the plants over-expressing the stable BdGAI were indifferent to the presence of GA - given that the DELLA domain had been removed from the protein - and showed no internode elongation. Leaf samples from these treated plants were also collected to quantify BdFTL2 levels after GA application, however, there was huge variability between biological replicates and so the analysis was not conclusive. Evans *et al* in 1990, however, highlighted that are both the chemical and structural properties of the different GA molecules that define their biological activity, i.e., their ability to stimulate stem elongation, flowering, or both. With that in mind, other GA molecules were tested; mainly the reportedly florigenic GA_1, GA_4, GA_5, and GA_6, in order to determine if a different GA molecule could be accounted as able to act as a florigen (Evans *et al*, 1990; King *et al*, 2006; Naor *et al*, 2004). The experimental settings were kept constant, with the exceptions that this time only Bd21 wt plants were used (10 plants per treatment), and the GA dosage was set to 20 μg/plant. The results obtained did not differ from the ones already mentioned for GA3, with consistently evident internode elongation (Figure 3.32) and no significant flowering response: only 1 plant out of 10 flowered in SD when GA3 and GA6 were applied (Ethan Stuart, personal communication). These unexpected results are difficult to explain, as the phenotype displayed by the responsive plants showed that the GAs were incorporated by the plant tissue, and that they were translocation across the vasculature down to the internodes, while remaining biologically active. In addition, the amount of applied GAs does not seem to be a limiting factor, given that the phenotype exhibited by the treated plants is consistent with the DELLA/GAI loss of function *slender* mutants from barley and rice (Chandler *et al*, 2002; Ikeda *et al*, 2001), and yet none of the GA molecules tested was able to induce flowering in Bd21 grown in SD the way it has been reported for other plant systems (Eriksson *et al*, 2006), including the monocots (Evans *et al*, 1990; King *et al*, 2006).

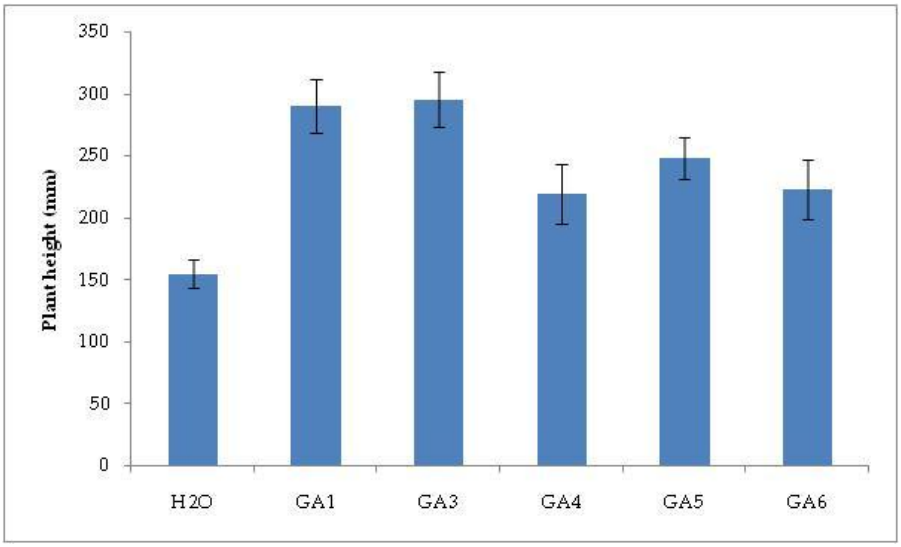


Figure 3.32: The effect of giberrellin application on plant architecture. Control plants were tested with water.

3.4 Discussion

Making use of the knowledge gathered from the research of flowering time in *Arabidopsis thaliana*, the study of the flowering transition in Brachypodium was initiated by the pursuit of "florigen", a regulatory element known to mediate the transport of the photoperiodic information across the plant and into the SAM, where it determines the developmental shift towards the reproductive fate. Through a genome wide BLAST search, it was possible to identify all the loci that are believed to encode the expression of proteins from the PEBP family and, more specifically, the ones that share high levels of amino-acid conservation with FT. From the expression data gathered from leaf samples of flowering induced plants, BdFTL2 (Bradi1g48830.1) stood out as the most significantly up-regulated FT orthologue candidate from the pool of potential PEBP encoding genes. Phylogenetically, when compared to other FT orthologues already identified in other plant systems, most notably the ones of temperate monocots, BdFTL2 amino-acid sequence displayed the highest levels of conservation among the other potential FT-like peptides. Its expression levels increased significantly in the leaves of Bd21 plants when exposed to flowering inductive long days, more precisely in the cells along the vasculature, and when constitutively expressed, BdFTL2 is highly florigenic. In fact, BdFTL2 is able to completely suppress the differentiation of all vegetative structures and to induce the re-programming of the *callus'* meristematic cells towards the specification of the inflorescence and its florets. In spite of the lack of loss of function mutants in Bd21, a targeted gene silencing strategy has been attempted through the use of the artificial micro RNAs technology. The results obtained by the constitutive expression of a specific amiRNA, with the purpose of singling out BdFTL2, have not been conclusive. The transgenic plants (T0 generation) displayed severe phenotypic defects in the development of a healthy inflorescence; however, it is early to attest if this is a true manifestation of the reduced levels of BdFTL2 gene. According to flowering time data on the T1 generation, the expression of such amiRNA did not significantly affected heading date compared to wt plants and, in addition, all these lines that were carried forward did not displayed the previously observed inflorescence phenotype. This fact could be due to low levels of silencing in the fertile lines that remained among the transgenic population, given that it is likely that the lines with 100% sterility rate were probably the ones that were heavily silenced (speculation, as no qPCR has been performed to assess BdFTL2 mRNA levels). In the future it would be pertinent to express this amiRNA, or any other silencing strategy such as hairpin constructs or RNAi, as part of an inducible system; this way the high infertility would have been accounted for and more precise studies would have been possible along with the maintenance of the lines along the generations. Still, it is possible to conclude that BdFTL2 is likely to be *Brachypodium distachyon*'s functional orthologue of Arabidopsis FT.

The up-regulation of the expression of "florigen" is known to be directly linked to the photoperiodic stimulus, conveyed through the output of the circadian clock. However, there seems to be little similarity between different plants systems on how the genetic scaffold of the photoperiod pathway is built. Reportedly associated to CO in Arabidopsis, in

wheat CO orthologues remain dissociated to any known photoperiod responsive QTL. With the interest in analysing if the Bd21 CO orthologue has a positive effect in the photoperiodic induction of flowering, in accordance with the reported data available on Arabidopsis flowering time, a genome wide BLAST search was performed using CO protein as query. The most phylogenetically conserved peptide was identified as BdCO1 (Bradi1g43670.1) and its genomic sequence cloned to generate constitutively expressing transgenic lines. From the over-expression of this genetic construct it was expected that, if the photoperiodic induction of flowering, in Bd21, was also channelled through the LD stabilization of BdCO1 protein leading to the activation of BdFTL2, as it is described for Arabidopsis, then the transgenic plants should exhibit an early flowering phenotype in LD, when compared to wt plants. The results, however, were inconsistent with this hypothesis. The ten random transgenic lines analysed displayed a consistently late flowering phenotype in LD, and although most of the transgenic lines flowered earlier in SD when compared to wt, this event was not correlated with increased levels of BdFT2 mRNA. In order to attest for the presence of the transgenic protein in SD grown plants, and exclude the possibility of transgene silencing, an immuno-detection assay was performed in a time course series. This analysis confirmed that the over-expression of BdCO1, in Bd21 background, yielded high levels of protein, *in vivo*, and that the lack of BdFTL2 expression in SD grown plants cannot be correlated to the absence, or low levels, of transgenic BdCO1 *in planta*. Interesting was the observation that, for certain periods of the 24h-day, the levels of transgenic BdCO1 dropped sharply below the limit of immuno-detection. This implies that independently of the amount of protein present in the leaves, BdCO1 protein must be actively removed from the system at specific times of the day (possibly through circadian regulation), independently of the light conditions; and this is probably mediated through different levels of regulation, including transcription control and protein stability. These observations totally contradict published results on Arabidopsis CO, and yet, data on Hd1 of rice are supportive of BdCO1 behaviour, as Hd1 is known to delay flowering when over-expressed under inductive conditions - a fact that is coincidently coupled with suppression of Hd3a expression – while its stability is not directly affected by the presence or absence of light. Curiously, this does not prevent Hd1 from being an essential flowering regulator that balances the levels of Hd3a according to the duration of the photoperiod, inducing Hd3a expression in inductive SD. However, it was the inconsistency between the behaviours of BdCO1 and of HvCO1, a closely related orthologue of another temperate monocot, that highlighted the fact that even if the over-expressed protein is being produced in high amounts *in vivo*, that does not exclude the possibility that the two tags are interfering with its proper folding, and that in this way a dominant-negative effect may have been generated, with the protein not being, in fact, functional. Therefore, other experiments were devised in order to assess the true effects of the over-expression of BdCO1 in Bd21. The over-expression of a non-tagged version of the protein would certainly clear all doubts, however, given the time restrictions it was not possible to clone, transform Bd21, and gather the flowering time data in order to be able to include it in this chapter. Alternatively, it was possible to assess the ability of the tagged BdCO1 protein to be functionally active, by transforming an Arabidopsis *co-9* mutant, as well as, the ability to induce phenotypical

changes in Col-0 background. Consistent with the results already obtained for Bd21, BdCO1 was able to slightly delay flowering in Col-0 background, at the same time it was able to partially rescue the late flowering phenotype of *co-9*, in LD. These observations lead to the conclusions that the c-terminus tagged protein is biologically active, that BdCO1 is likely not to be the mediator of the photoperiodic induction of flowering in Bd21 as it has been reported for Arabidopsis, and that the scaffold of the photoperiod pathway in Bd21 probably shares more similarities with the one of wheat: with TaCO1 not being at all associated to any known flowering QTL in response to photoperiod, but being replaced by another potential clock output, the Ppd1 locus. Nevertheless, the molecular regulations and the physiological functions of BdCO1 protein in Bd21 remain, in this way, fairly obscured. Future work exploring the biological context in which BdCO1 is required to intervene should include an extended time course characterization of BdCO1 endogenous protein levels in SD and LD, along with the correspondent determination of its mRNA levels. This can be achieved by generating transgenic lines that express a tagged version of BdCO1 under the control of its endogenous promoter. Also, it would be relevant to analyse the flowering time of the lines constitutively expressing the native version of BdCO1, in order to consolidate the results already discussed; and even the design a silencing construct to assess the effect of reduced levels of BdCO1 in the photoperiod-dependent flowering response.

Recently published data on the CO-independent induction of flowering via a phytochrome interacting protein in Arabidopsis, and the involvement of PHYB in the regulation of Hd3a levels in rice pointed out new and important genetic relations involving the transmission of the inductive light signal, that were worth exploring in the context of the flowering transition of temperate cereals. Using PIF4 amino-acid sequence as a query, a genome wide BLAST search was performed and the potential BdPIF-like proteins were identified, cloned, and constitutive expressed in Bd21. From these, two independent constructs, expressing the loci Bradi2g11100.1 (BdPIF1) and Bradi3g33170.1 (BdPIF7), induced early flowering in SD conditions across the population of transgenic plants, replacing the need for a photoperiodic cue. This observation was particularly significant for BdPIF1, as the heading date was comparable to Bd21 grown in LD. Following this observations it was hypothesized that this precocious flowering in SD could be the result of the anticipation of the up-regulation of BdFTL2, via constitutive expression of BdPIFs. PIFs are known to be regulated at the protein level through the interaction with active phytochromes, and this regulation may happen independently of transcription. Unfortunately, given the time limitations, it was not possible to assess the BdPIFs protein dynamics across the time course series in both SD and LD. Still, this experiment could provide circumstantial support for the effect that its expression has on the BdFTL2 up-regulation, and its relation with the induction of flowering. It would, also, be pertinent to quantify BdFTL2 mRNA levels through a qPCR run in the constitutively expressing lines, so to confirm that the early flowering phenotype in SD is correlated with the ectopic activation of BdFTL2 expression. The current working hypothesis for the involvement of BdPIFs in the flowering response of Bd21 follows the precept that has been recently defined for PIF4 in Arabidopsis (Kumar and Lucyshyn *et al*, 2012), with BdPIFs being regulators of BdFTL2 through direct binding to its promoter; an

analysis that could, also, be performed in a more opportune time through a chromatin immuno-precipitation (ChIP), in an analogous experiment to the one performed by Kumar and Lucyshyn *et al* in 2012.

The inactivation PHYB is also known to feed into the gibberellin-mediated flowering pathway, by inducing the up-regulation of gibberellin biosynthesis, and releasing DELLA´s direct repression on PIF activity (Feng *et al*, 2008; de Lucas *et al*, 2008). In Bd21, the GAI protein orthologue was identified through a genome wide BLAST search as a single protein family - similarly to what happens in other cereal crops. To confirm that Bd21's GAI orthologue was functional, able to repress growth, and eventually involved in the regulation of BdPIF activity, the protein was constitutively expressed in its native form and, also, with a DELLA domain mutation that left it insensitive to GA-mediated degradation. As a result, the architecture of the mutated *della* over-expresser was consistent with the dwarf wheat and barley mutants (Peng *et al*, 1999; Chandler *et al*, 2002), while the native DELLA transgenics, still vulnerable to GA-mediated degradation, displayed a wt phenotype. The head emergence date of the mutated *della* over-expressing lines appeared to be delayed, but due to the lack of internode elongation, it was not considered the best way to score flowering time; a more precise analysis must be carried out, in the future, through the dissection of the SAM under a binocular microscope in order to account for this limitation. However, the BdFTL2 mRNA levels were quantified at ZT=2 (to coincide with BdFTL2 morning peak of expression), for both this line and in wt plants that were florally induced for 7 LD, and the results demonstrated a significant reduction of BdFTL2 mRNA levels in the mutated *della* over-expresser. This result supports the hypothesis of the BdGAI interacting with BdPIFs and limiting its activity through the possible (direct) up-regulation of BdFTL2; but to better test this hypothesis it would be pertinent to cross the pUBI::ΔDELLA-BdGAI plants with the pUBI::BdPIF in order to test if the presence of stable mutated *della* would prevent BdPIF activity and, therefore, eliminate the ability for pUBI::BdPIF to flower in SD.

Gibberellins are known to relieve DELLA repression by targeting these proteins for degradation. Also, it is known that the levels of GA biosynthetic enzymes from the GA20ox group increase upon transition from SD to LD (Dr Scott Boden personal communication; King *et al*, 2006). It was, therefore, hypothesized that there is a relation between photoperiod perception, the GA levels, the stability of BdGAI, and the ability of BdPIFs to induce flowering via BdFTL2; in other words, in SD, plants sustain low GA levels allowing BdGAI to remain stable and seize BdPIFs, preventing them to activate BdFTL2 expression and inducing flowering. In LD, though, the scenario would be inverted as GA levels would increase - via the up-regulation of GA20ox biosynthetic enzymes - the BdGAI would be targeted for ubiquitination followed by proteasomal degradation, releasing BdPIFs to perform the activation of gene expression, leading to a coordinated physiological response involving flowering transition at the SAM (most likely through preliminary up-regulation of BdFTL2) and internode elongation, culminating into head emergence. Being true, it was anticipated that in SD grown wt plants, exogenous applications of GA could replace the photoperiodic stimulus and set off flowering transition by releasing BdGAI repression on

BdPIFs; while in SD grown BdPIFs constitutive expressers, the exogenous GA applications could advance even further the heading date. With that in mind, four consecutive leaf-blade applications of GA3 (0, 5, and 50 μg) were made, at dusk (according to Evans *et al*, 1990), to SD grown wt, the constitutive expressing BdPIF1, BdPIF7, and the GA insensitive *della* transgenic plants. The results, however, did not fully match with the envisaged predictions. After the treatment all the plants treated with 5 or 50 μg of GA3, excluding the mutated *della* over-expressers, displayed a massive internode elongation phenotype similar to the slender rice and barley mutants (Ikeda *et al*, 2001; Chandler *et al*, 2002), but no flowering phenotype. This expected architecture phenotype confirmed that GA3 was absorbed and transported across the plant down to the basal internodes, while retaining it bioactivity. Once at the internodes, GA3 was able to release BdGAI's repression on growth for both wt and BdPIFs plants; however, GA3 was not florigenic even after a massive 50 μg/plant application. Leaf samples were harvested to determine BdFTL2 mRNA levels, as a consequence of the GA3 treatment; however, the results were not conclusive. The stable mutated *della* overexpressing plants remained insensitive to the GA3 treatment, as expected, given the lack of the protein's GA recognition DELLA domain.

In Bd21, as probably all grasses, the inflorescence development initiation actually happens prior to the increase of stem expansion, and not as a consequence of internode elongation (possibly due to an evolutionary survival strategy against grazing from herbivores); and the fact that GA3 wasn't able to trigger flowering is probably due to what Evans and colleagues pointed out in 1990, which are both the chemical and structural properties of the gibberellin molecules that define their biological activity, i.e., their ability to stimulate flowering, stem growth, or both. In fact, it has been published that different plants respond differently to different GA molecules, with Arabidopsis displaying a flowering response to GA4 (Eriksson *et al*, 2006), Calla lily to GA1, GA3 and GA4 (Naor *et al*, 2004), while for Lolium it's GA5 and GA6 that confer the higher florigenic response, without causing much internode elongation (King *et al*, 2006). Since GA3 was not able to induce flowering in Bd21 under non-inductive conditions, other reportedly florigenic molecules were tested using a similar experimental set-up. This time, standard 20 μg/plant applications of GA1, GA4, GA5 and GA6 were carried out, at dusk, on the centre of the leaf blade of ten SD grown wt plants. Once again, none of the GA molecules was able to induce flowering as it has been reported for other plant systems but, nonetheless, they all exhibited the internode elongation phenotype characteristic of the slender mutants mentioned previously. On the face of this phenotype it's possible to, once again, conclude that the different GA molecules were absorbed by the plants, and that they remained biologically active while travelling throughout the vasculature, down to the internodes where they released the BdGAI repression on growth. However, none of them seems to be florigenic in Bd21, in spite of the generous dosage applied.

DELLA proteins are known to be nucleus localized transcription factors involved in the regulation of germination, growth, and flowering as part of the gibberellins signalling pathway; however, contrasting to the coordinated sub-functionalization of Arabidopsis' five protein members, similarly to other monocots, Bd21 only has one identifiable DELLA

protein. The biological consequences of this different genetic arrangement are yet unknown, however it is possible that DELLA activity in monocots has, alternatively, evolved through regulatory plasticity according to tissue specificity and/or developmental phase. In Bd21's case, one could speculate that probably the GA-mediated flowering response initiated in the leaves is more tightly regulated when compared to overall growth (via the cell expansion and internode elongation), and that BdGAI degradation in the leaf may, physiologically, involve extra layers of complexity, possibly through additional molecular elements that are dispensable at the internode level. This hypothesis is not completely implausible, as it has been shown that the degradation of DELLA proteins in Arabidopsis is dependent on factors such as SLY1 availability, on the strength of the interaction between SLY1 and the DELLA target, and on the promotion of the SCF^{SLY1}-DELLA interaction by DELLA phosphorylation (Fu *et al*, 2004). Also, it has been reported that not all DELLA proteins of Arabidopsis (specifically GAI) disappear from the nucleus, releasing their regulation on gene expression, after a GA3 treatment (Fleck and Harberd, 2002). This demonstrates that the regulation of DELLA proteins is a more complicated affair than simply being dependent on GA availability; and it is possible that Bd21 also uses this complex multitude of regulatory checkpoints, like the fluctuation of SCF^{SLY1} E3 Ubiquitin ligase activity, for example, to control DELLA protein availability and, through it, differentially regulate growth and development.

It would be important, however, to assess if nuclear BdGAI protein levels decrease upon GA application on leaf tissue where BdFTL2 is up-regulated. This could be achieved by producing pBdGAI::BdGAI-reporter lines that could be treated with GA and analysed in vivo through confocal microscopy, as described by Fleck and Harberd in 2002. Such an experiment could provide a more clear idea of the consequences of GA application on BdGAI levels, especially concerning the ability to release BdGAI's possible repressive action on flowering induction. Additionally, it would also be interesting to perform a protein pull down assay or a FRET analysis to prove, *in planta*, the direct interaction between BdGAI and BdPIFs.

Chapter 4

Transitioning into the reproductive phase – Part I

4.1 Introduction

In order to be able to synchronize floral transition with favourable environmental conditions and successfully reproduce - a particularly important trait to monocarpic species (Bernier and Périlleux, 2005) - plants rely extensively on the constant monitoring of their surroundings, and on the simultaneous coordination of this external information with its internal physiological networks. As a result of the flowering inductive cue being sensed, a mobile florigenic signal is produced in the leaves, translocated across the plant, and purposely delivered to the shoot apical meristem. At the SAM, the florigen molecule triggers a series of metabolic and morphological modifications that will lead to the initiation of the flowering process and, ultimately, the differentiation of the reproductive organs.

Evidence from the previous chapter have highlighted that not all genetic elements of the photoperiodic pathway remain functionally conserved between both the dicot and the monocot plant systems. In the context of grasses exhibiting a significantly different type of both flower and inflorescence, this raises the following questions: are the downstream meristem floral identity genes known from work done in Arabidopsis present in Brachypodium? Is their biological function conserved? Where do they act and when? And what are the consequences of their dysfunction?

4.1.1 Flowering transition of cereal crops

Wheat, barley, rye, triticale, and oats are cool-season cereals that are well-adapted to temperate climates. Winter varieties are sown in the autumn, germinate and grow vegetatively, then become dormant during winter. They resume growing in the springtime and mature in late spring or early summer (Figure 4.1). These varieties do not flower until springtime because they require vernalization (exposure to low temperature for a genetically determined length of time) (Trevaskis *et al*, 2007). Where winters are too warm for vernalization or exceed the hardiness of the crop (which varies by species and variety), farmers grow spring varieties. Spring cereals are planted in early springtime and mature later that same summer, without vernalization.

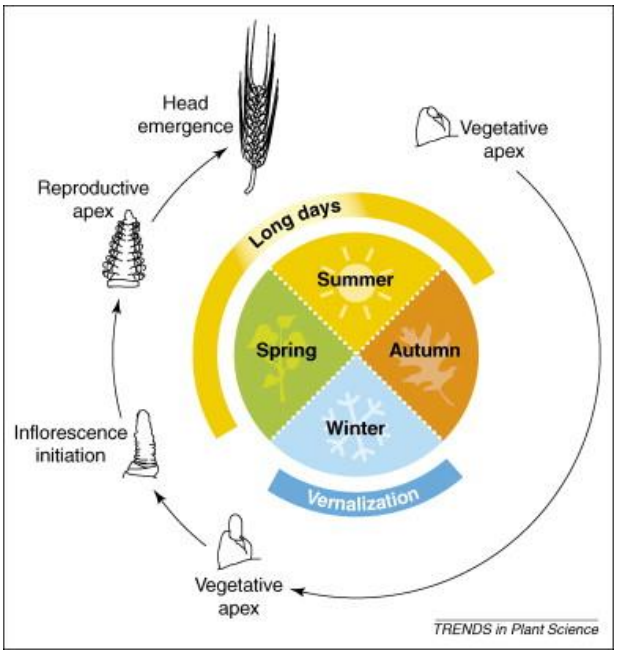


Figure 4.1: The influence of seasonal cues on shoot apex development in the temperate cereals (Adapted from Trevaskis *et al*, 2007).

Wheat (*Triticum aestivum*) and barley *(Hordeum vulgare*) plants sown in late summer or autumn do not flower before winter because both the vernalization and photoperiod response pathways are inactive (Figure 4.2): the expression of the *APETALA1*-like MADS box gene *VERNALIZATION 1* (*VRN1*) is low and *VERNALIZATION 2* (*VRN2*) diurnal activity represses LD induction of *VERNALIZATION 3* (*VRN3*), the temperate cereals homologue of Arabidopsis *FT* (Hemming *et al*, 2008; Trevaskis *et al*, 2007). The nature of VRN2 repression over VRN3 is still unknown, but it may involve barley's *PHOTOPERIOD1* (*PPD-H1*) gene, a pseudo-response regulator with diurnal expression pattern, characterized by a pseudo-receiver domain, implicated in signal transduction, and a CCT (CONSTANS, CONSTANS-like and TOC1) domain, that moderates day-length expression of *VRN3* by controlling *HvCO* activity (Turner *et al*, 2005). Low temperatures during autumn, when plants are still under LD, repress *VRN2* gene expression (Dubcovsky, 2006). During the short and cold days of winter, *VRN1* is up-regulated, directly targets *VRN2* for repression

(Trevaskis *et al*, 2006; Loukoianov *et al*, 2005) and induces flowering competency. (Yan *et al*, 2003, 2004, 2006; Danyluk *et al*, 2003; Trevaskis *et al*, 2003; Preston and Kellogg, 2007). As daylength and ambient temperatures rise towards the beginning of spring, the mutually conserved backbone of the photoperiod signalling pathway, released from the repression of *VRN2*, is set off to induce *VRN1,* through the heterologous protein complex *VRN3-TaFDL2* (as FT-FD in Arabidopsis) (Li and Dubcovsky, 2008) and promote floral transition. In spring varieties, expression of *VRN1* increases during inflorescence initiation and remains high through subsequent stages of apex development (Trevaskis *et al*, 2007).

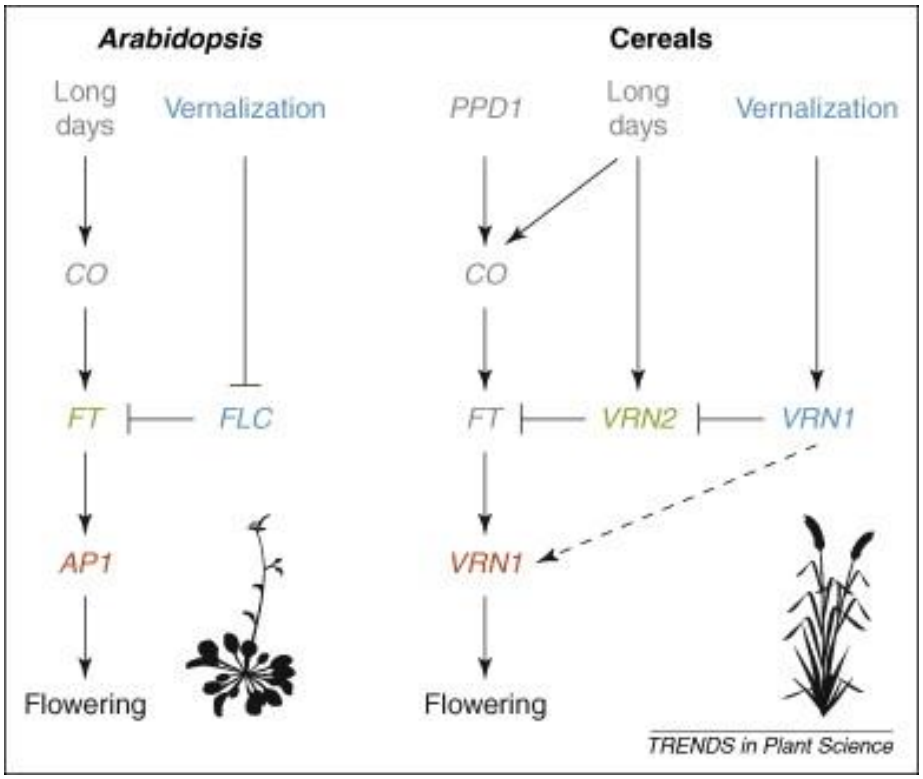


Figure 4.2: Flowering pathways in Arabidopsis and cereal crops: The photoperiod pathway is conserved (grey). *CO* senses LD and activates *FT* expression. This requires *PPD-H1* in cereals. The vernalization pathway (blue) evolved independently in both *Arabidopsis* and cereals. In *Arabidopsis*, the vernalization and photoperiod pathway intersect at *FT*, which can be described as a floral integrator gene. In cereals, *VRN2* is a floral integrator gene. In both plants, activation of flowering causes expression of genes that promote inflorescence meristem identity (red), such *AP1* in *Arabidopsis*. *VRN1* acts as both a flowering time gene in the vernalization response pathway and as a meristem identity gene during reproductive development red). Adapted from Trevaskis *et al*, 2007.

Most of the natural allelic variation in vernalization requirement of Arabidopsis is associated with *FRI* and *FLC* genes (Gazzani *et al*, 2003), while in wheat and barley, that is not the case. Mutations in the *VRN1* promoter and its first intron (Yan *et al* 2003; Yan *et al* 2004; Fu *et al*, 2005; von Zitzewitz *et al* 2005) result in dominant spring alleles that have high basal levels of expression, capable of repressing *VRN2* and accelerating inflorescence initiation, bypassing the need of vernalization (Trevaskis *et al*, 2006; Loukoianov *et al*, 2005). This allows LD to induce expression of *VRN3* and further accelerate floral development in day-length sensitive varieties. A more significant difference between Arabidopsis and the temperate cereals relies on the vernalization gene involved in flowering repression. The central repressor in the vernalization pathway of Arabidopsis is the MADS-box gene *FLC* (Michaels and Amasino, 1999), whereas in temperate cereals is the zinc finger- CCT domain transcription factor *VRN2* (Yan *et al* 2004) (Figure 4.3).

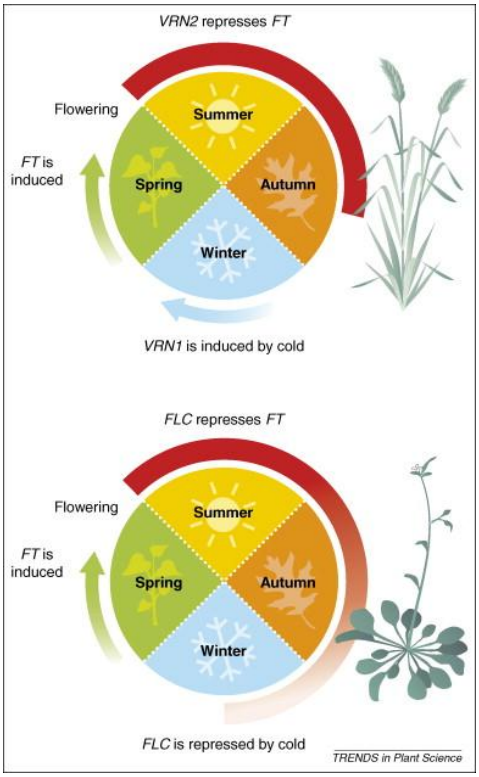


Figure 4.3: Molecular basis of vernalization-induced flowering in cereals versus Arabidopsis. In the temperate cereals (top), VRN2 represses FT and blocks long-day promotion of flowering before winter. *VRN2* is not expressed in the short days of winter, when *VRN1* is induced by prolonged exposure to cold. After winter, *VRN1* expression remains high. This promotes inflorescence initiation and represses VRN2, to allow long-day induction of FT to accelerate reproductive development. When flowering occurs, VRN1 expression is reset to establish the vernalization requirement in the next generation. In Arabidopsis (bottom), FLC is expressed before winter and represses FT. Vernalization represses FLC, and this allows long-day induction of FT (and SOC1) to promote flowering in spring. FLC expression is reset during meiosis to establish the vernalization requirement in the next generation. Adapted from Trevaskis *et al*, 2007.

No clear homologue of Arabidopsis FLC has been identified in wheat, and the same is true for *VRN2* outside the temperate cereals (Yan *et al*, 2004). Varieties of diploid wheat and barley with loss-of-function point mutations at the *VRN2* locus (vrn2a allele) or complete gene deletions (vrn2b allele) are associated with a recessive spring growth habit and, therefore, flower earlier in LD but not in SD (Karsai *et al*, 2005), and do not require vernalization to flower (Yan *et al*, 2006, Yan *et al*, 2004; Fu *et al*, 2005; Dubcovsky and Loukoianov *et al*, 2006). In some photoperiod-sensitive winter varieties of hexaploid wheat, the vernalization requirement can be eliminated or greatly reduced by exposing the plants to SD for several weeks and then transferring them back to LD. The SD replacement of vernalization in wheat is associated to the down regulation of *VRN2* in SD at room

temperature (Dubcovsky and Loukoianov *et al*, 2006). Additionally, the down regulation of *VRN2* is not followed by an up-regulation of the meristem identity gene *VRN1* until plants are transferred to LD, which contrasts with that observed after the vernalization treatment, implying the existence of a second *VRN1* repressor (Dubcovsky and Loukoianov *et al*, 2006). There are also alleles of *VRN3* that are expressed without vernalization bypassing the requirement for a level of *VRN1* activity to allow a LD flowering response. In barley, these alleles of *VRN3* have polymorphisms in the first intron and in wheat, there is a retroelement insertion in the promoter of *VRN3* (Yan *et al*, 2006). These regions in the promoter or first intron of the *VRN3* gene are presumably important for repression of *VRN3* before vernalization, and might contain binding sites for the VRN2 protein.

Contrary to the reports of LEAFY (LFY) from Arabidopsis having a fundamental role in the flowering transition, in wheat, *Lolium*, and rice the expression of *WFL*, *LtLFY*, and *RFL*, respectively, is associated with the formation of the spikelet and panicle, more precisely in the specification of the palea, glumes and lemma (Shitsukawa *et al*, 2006; Gocal *et al*, 2001); and with panicle branching, suppressing the transition from inflorescence meristem to floral meristem (Ikeda-Kawakatsu *et al*, 2012; Rao *et al*, 2008). TERMINAL FLOWER 1 (TFL1)/CENTRORADIALIS (CEN) homologues in rice, *Lolium*, and maize, on the other hand, have shown a higher degree of functional conservation, been associated with delay in flowering transition and inflorescence architecture through the maintenance of meristem indeterminacy. This mobile protein expressed in the vasculature has also been detected in the spikelet differentiating SAM of *Lolium* (Danilevskaya *et al*, 2010; Nakagawa *et al*, 2002; Jensen *et al*, 2001)

4.2 Rational

AP1, LFY, TFL1, and FD are genes know to be at the epicentre of the control of floral meristem identity in Arabidopsis (Figure 4.4), either by being the recipients of the florigen signal, by counteracting its activity, or by interacting with it and directly participate in the meristem's fate change. Several reports have indicated the existence of homologous proteins in other dicot and monocot plants species; however, the functional conservation is not always maintained. In a new plant system it is important to lay down the foundations of the genetics of flowering time, and this means to identify Bd21 orthologues for these genetic elements at the core of the SAM's floral network and to try to understand the extent of their involvement in the transitional process. With that in mind, these genes were searched, cloned, expressed and silenced, and their profile examined in comparison to their related counterparts. For practical reasons regarding workflow and the best use of time, the experiments were planned and executed giving priority to tissue culture-mediated plant transformation followed by the expression profile analysis although, ideally, the expression profile should have been assessed first and, only then, the function of the candidate gene should have been analysed.

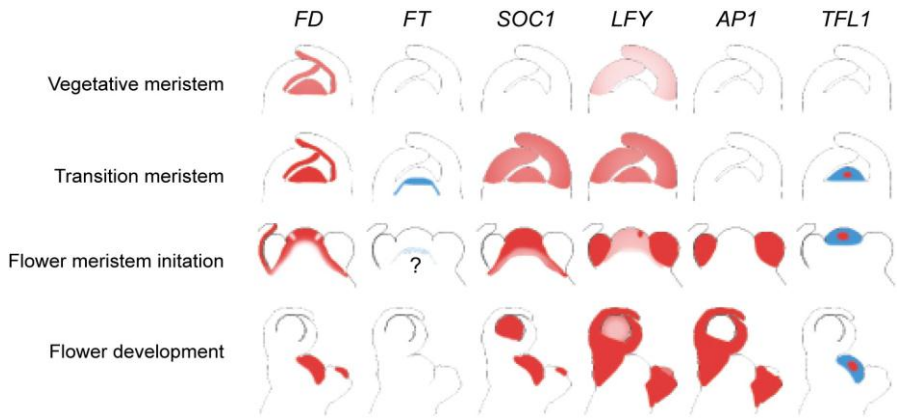


Figure 4.4: Dynamics of gene expression in the SAM upon floral transition in Arabidopsis thaliana. From top to bottom: progression of the apical meristem from a fully vegetative state (vegetative meristem) to a reproductive state (flower development). Two intermediate steps are shown: the transition meristem stage, where morphological changes are not yet visible but induction is taking place, and the floral meristem initiation stage, where the first flower primordia are arising on the flanks of the inflorescence meristem. The mRNA (red) and protein (blue) patterns of key regulators of floral transition are shown. The varying intensity of the colours accounts for the expression level of the gene. The ? referring to FT in later stages indicates that the pattern is inferred. (Adapted from Turck *et al*, 2008)

4.3 Results

4.3.1 Brachypodium's LEAFY (BdLFY) is not involved in the transition into reproductive development, but may be involved in floral organ specification

The Brachypodium's genome wide search for LFY's orthologue, using its protein sequence as query (http://www.modecrop.org, v1.0 genome assembly), yielded a single genetic element under the reference of Bradi5g20340.1 (Figure 4.5-A) (Higgins *et al*, 2010). The retrieved protein sequence was aligned with other well identified LFY orthologues in order to determine amino acid conservation (Figure 4.5-B).

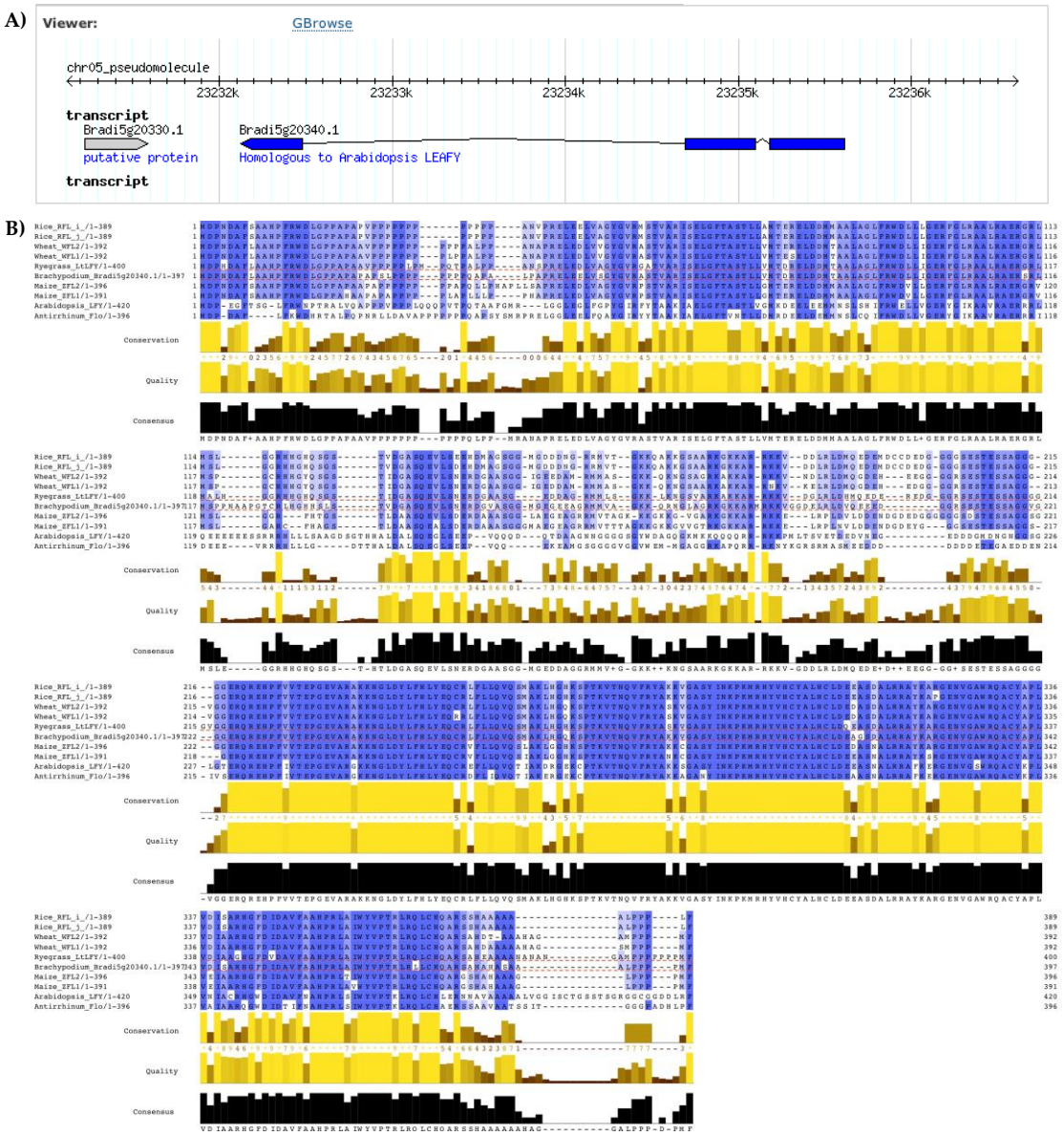


Figure 4.5: A) Schematic representation of Bradi5g20340 locus, (Adapted from http://mips.helmholtz-muenchen.de/plant/brachypodium/). **B) ClustalW alignment of LFY and the correspondent orthologues in different cereal species**. Bradi5g20340.1 identified by the dashed-red box. Blue highlights represent aminoacid residue conservation amongst the aligned proteins: highly conserved aminoacid residues in dark blue and conserved aminoacid in light blue. Dashes represent residue insertions or deletions amongst orthologue peptide sequences. Yellow bars represent higher levels of aminoacid conservation between LFY orthologues, while brown bars represent regions with lower levels of aminoacid conservation between the aligned sequences. Black bars represent the conservation frequency of the aminoacid residues that make up the consensus sequence.

4.3.1.1 The constitutive expression of BdLFY has no effect in Brachypodium's flowering time

Arabidopsis LFY is known to be a defining meristem identity gene that mediates the induction of floral organ identity genes at the SAM, and is able to accelerate flowering transition when constitutively expressed by the 35S promoter (Weigel *et al*, 1992; Blázquez *et al*, 1997). In order to determine if the constitutive expression of BdLFY on Brachypodium would have an effect on Bd21's flowering time, the full genetic sequence of Bradi5g20340, was cloned into a Gateway compatible plasmid, in front of the rice actin constitutive promoter and leading intron (Himmelbach *et al*, 2007), and transformed into Bd21 according to Alves *et al* (2009). Independent T1 plants grown, in LD, flowered at the same time and displayed the same amount of vegetative biomass as the control wt plants, demonstrating that the ectopic expression of BdLFY does not accelerates flowering in Brachypodium in LD, as it does in other dicot plant species (Weigel *et al*, 1992; Coen *et al*, 1990). This result is consistent with the account that the function of rice's RFL differs from that of Arabidopsis' LFY (Kyozuka *et al*, 1998). In addition, the constitutive expressers showed no detectable differences in regards to the spike morphology or seed fertility when compared with wt plants (Figure 4.6).

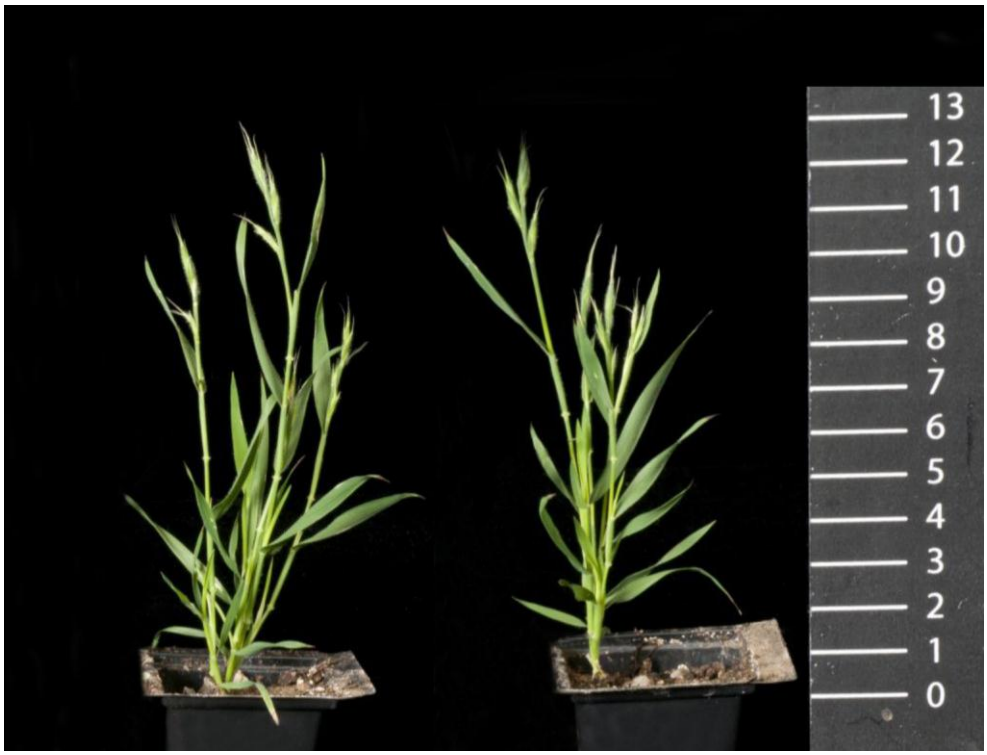


Figure 4.6: Constitutive expression of BdLFY under LD does not have an effect on flowering transition: Wild-type (left), pOsAct1::BdLFY (right).

4.3.1.2 Silencing BdLFY through the over-expression of a amiRNA may compromise inflorescence and floret development

With no Bd21 mutants available to ascertain if the absence of BdLFY expression, during the induction of flowering, can delay flowering transition, an mRNA silencing approach was set up using the artificial microRNAs silencing strategy described in the previous chapter (Figure 4.7) (Ossowski, Fitz, Schwab, Riester, and Weigel, personal communication; Warthmann *et al*, 2008; Himmelbach *et al*, 2007; Alves *et al*, 2009). The results, summarised in Figure 4.8 correspond to the T0 generation, and show in the most extreme cases an aberrant spike phenotype where spikelet position has been defined at the top of the culm but no florets have differentiated (Figure 4.8-B1, C1). It is difficult to attest if this lack of floret formation is due to termination of the meristem as a result of BdLFY silencing, or if is due to a silencing-unrelated desiccation of the meristem derived from tissue culture regeneration. This lack of certainty comes from the fact that the few florets developed were able to correctly differentiate all floral organs in the appropriate verticil, and at the same time exhibit extensive cell collapse consistent with severe dehydration (Figure 4.8-D1, E1). This dramatic phenotype was not carried through the next generation, and no difference in flowering time was detected between the T1 amiRNA lines and the control plants; the levels of vegetative biomass also remained equal between the transgenics and the wt. This can be explained by the possibility that only the lines with residual levels of gene silencing were able to produce fertile seed; however, given time restrictions it was not possible to repeat the transformation in order to confirm the mutant phenotype and measure BdLFY mRNA levels.

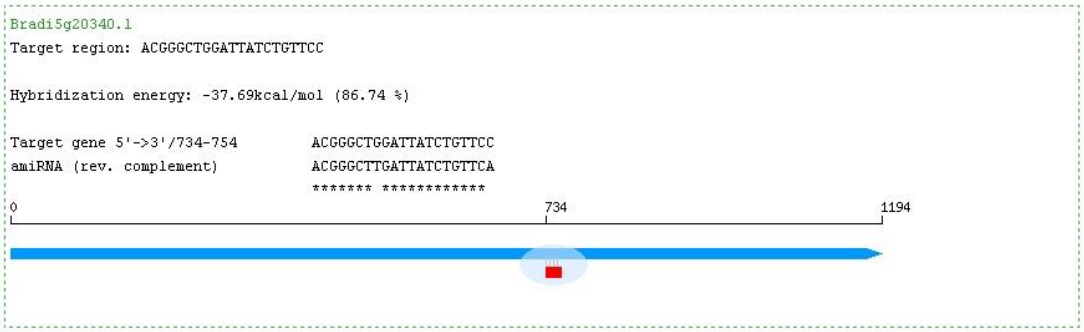


Figure 4.7: Schematic representation of the 21mer designed by the WMD3, and the correspondent target sequence in Bradi5g20340.1. Designed 21mer (red), targeted sequence (light blue) in Bradi5g20340.1's ORF of 1194 bp (dark blue) (Ossowski, Joffrey, Schwab, Riester and Weigel personal communication).

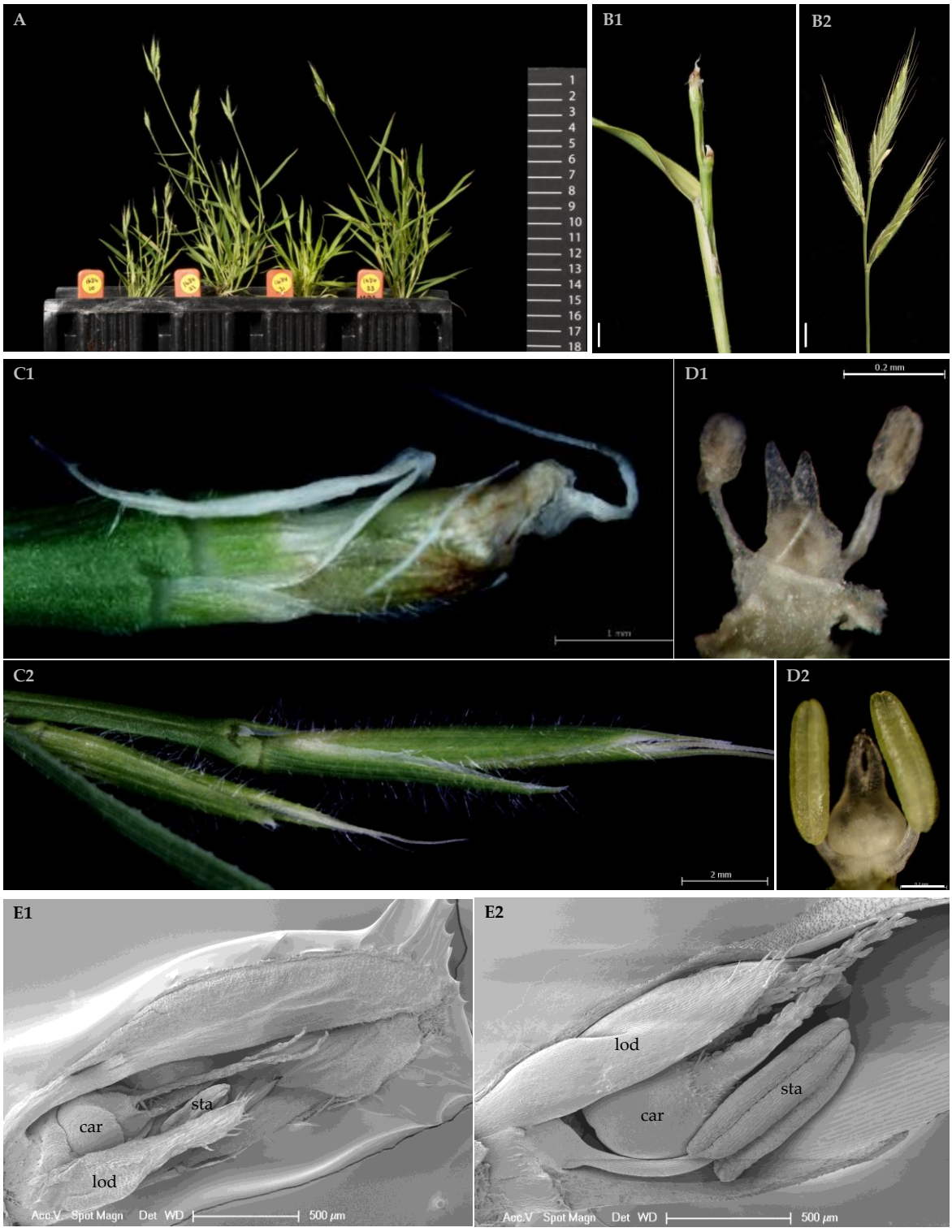


Figure 4.8: Transgenic plants expressing an artificial microRNA sequence that specifically targets BdLFY mRNA for degradation (pOsAct1::amiRNA-BdLFY) [A]; Spike development detail [B1, pOsAct1::amiRNA-BdLFY (scale bar: 0,4 cm) - B2, wt control (scale bar: 0,2 cm)]; Spikelet development detail [C1, pOsAct1::amiRNA-BdLFY (scale bar: 1 mm) - C2, wt control (scale bar: 2 mm)]; Dissected floret exhibiting atrophied reproductive organs (carpel and stamens) [D1, pOsAct1::amiRNA-BdLFY (scale bar: 0,2 mm) – D2, wt control (scale bar: 0,2 mm)]; SEM microscopy of a dissected floret: lod, lodicule; car, carpel; sta, stamen [E1, pOsAct1::amiRNA-BdLFY (scale bar: 100 µm) - E2, wt control (scale bar: 500 µm)]

4.3.1.3. BdLFY is expressed 14 days after flowering induction in developing floral meristems

Given that BdLFY over-expression showed no effect on Bd21's flowering time, and the silencing approach indicated possible floral defects, it was necessary to determine if BdLFY would be up-regulated in the SAM at the time of flowering induction and/or floral development. In order to assess the spatial and temporal expression of BdLFY, SAM samples of plants undergoing flowering transition were harvested before photoperiod shift, and then after 2 LD, 8 LD, and 14 LD to perform a transcript *in situ* hybridization. With a specific probe of the full cDNA sequence, the expression of the BdLFY transcript was only detected in the 14 LD sample, indicating that BdLFY, in Brachypodium, is not involved in either the transition from vegetative to reproductive growth or in the inflorescence meristem development (Figure 4.9). However, BdLFY appears to be necessary for the posterior specification of the floral organs during the floret morphogenesis, as it is detectable in the floral primordia (Figure 4.9-D). This results contrast significantly to the behaviour of Arabidopsis LFY but, on the other hand, it is consistent with the 12 LD expression profile described for *Lolium*'s LtLFY, where it has been detected in the glume and lemma primordia (Gocal *et al*, 2001). WHEAT FLORICAULA/LEAFY (WFL) has also been localized through *in situ* hybridization in the developing palea (Shitsukawa *et al*, 2006), which further supports BdLFY's role in the floral meristem.

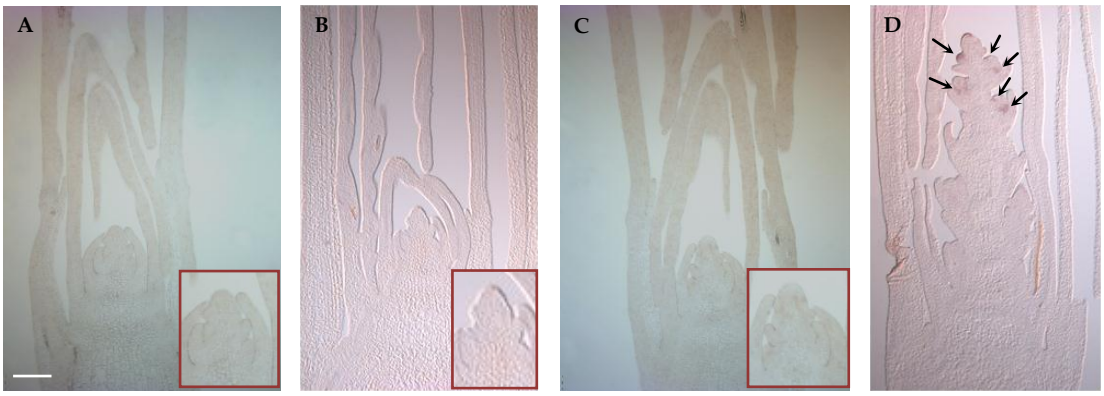


Figure 4.9: BdLFY transcript immuno-localization in cross-sections of the shoot apical meristem (enclosed by layers of leaves) undergoing flowering transition: A) 0 LD; B) 2 LD; C) 8 LD; D) 14 LD. BdLFY transcript is only detectable 14 days after flowering induction (arrows) in the floral primordia. Enclosed SAM close up in the lower-right corner. Scale bar: 0.2 mm.

4.3.2 Brachypodium's BdVRN1/BdFUL1 is the closest homologue to VERNALIZATION1 (VRN1)/FRUITFULL1 (FUL1) of temperate cereals and of Arabidopsis APETALA1 (AP1), but it is not a floral meristem identity gene

The MADS-box protein VRN1/FUL1 of grasses has been identified as the orthologue of Arabidopsis AP1 through its high protein sequence homology, phylogenetic analysis and its expression profile at the florally induced SAM (Yan *et al*, 2003; Preston and Kellogg, 2007; Higgins *et al*, 2010). VRN1 is, therefore, considered the flowering transition marker in temperate cereals, given its presumed meristem identity functions, and the positive correlation between its expression levels and the ones of VRN3/FT (Trevaskis *et al*, 2007). The Bd21 orthologue of AP1/VRN1/FUL1 with higher level of protein sequence homology was found in a whole genome blast search, under the reference Bradi1g08340.1 (Figure 4.10, Figure 4.11, Figure 4.12) (Higgins *et al*, 2010).

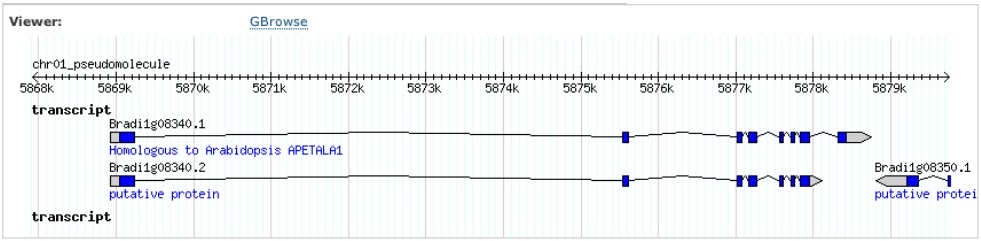


Figure 4.10: Schematic representation of Bradi1g08340 locus (Adapted from http://mips.helmholtz-muenchen.de/plant/brachypodium/).

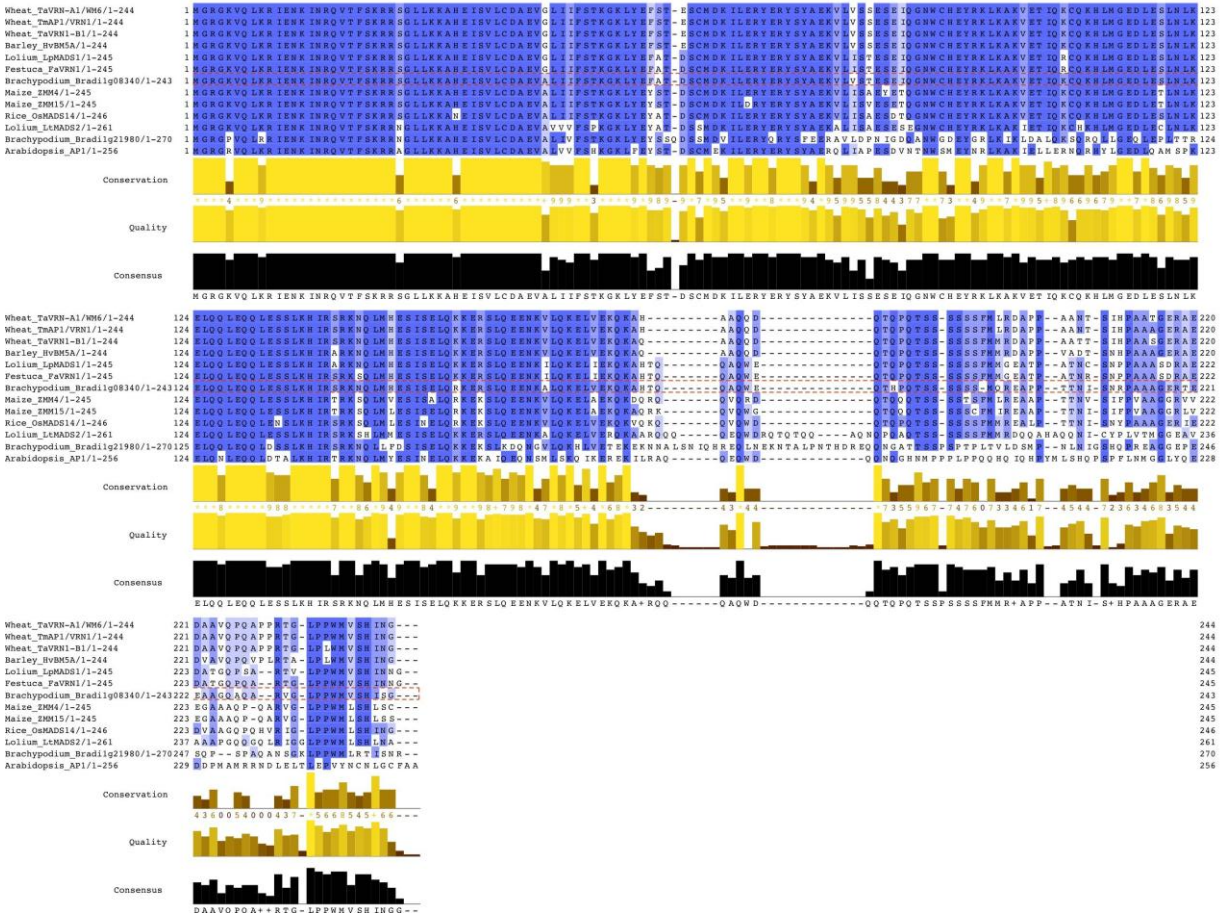


Figure 4.11: ClustalW alignment of AP1 and the correspondent orthologues in different cereal species. Bradi1g08340.1 identified by the dashed-red box. Blue highlights represent aminoacid residue conservation amongst the aligned proteins: highly conserved aminoacid residues in dark blue and conserved aminoacid in light blue. Dashes represent residue insertions or deletions amongst orthologue peptide sequences. Yellow bars represent higher levels of aminoacid conservation between FT orthologues, while brown bars represent regions with lower levels of aminoacid conservation between the aligned sequences. Black bars represent the conservation frequency of the aminoacid residues that make up the consensus sequence.

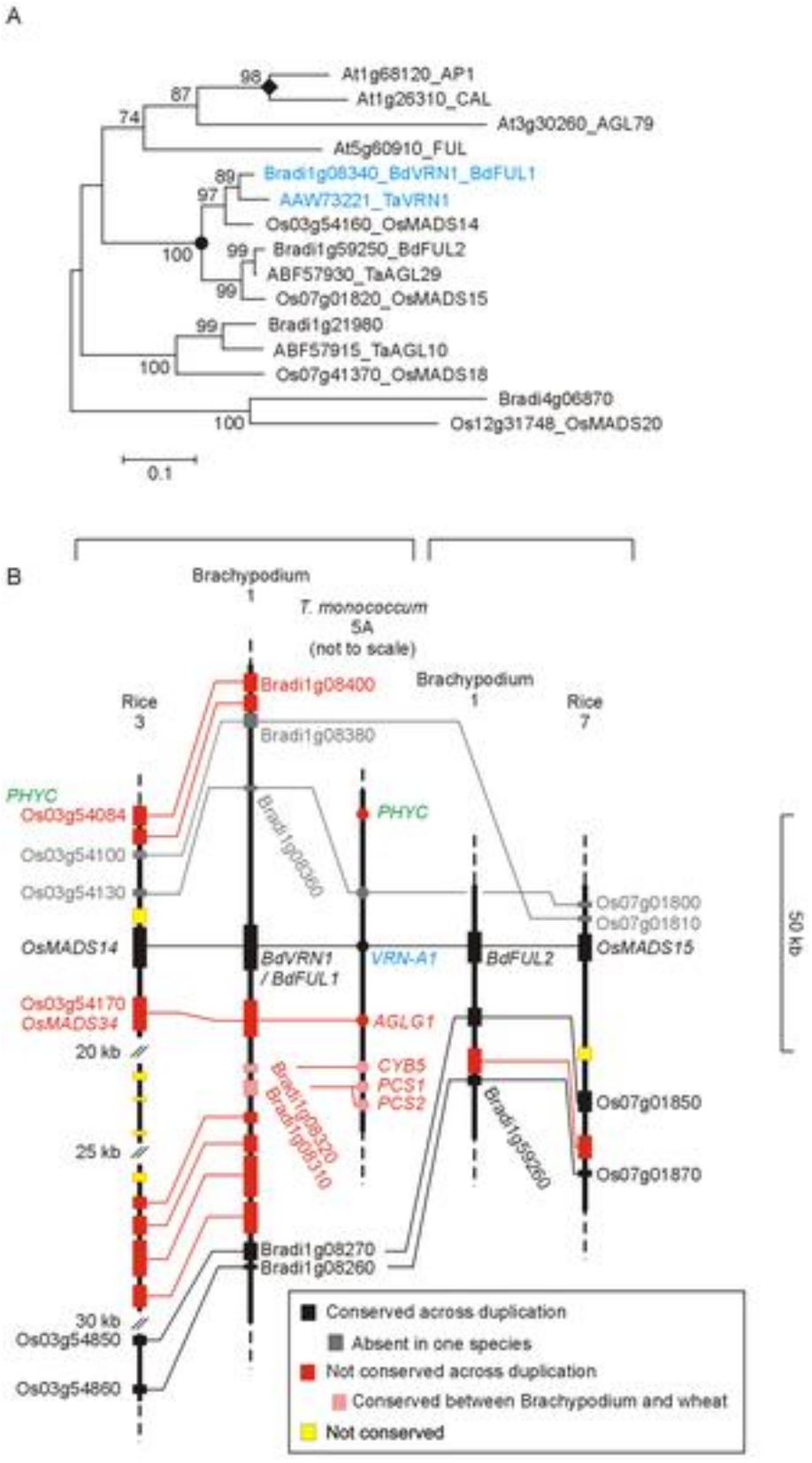


Figure 4.12: A) NJ tree of VRN1 and related proteins (whole protein alignment). B) Structure of the segmental duplication containing the FUL1 (VRN1; OsMADS14) and FUL2 (OsMADS15) genes of the grasses. (Adapted from Higgins *et al*, 2010)

4.3.2.1 The constitutive expression of BdVRN1/BdFUL1 does not accelerate flowering under inductive LD

Arabidopsis AP1 protein is known to accelerate flowering transition when constitutively expressed by inducing the expression of floral organ identity genes at the SAM. In order to confirm the expected floral meristem identity function of Brachypodium's AP1/VRN1/FUL1 orthologue, as the downstream target to BdFTL2, the full genetic sequence of Bradi1g08340.1 was cloned into a Gateway compatible plasmid, in front of the rice actin constitutive promoter (Himmelbach *et al*, 2007), and transformed into Bd21 according to Alves *et al* (2009). As the supposed downstream target of BdFTL2, it was expected that BdVRN1/BdFUL1 over-expression would replicate the dramatic flowering phenotype of BdFTL2 presented in Chapter 3. However, the result of the over-expression of BdVRN1/BdFUL1 was not as predicted, as it did not accelerated flowering transition of Brachypodium in LD (Figure 4.13). This result in Bd21 indicates that BdVRN1/BdFUL1 is not a floral meristem identity gene and so, in regards to this trait, it's not a functionally conserved orthologue of Arabidopsis AP1.

Figure 4.13: Constitutive expression of BdVRN1 does not accelerate flowering transition in LD: Wild-type (left), pOsAct1::BdVRN1 (right).

4.3.2.2 Silencing BdVRN1/BdFUL1 through the over-expression of a amiRNA may compromise inflorescence and floret development

To determine the phenotypical consequences of a BdVRN1/BdFUL1 loss of function mutation in the induction of flowering and spike development, an mRNA silencing approach was set up using the artificial microRNAs silencing strategy described before (Figure 4.14)(Ossowski, Fitz, Schwab, Riester, and Weigel personal communication; Warthmann *et al*, 2008; Himmelbach *et al*, 2007; Alves *et al*, 2009). The results, summarised in Figure 4.15, correspond to the T0 generation, and show in the most extreme cases a repetition of the aberrant spike phenotype, already mentioned for pOsAct1::amiRNA-BdLFY, where spikelet position has been defined at the top of the culm but no florets have differentiated (Figure 4.15-B1, C1). It is difficult to attest if this lack of floret formation is due to termination of the meristem as a result of BdVRN1/BdFUL1 silencing or if, again, it's due to a silencing-unrelated desiccation of the meristem derived from tissue culture regeneration. The few florets developed were able to correctly differentiate all floral organs in the appropriate verticil, and at the same time exhibit extensive cell collapse consistent with severe dehydration (Figure 4.15-D1, E1). The plant's architecture was affected with the normal elongation of the upper-most internode significantly increased (Figure 4.15-A). The spike phenotype wasn't carried through the next generation, and no difference in flowering time was detected between the amiRNA lines and the control plants; the levels of vegetative biomass also remained equal between the transgenics and the wt. The explanation for this loss of phenotype in the T1 is the same as for pOsAct1::amiRNA-BdLFY, only the lines with residual levels of gene silencing were able to produce fertile seed; however, given time restrictions it was not possible to repeat the transformation in order to confirm the mutant phenotype and measure BdVRN1/BdFUL1 mRNA levels.

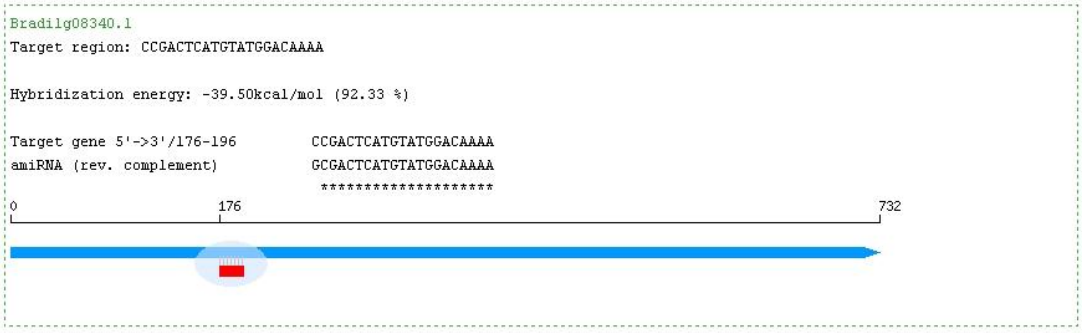


Figure 4.14: Schematic representation of the 21mer designed by the WMD3, and the correspondent target sequence in Bradi1g08340.1. Designed 21mer (red), targeted sequence (light blue) in Bradi1g08340.'s ORF of 732 bp (dark blue) (Ossowski, Joffrey, Schwab, Riester and Weigel personal communication).

Figure 4.15: Transgenic plants expressing an artificial microRNA sequence that specifically targets BdVRN1/BdFUL1 mRNA for degradation (pOsAct1::amiRNA-BdVRN1/BdFUL1) [A]; Spike development detail [B1, pOsAct1::amiRNA-BdVRN1/BdFUL1 (scale bar: 0,4 cm) - B2, wt control (scale bar: 0,2 cm)]; Spikelet development detail [C1, pOsAct1::amiRNA-BdVRN1/BdFUL1 (scale bar: 1 mm) - C2, wt control (scale bar: 2 mm)]; Dissected floret exhibiting atrophied reproductive organs (carpel and stamens) [D1, pOsAct1::amiRNA-BdVRN1/BdFUL1 (scale bar: 0,2 mm) – D2, wt control (scale bar: 0,2 mm)]; SEM microscopy of a dissected floret: lod, lodicule; car, carpel; sta, stamen [E1, pOsAct1::amiRNA-BdVRN1/BdFUL1 (scale bar: 100 µm) - E2, wt control (scale bar: 500 µm)].

4.3.2.3 BdVRN1/BdFUL1 is expressed 8 days after flowering induction in inflorescence meristems

Given that BdVRN1 over-expressing lines showed an unexpected flowering phenotype, and the silence approach indicated possible floral defects, it was necessary to determine BdVRN1/BdFUL1 expression profile in the SAM at the time of flowering induction and/or floral development. In order to assess the spatial and temporal expression of BdVRN1/BdFUL1, SAM samples like the ones used to perform a transcript *in situ* hybridization in BdLFY were used, this time to localize BdVRN1/BdFUL1 expression. The expression results reveal that the BdVRN1 transcript is strongly up-regulated after 8 LD, with mRNA levels increasing even further at 14 LD (Figure 4.16). This indicates that BdVRN1/BdFUL1 is not involved in the transition from vegetative to reproductive growth, but it is necessary for proper inflorescence meristem development (Figure 4.16-C). Additionally, BdVRN1/BdFUL1 appears to be necessary for the posterior specification of the floral organs during the floret morphogenesis, as it is detected in the floral primordia (Figure 4.16-D). This results contrast significantly to the meristem identity behaviour of Arabidopsis AP1 but, on the other hand, BdVRN1/BdFUL1 and AP1 may share the floral homeotic functions.

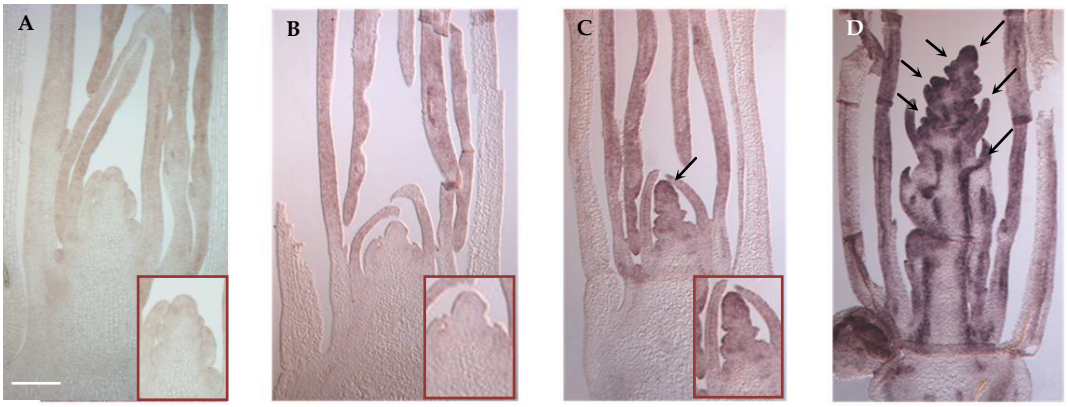


Figure 4.16: BdVRN1/BdFUL1 transcript immuno-localization at the shoot apical meristem (enclosed by layers of leaves) undergoing flowering transition: A) 0 LD; B) 2 LD; C) 8 LD; D) 14 LD. BdVRN1/BdFUL1 transcript is only detectable 8 days after flowering induction in the inflorescence meristem and in the floral primordial (arrows). Enclosed SAM close up in the lower-right corner. Scale bar: 0.2 mm:

4.3.3 Brachypodium's TERMINAL FLOWER 1 (BdTFL1) is a flowering repressor associated with the development of the floral meristem

The PEBP protein of Brachypodium TERMINAL FLOWER 1 (BdTFL1) was identified in a whole genome blast search, under the reference Bradi4g42400.1 (Figure 4.17). From the protein sequence alignment it's very evident the high level of conservation of TFL1-like between grass species and Arabidopsis (Figure 4.18).

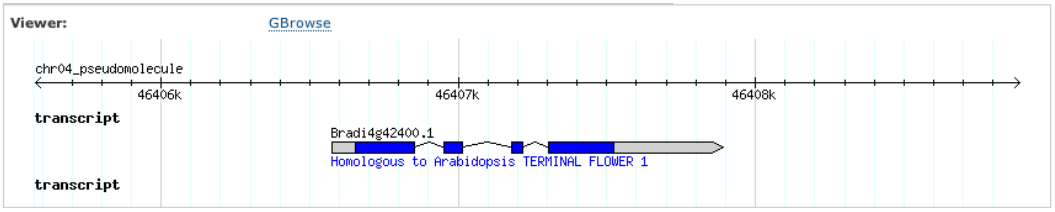


Figure 4.17: Schematic representation of Bradi4g42400.1 locus (Adapted from http://mips.helmholtz-muenchen.de/plant/brachypodium/)

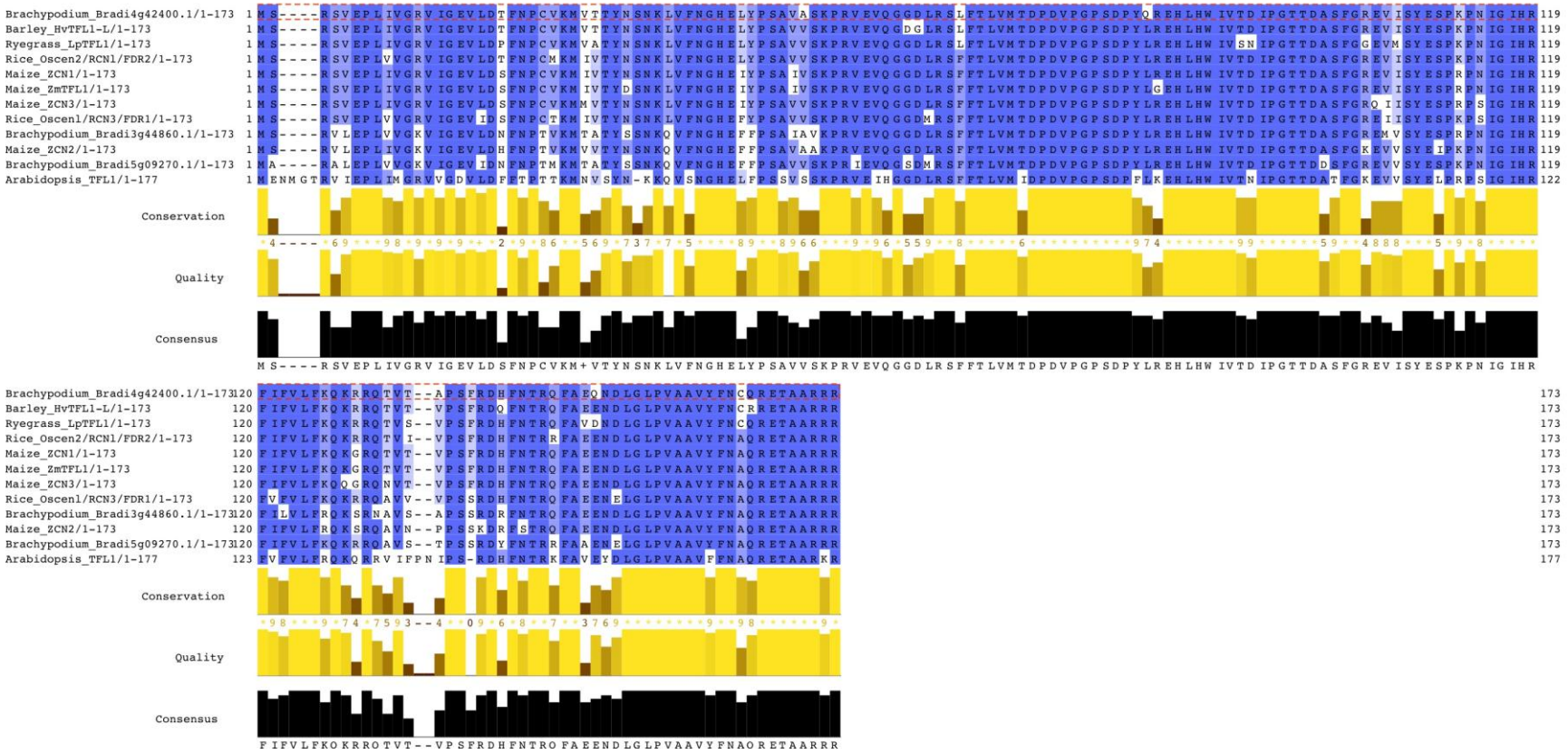


Figure 4.18: ClustalW alignment of TFL1 and the correspondent orthologues in different cereal species. Bradi4g42400.1 identified by the dashed-red box. Blue highlights represent aminoacid residue conservation amongst the aligned proteins: highly conserved aminoacid residues in dark blue and conserved aminoacid in light blue. Dashes represent residue insertions or deletions amongst orthologue peptide sequences. Yellow bars represent higher levels of aminoacid conservation between TFL1 orthologues, while brown bars represent regions with lower levels of aminoacid conservation between the aligned sequences. Black bars represent the conservation frequency of the aminoacid residues that make up the consensus sequence.

4.3.3.1 The constitutive expression of BdTFL1 delays flowering under inductive LD

Arabidopsis TFL1 protein is known to delay flowering transition when constitutively expressed by repressing the expression of floral organ identity genes, activated by FT (Hanano and Goto, 2011). In order to confirm if Brachypodium's TFL1 orthologue would be able to repress flowering transition, the full genetic sequence of Bradi4g42400.1 was cloned into a Gateway compatible plasmid, in front of the rice actin constitutive promoter (Himmelbach *et al*, 2007), and transformed into Bd21 according to Alves *et al* (2009). The over-expression of BdTFL1 resulted in a significant delay in flowering transition under inductive LD, widened the vegetative period (Danilevskaya *et al*, 2010), and produced bushier plants than the wt control (Figure 4.19) - a phenotype very much consistent with being the orthologue of Arabidopsis TFL1.

Figure 4.19: Constitutive expression of BdTFL1 delays flowering transition in LD: Wild-type (left), pOsAct1::BdTFL1 (right).

4.3.3.2 Silencing BdLTFL1 through the over-expression of a amiRNA may compromise inflorescence and floret development

As a flowering repressor that competes with FT for the genetic control over the inflorescence meristem identity genes, it was hypothesized that if BdTFL1 lost its function, the plants would transition into the reproductive development similarly to the BdFTL2 over-expression lines. With this in mind, an mRNA silencing approach was set up using the artificial microRNAs silencing strategy described before (Figure 4.20)(Ossowski, Fitz, Schwab, Riester, and Weigel personal communication; Warthmann *et al*, 2008; Himmelbach *et al*, 2007; Alves *et al*, 2009). The results, summarised in Figure 4.21 correspond to the T0 generation, and show in the most extreme cases a repetition of the aberrant spike phenotype, already mentioned for pOsAct1::amiRNA-BdLFY and pOsAct1::amiRNA-BdVRN1/BdFUL1, as well as for pOsAct1::amiRNA-BdFTL2 (see Chapter 3) where spikelet position has been defined at the top of the culm but no florets have differentiated (Figure 4.21-B1, C1). It is difficult to attest if this lack of floret formation is due to termination of the meristem as a result of BdTFL1 silencing or if, once again, it's due to a silencing-unrelated desiccation of the meristem derived from tissue culture regeneration. The few florets developed were able to correctly differentiate all floral organs in the appropriate verticil, and at the same time exhibit extensive cell collapse consistent with severe dehydration (Figure 4.21-D1, E1). The plant's architecture was affected with the normal elongation of the upper-most internode significantly suppressed (Figure 4.21-A). The spike phenotype wasn't carried through the next generation, and no difference in flowering time was detected between the amiRNA lines and the control plants; the levels of vegetative biomass also remained equal between the transgenics and the wt. The explanation for this loss of phenotype in the T1 is the same as for the other silenced genes already mentioned, only the lines with residual levels of gene silencing were able to produce fertile seed; however, given time restrictions it was not possible to repeat the transformation in order to confirm the mutant phenotype and measure BdTFL1 mRNA levels.

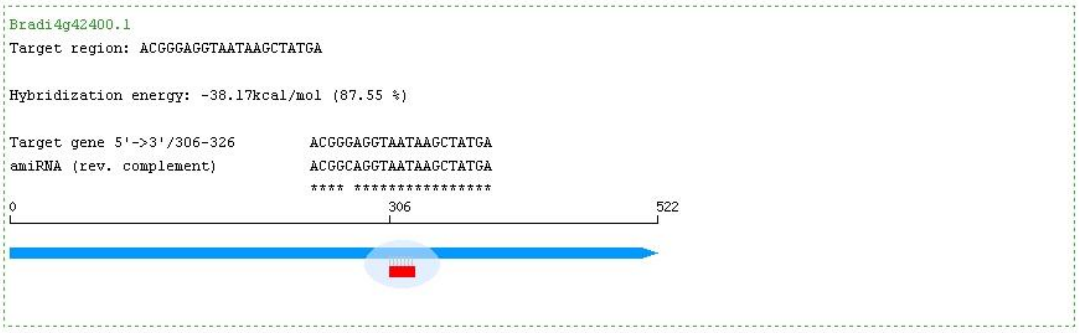


Figure 4.20: Schematic representation of the 21mer designed by the WMD3, and the correspondent target sequence in Bradi4g42400.1. Designed 21mer (red), targeted sequence (light blue) in Bradi4g42400.1's ORF of 522 bp (dark blue) (Ossowski, Joffrey, Schwab, Riester and Weigel personal communication).

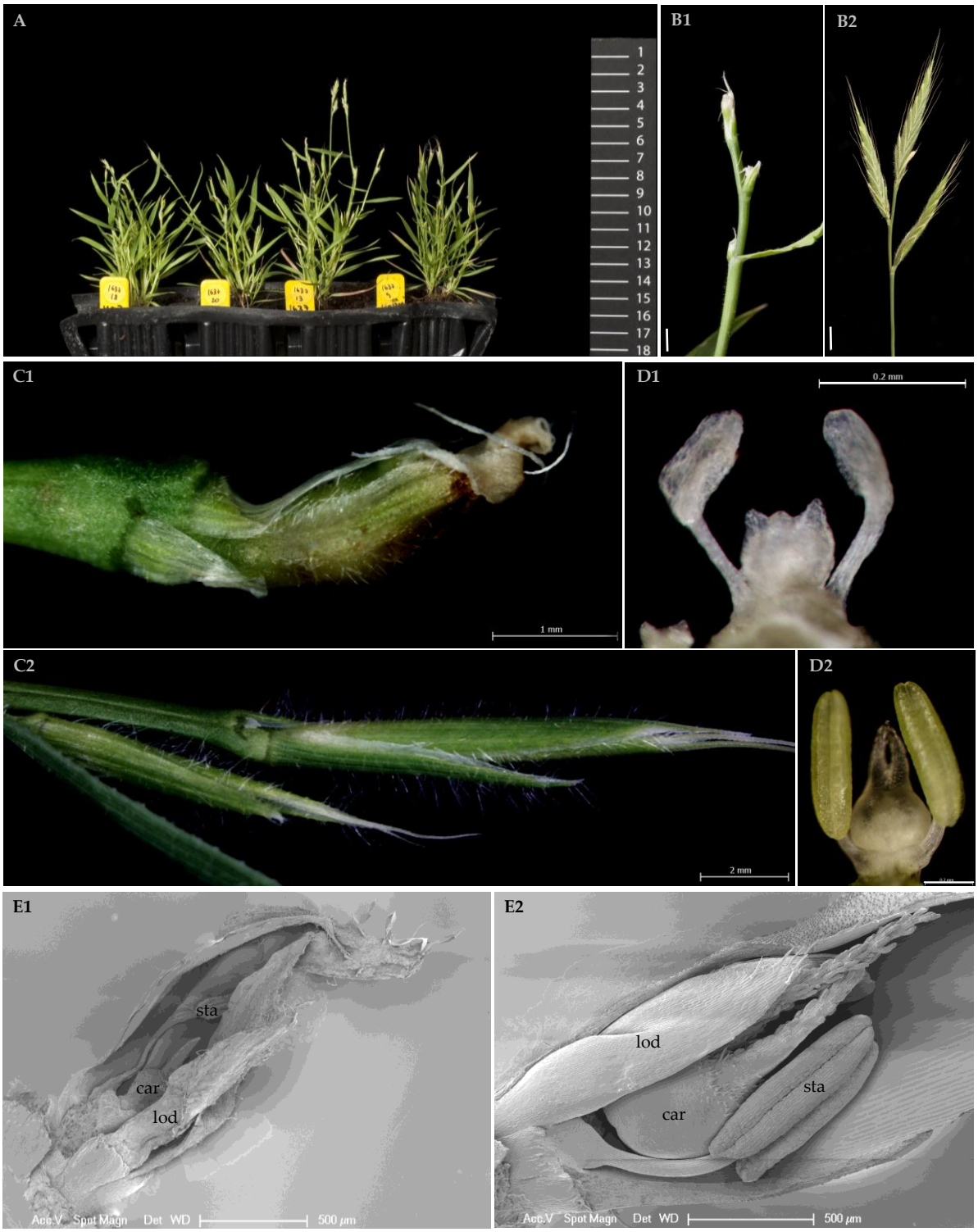


Figure 4.21: Transgenic plants expressing an artificial microRNA sequence that specifically targets BdTFL1 mRNA for degradation (pOsAct1::amiRNA-BdTFL1) [A]; Spike development detail [B1, pOsAct1::amiRNA-BdTFL1(scale bar: 0,4 cm) - B2, wt control (scale bar: 0,2 cm)]; Spikelet development detail [C1, pOsAct1::amiRNA-BdTFL1 (scale bar: 1 mm) - C2, wt control (scale bar: 2 mm)]; Dissected floret exhibiting atrophied reproductive organs (carpel and stamens) [D1, pOsAct1::amiRNA-BdTFL1 (scale bar: 0,2 mm) – D2, wt control (scale bar: 0,2 mm)]; SEM microscopy of a dissected floret: lod, lodicule; car, carpel; sta, stamen [E1, pOsAct1::amiRNA-BdTFL1 (scale bar: 100 µm) - E2, wt control (scale bar: 500 µm)]

4.3.3.3 BdTFL1 is expressed 14 days after flowering induction in the vasculature and apical dome of spikelet meristems

The BdTFL1 over-expressing lines showed a late flowering phenotype, possibly by repressing the inflorescence meristem identity genes at the SAM. The amiRNA silence strategy, on the other hand, produced plants with possible floral defects, consistent with the silencing data presented for BdLFY and BdVRN1/BdFUL1. These results were contradictory to the studies of Hanano and Goto (2011) in Arabidopsis, since TFL1 is thought to compete with FT for the regulation of the inflorescence meristem identity genes. However, as a gene that acts in the SAM but has been identified as a mobile signal it was necessary to determine if BdTFL1 has an mRNA expression profile in the SAM at the time of flowering induction and/or floral development or if only the mobile protein reaches the apex. In order to assess the spatial and temporal expression of BdTFL1, SAM samples like the ones used to perform a transcript *in situ* hybridization in BdLFY and BdVRN1/BdFUL1 were used, this time to localize BdTFL1 expression. The expression results revealed that the BdTFL1 transcript is strongly up-regulated after 14 LD (Figure 4.22-D). This indicates that BdTFL1 is not expressed in the SAM at the time of the transition from vegetative to reproductive growth (Figure 4.22-A to C), but it is up-regulated in the vasculature and apical domes of floral meristems (Figure 4.22-D), consistent with the work publish by Danilevskaya *et al*, 2010. BdTFL1 appears to be necessary for the maintenance of meristem indeterminacy during the floret morphogenesis (Figure 4.22-D) (Danilevskaya *et al*, 2010). Also consistent with the profile of TFL1, as a mobile signal, is the result of the reporter studies performed on transgenic Bd21 plants, using a GUS protein fusion under BdTFL1's native promoter. The results of this experiment revealed the presence of BdTFL1-GUS protein across the vasculature of Bd21 plants (Figure 4.22-E to G).

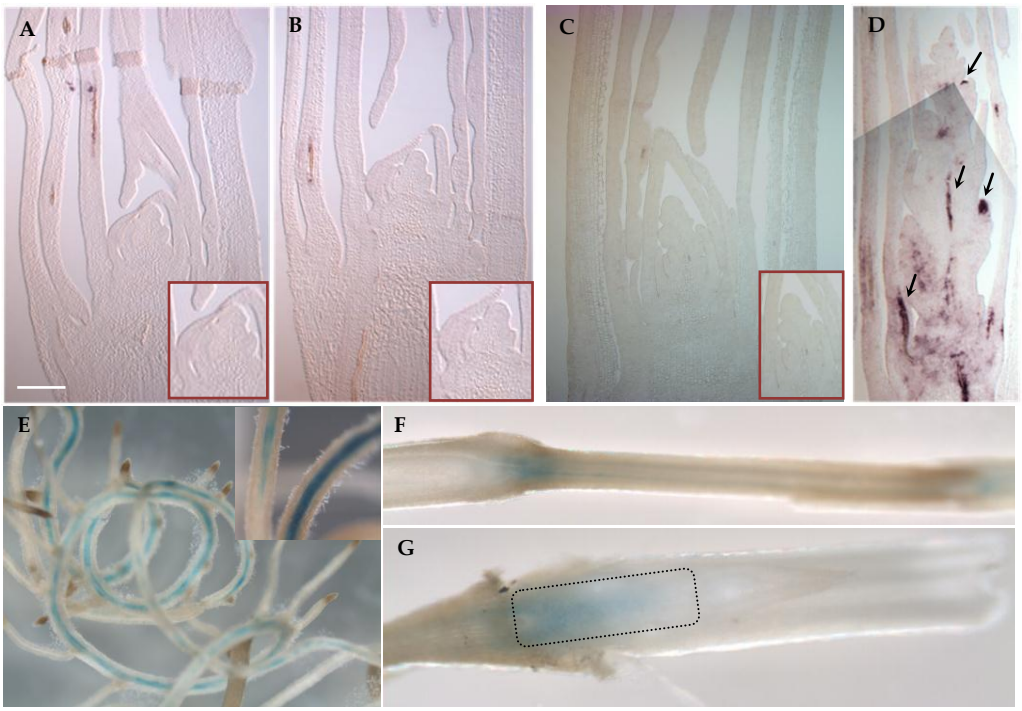


Figure 4.22: BdTFL1 transcript immuno-localization at the shoot apical meristem (enclosed by layers of leaves) undergoing flowering transition: A) 0 LD; B) 2 LD; C) 8 LD; D) 14 LD. BdTFL1 transcript is only detectable 14 days after flowering induction (arrows) in the vasculature and apical domes; scale bar: 0.2 mm. Enclosed SAM highlighted by the dashed-box. **Reporter studies of pBdTFL1::BdTFL1: GUS protein localization**: E) root; F) stem (node and internode); G) shoot apical meristem-dashed-box. Enclosed SAM close up in the lower-right corner.

4.3.4 BdFDL1 and BdFDL2 are both meristem specific bZIP proteins involved in reproductive development

Protein conservation amongst the members of the bZIP family of protein is quite low and restricted to the bZIP domain, making the *in silico* search for the BdFD orthologue very difficult (Figure 4.23, Figure 4.24, and Figure 4.25). There is one feature, however, that has been associated with FD's ability to heterodimerize with FT, and that has to be present in a potential FD-like orthologue, the CDPK site. On this site, it is imperative that residue 282 is occupied by a threonine or alternatively a serine. Mutations leading to the substitution of this residue with an alanine will abrogate CDPK function and prevent FD/FT interaction (Abe *et al*, 2005). When blasted against Bd21 genome annotation, the locus output for FD-like sequences producing high score segment pairs was Bradi3g00300 (BdFDL1). However, according to the amino acid sequence, Bradi2g21820 (BdFDL2) is much more closely related to wheat's FD orthologue, TaFDL2 (Higgins *et al*, 2010). After analysing the amino acid sequence of both BdFDL1 and BdFDL2 proteins, it was confirmed that both exhibit a serine at 282-equivalent position on the CDPK site, so without further discriminating features available both remain as potential partners to BdFTL2.

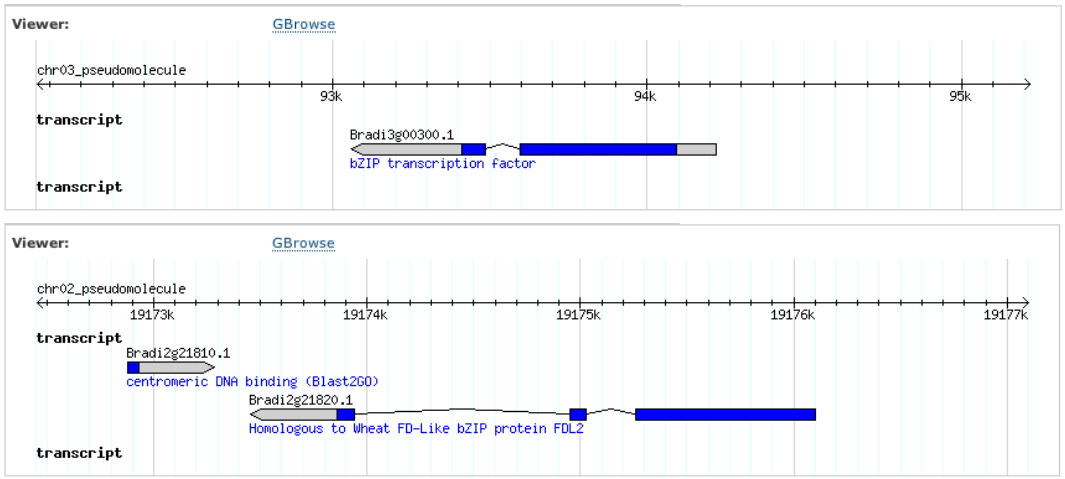


Figure 4.23: Schematic representation of Bradi3g00300.1 and Bradi2g21280.1 loci (Adapted from http://mips.helmholtz-muenchen.de/plant/brachypodium/).

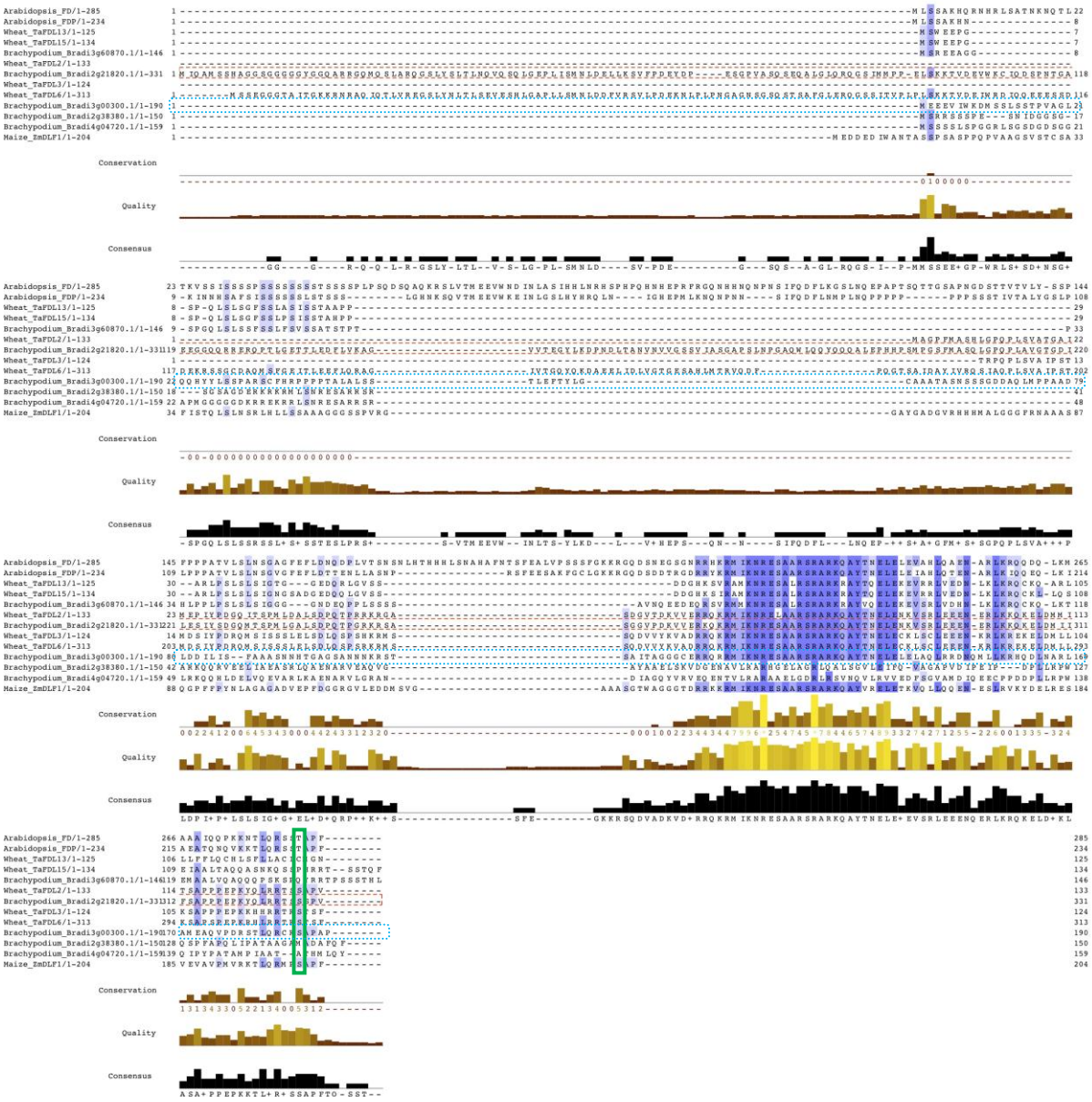


Figure 4.24: ClustalW alignment of FD and the correspondent orthologues in different cereal species. Highlighted in a green box is the amino acid residue 282 on the CDPK site, essential for heterodimerization with FT. Bradi3g00300.1 (BdFDL1) identified by the dashed-turquoise box, Bradi2g21820.1 (BdFDL2) identified by the dashed-red box. Blue highlights represent aminoacid residue conservation amongst the aligned proteins: highly conserved aminoacid residues in dark blue and conserved aminoacid in light blue. Dashes represent residue insertions or deletions amongst orthologue peptide sequences. Yellow bars represent higher levels of aminoacid conservation between FD orthologues, while brown bars represent regions with lower levels of aminoacid conservation between the aligned sequences. Black bars represent the conservation frequency of the aminoacid residues that make up the consensus sequence.

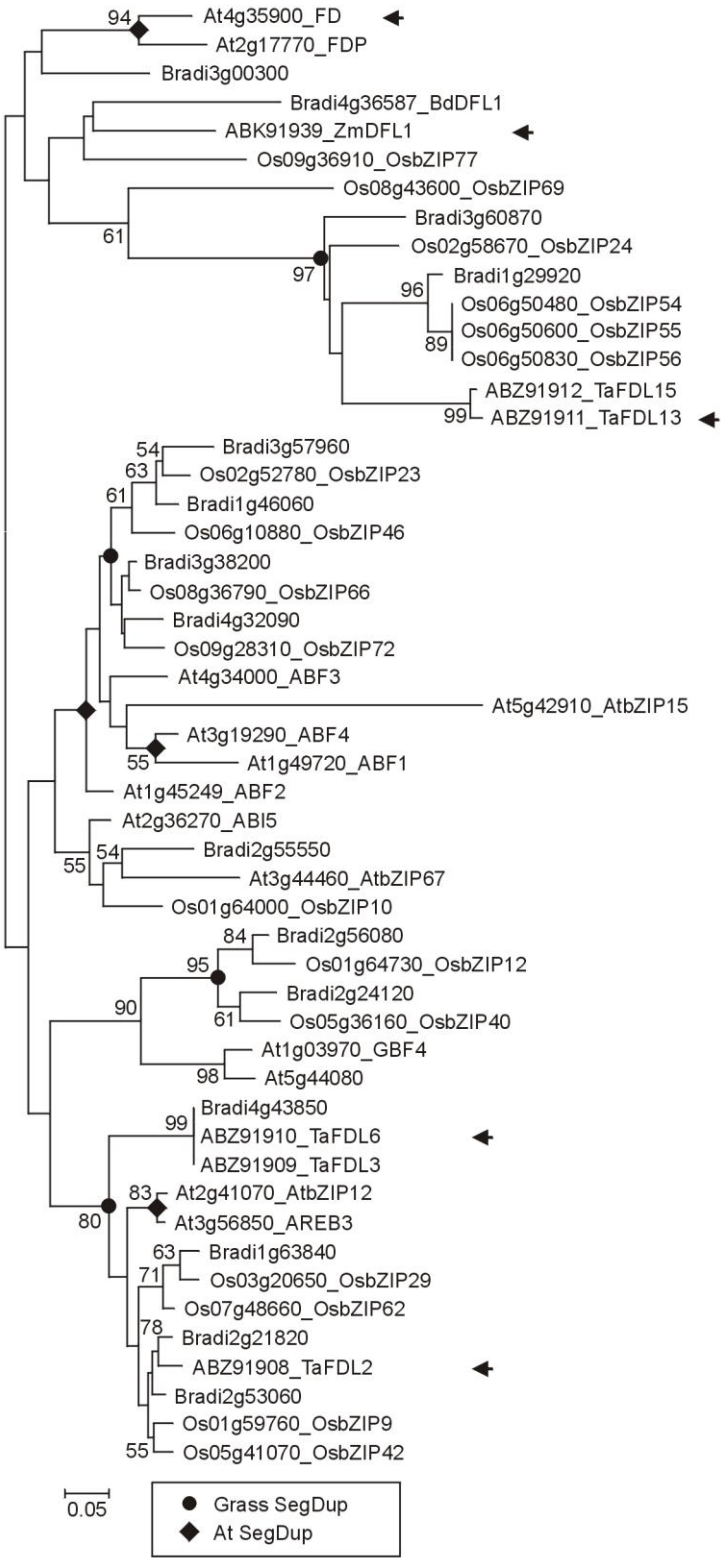


Figure 4.25: Phylogenetic relationship of FD and related bZIP proteins. NJ tree of FD and related basic region leucine zipper domain proteins domain alignment. The bZIP clade was identified with the online interrogatory tree using known Arabidopsis flowering-time genes as query. Black arrows show genes with roles in flowering (Adapted from Higgins *et al*, 2010).

4.3.4.1 The constitutive expression of either BdFDL1 or BdFDL2 is likely to delay flowering transition under inductive LD and compromise the development of the reproductive structures

Arabidopsis FD protein is known to accelerate flowering transition in both SD and LD when constitutively expressed by inducing the expression of floral organ identity genes at the SAM (Wigge *et al*, 2005). In order to determine if any of the of Brachypodium's FD-like proteins, would anticipate flowering transition, the full genetic sequence of both Bradi3g00300.1 and Bradi2g21820.1 were cloned into a Gateway compatible plasmid, in front of the rice actin constitutive promoter (Himmelbach *et al*, 2007), and transformed into Bd21 according to Alves *et al* (2009). The results presented in Figure 4.26 summarize the phenotype of T0 plants over-expressing BdFDL1 and BdFDL2. The flowering time of these transgenics has not been determined, but from the amount of vegetative biomass produced it is likely that the constitutive expression of both genes delay flowering transition in LD (Figure 4.26). However, this does not necessarily mean that these genes are flowering repressors and are not the co-activation partners of BdFTL2 and co-repression partners of BdTFL1. It is possible that as a consequence of their ectopic expression, BdFTL2 remained sequestered in the leaves and was unable to reach the SAM in order to activate flowering transition. The phenotype of these lines also included spike developmental defects and a degree of suppression of internode elongation (Figure 4.26).

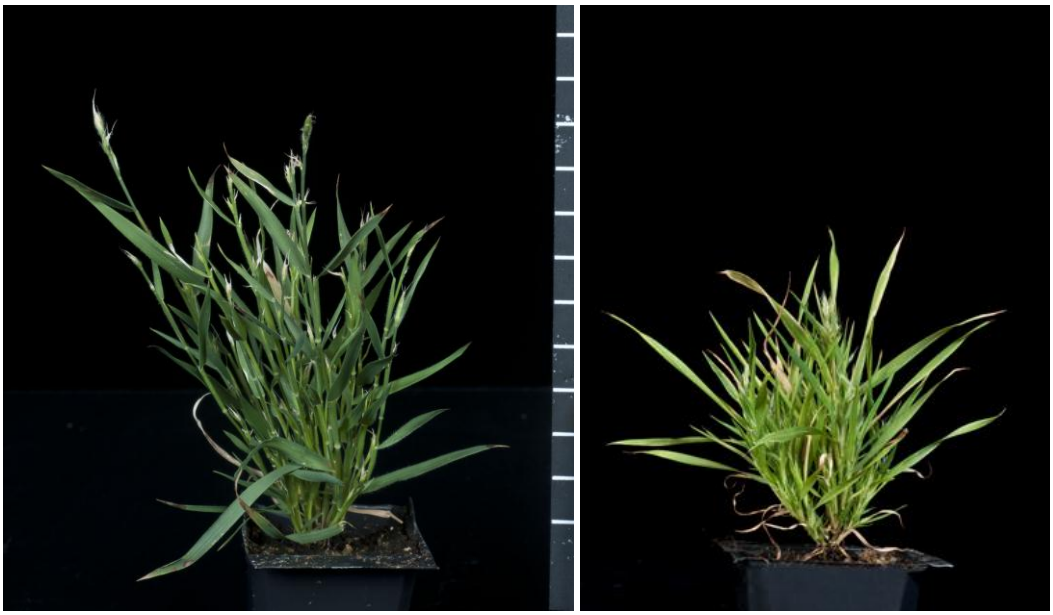


Figure 4.26: Constitutive expression of Bradi3g00300.1 (BdFDL1) and Bradi2g21820.1 (BdFDL2) in LD, T0: pOsAct1::BdFDL1 (left), pOsAct1::BdFDL2 (right).

4.3.4.2 Silencing BdFDL1 or BdFDL2 through the over-expression of a amiRNA may compromise inflorescence and floret development

Loss of function mutants of FD flower very late in LD, due to the inability of FT to bind AP1 promoter and trigger flowering transition (Wigge *et al*, 2005; Abe *et al*, 2005). In order to test if BdFDL1 and BdFDL2 loss of function transgenic plants would have a late transition into the reproductive development, as expected from FD of Arabidopsis, an mRNA silencing approach was set up using the artificial microRNAs silencing strategy described before (Figure 4.27) (Ossowski, Fitz, Schwab, Riester, and Weigel personal communication; Warthmann *et al*, 2008; Himmelbach *et al*, 2007; Alves *et al*, 2009). The results, summarised in Figure 4.28 and Figure 4.29 correspond to the T0 generation, and show in the most extreme cases a repetition of the aberrant spike phenotype, already mentioned for all the previously presented silence transgenic lines, where spikelet position has been defined at the top of the culm but no florets have differentiated (Figure 4.28-B1, C1; Figure 4.29-B1, C1). The pOsAct1::amiRNA-BdFDL2 plant's architecture was affected with the normal elongation of the upper-most internode significantly suppressed (Figure 4.29-A). The spike phenotypes of both silenced genes weren't carried through the next generation, and no difference in flowering time was detected between the amiRNA lines and the control plants; the levels of vegetative biomass also remained equal between the transgenics and the wt. The explanation for this loss of phenotype in the T1 is the same as for the other silenced genes already mentioned, only the lines with residual levels of gene silencing were able to produce fertile seed; however, given time restrictions it was not possible to repeat the transformation in order to confirm the mutant phenotype and measure BdFDL1 and BdFDL2 mRNA levels.

Figure 4.27: Schematic representation of the 21mers designed by the WMD3, and the correspondent target sequence in Bradi3g00300.1 and Bradi2g21820.1. Designed 21mers (red), targeted sequences (light blue) in Bradi3g00300.1's ORF of 573 bp [top] and Bradi2g21820.1's ORF of 996 bp [bottom] (dark blue) (Ossowski, Joffrey, Schwab, Riester and Weigel personal communication).

Figure 4.28: Transgenic plants expressing an artificial microRNA sequence that specifically targets BdFDL1 mRNA for degradation (pOsAct1::amiRNA-BdFDL1) [A]; Spike development detail [B1, pOsAct1::amiRNA-BdFDL1(scale bar: 0,4 cm) - B2, wt control (scale bar: 0,2 cm)]; Spikelet development detail [C1, pOsAct1::amiRNA-BdFDL1 (scale bar: 1 mm) - C2, wt control (scale bar: 2 mm)]; Dissected floret exhibiting atrophied reproductive organs (carpel and stamens) [D1, pOsAct1::amiRNA-BdFDL1 (scale bar: 0,2 mm) – D2, wt control (scale bar: 0,2 mm)]; SEM microscopy of a dissected floret: lod, lodicule; car, carpel; sta, stamen [E1, pOsAct1::amiRNA-BdFDL1 (scale bar: 100 µm) - E2, wt control (scale bar: 500 µm)].

Figure 4.29: Transgenic plants expressing an artificial microRNA sequence that specifically targets BdFDL2 mRNA for degradation (pOsAct1::amiRNA-BdFDL2) [A]; Spike development detail [B1, pOsAct1::amiRNA-BdFDL2 (scale bar: 0,4 cm) - B2, wt control (scale bar: 0,2 cm)]; Spikelet development detail [C1, pOsAct1::amiRNA-BdFDL2 (scale bar: 1 mm) - C2, wt control (scale bar: 2 mm)]; Dissected floret exhibiting atrophied reproductive organs (carpel and stamens) [D1, pOsAct1::amiRNA-BdFDL2 (scale bar: 0,2 mm) – D2, wt control (scale bar: 0,2 mm)]; SEM microscopy of a dissected floret: lod, lodicule; car, carpel; sta, stamen [E1, pOsAct1::amiRNA-BdFDL2 (scale bar: 100 µm) - E2, wt control (scale bar: 500 µm)].

4.3.4.3 BdFDL1 is expressed in both vegetative and reproductive meristems, while BdFDL2's expression is only initiated 8 days after flowering induction

The results from both the constitutive expression and the gene silencing strategy were not very elucidative about which one of these genes is the functional orthologue of FD. However, FD is required at the SAM to be able to interact with FT and TFL1 and regulate the expression of AP1. In fact, FD is expressed in the vegetative meristem and remains strongly up-regulated after flowering induction in the floral anlagen, and down-regulated in the floral primordia (Wigge *et al*, 2005). The BdFDL1 and BdFDL2 expression profiles in the SAM at the time of flowering induction and/or floral development could, therefore, provide important information that would contribute to the identification of Brachypodium's FD functional orthologue. In order to assess the spatial and temporal expression of BdFDL1 and BdFDL2, SAM samples like the ones used to perform previous transcript *in situ* hybridization were used, this time to localize the mRNA expression of this pair of genes (Figure 4.30). The expression results reveal that the BdFDL1 transcript is highly similar to the one of Arabidopsis FD, i.e., it is already expressed at the vegetative meristem, becoming strongly up-regulated after flowering induction in all tested samples, while not expressed in the floral primordia (Figure 4.30, top). BdFDL2, on the other hand, was also up-regulated in the SAM, but only 8 days after flowering induction (Figure 4.30, bottom), which indicates that it is likely not to be involved in the BdFTL2 mediated flowering induction process. This indicates that, BdFDL1 is likely to mediate the transition from vegetative to reproductive growth, as well as being involved in the proper inflorescence and floral meristem development, while BdFDL2 activity seems to be restricted to the inflorescence and the floral meristem development.

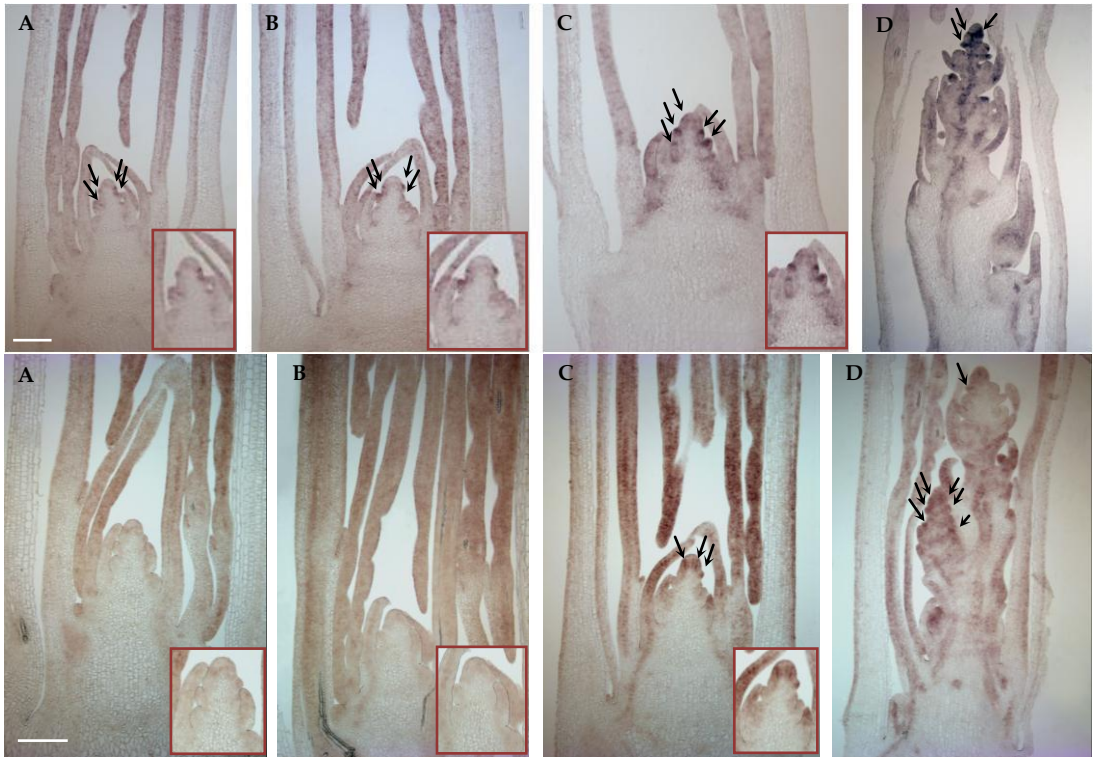


Figure 4.30: BdFDL1 (top) and BdFDL2 (bottom) transcript immuno-localization at the shoot apical meristem (enclosed by layers of leaves) undergoing flowering transition: A) 0 LD; B) 2 LD; C) 8 LD; D) 14 LD. BdFDL1 transcript is detectable in all tested samples (arrows). BdFDL2 transcript is only detectable 8 days after flowering induction in the inflorescence meristem and floral primordia (arrows). Enclosed SAM close up in the lower-right corner. Scale bars: 0.2 mm.

4.4 Discussion

LFY-like genes can be found in all kinds of plant genomes, including mosses and gymnosperms, and they exist as a highly conserved single (or low copy) locus that codifies for a peptide with no resemblance to any other known protein sequence (Reviewed by Moyroud *et al*, 2009); Brachypodium is no exception and in its proteome only one peptide can be found with high homology to Arabidopsis LFY. The involvement of BdLFY in the reproductive development of Bd21 was, firstly, investigated through the constitutive expression of its full genomic sequence, and the result were transgenic plants with no abnormal phenotype, i.e., it was not able to accelerate flowering, or interfere in the normal inflorescence and floral development. Although this result is highly inconsistent to Arabidopsis' LFY behaviour, there have been reports describing the lack of functional conservation between orthologues, of which the rice RFL is good example. Also, in petunia, the over-expression of ARABIDOPSIS LEAFY (ALF) does not produce any noticeable phenotype but, on the other hand, the over-expression of DOUBLE TOP (DOT), ALF's co-regulator, leads to early flowering and the transformation of the inflorescence into a single flower (Reviewed by Moyroud *et al*, 2009). In contrast, the silencing strategy rendered a very aberrant phenotype to the inflorescence and floret morphology on the T0 generation, leading to a massive degree of sterility among the transgenic population. Such dramatic features were not passed onto the next generation, probably because only weekly silenced lines were actually fertile. But, this justification is not completely satisfactory as the few florets produced by the most affected plants displayed extensive cell collapse consistent with desiccation, and coincidently this phenotype is shared across all the other differently silenced transgenics. Since all the silencing events were transformed into the same pool of calli, one cannot rule out the possibility of somaclonal variation within the callus cells prior to transformation as the cause for such drastic phenotype. It would be necessary to, in a future opportunity, repeat those transformations with separate batches of calli, along with the regeneration of untransformed shoots to be able to confirm the silencing phenotype. However, with the transcript immuno-localization assay it was possible to contextualize the spatiotemporal expression profile of BdLFY with the development of the floral meristem, and rule out any BdLFY participation in the transition from vegetative into reproductive growth. However, there is a definitive involvement of BdLFY in the specification of the floral organs, therefore, it would be pertinent to perform a ChIP-on-chip or ChIP-seq experiment to determine the downstream targets of BdLFY in the context of Bd21 floral development.

The VRN1 gene of cereals is currently associated with AP1 of Arabidopsis through the evidences of protein sequence homology, and the correlation between its expression levels and the presence of inductive stimulus and florigenic signals. The Bd21 VRN1 gene was easily identified and cloned to be over-expressed with the purpose of inducing precocious flowering and, thereby, confirming its meristem identity function as the downstream target of BdFTL2. The results, however, were not as predicted. The transgenic plants did not flowered ahead of wt plants, indicating that independently of the previously mention facts, functionally BdVRN1 is not able to induce flowering. By silencing its expression under

inductive conditions it was thought that the meristem would remain vegetative for longer, and so flowering would be delayed in relation to non-silenced plants. This, however, did not happen but, instead, there were severe inflorescence and floret morphological defects that were not carried through the next generation, and require further confirmation. It was with the in situ hybridization technique that became possible to determine that BdVRN1 transcript is only actually up-regulated after floral evocation, and is strongly expressed in floral meristems; this means that BdVRN1 cannot serve as the inflorescence meristem identity gene, recipient of the florigenic message, as initially supposed. Still, this gene is expected to have a very important role in the development of the floral organs and, therefore it would be very interesting to study its function and its downstream targets. Again, this could be achieved by determining the all promoter binding sites through a ChIP-on-chip or ChIP-seq experiment.

Bd21 has three very well conserved TFL1-like proteins, one of which with higher levels of amino acid similarity to the Arabidopsis homologue. This protein was cloned and over-expressed in order to confirm its ability to delay flowering. The resulting transgenics, as expected, flowered significantly later than wt and developed a vast amount of vegetative biomass resembling wt plants grown on SD. The silenced lines, however, developed the very same sterility defects, as previously mentioned, that require further confirmation. The expression studies were consistent with the presence of mRNA and protein in the vasculature of the plant, including the apical dome of the floral meristem, 14 days into the induction of flowering. These results indicate high level of functional conservation towards the TFL1 family of PEBPs across plants, in the maintaining of the indeterminacy of the meristem (Olsen *et al*, 2006).

The *in silico* search for the most likely candidate to be the FD homologue in Brachypodium was particularly difficult. This because when compared with other related peptides of the same species or different species , all family members of the bZIP class of proteins have little homology between them, being related only by one highly conserved DNA binding domain. According to the literature, it is known that FD protein heterodimerizes at the shoot apical meristem with both FT and TFL1 proteins, before binding to the DNA to exercise its regulatory function upon downstream target genes. It has also been reported that there is a very important calcium-dependent protein kinase (CDPK) site at the C-terminus of FD that if mutated prevents the interaction with FT. This feature was considered when analysing the list of potential candidates to be BdFD, and there were two proteins that not only contained a potential CDPK site with the correct amino acid residue, but also were phylogenetically related to two FT interacting peptides that have been described before, FD from Arabidopsis and TaFDL2 from wheat. Experiments were performed on both candidates to evaluate their expression profile and biological function, in order gather information that could help set them apart. From the constitutive expression of both genes very little is possible to conclude at this point in time, given that the results presented concern the T0 generation, however, the (preliminary) phenotype of those plants indicates that ectopic expression of these bZIP proteins may delay flowering transition in LD and possibly cause defects on the inflorescence development and on the elongation of

the upper-most internodes. These observations relate to the high level of vegetative biomass, the low height of the plant at maturity, and the abnormal spikelet formation. The possibly late flowering phenotype could be explained by the ectopic levels of BdFDs could sequester BdFTL2 in the leaves and preventing the florigenic signal from reaching the meristem, while endogenous levels of BdTFL1 would be free to bind BdFDs in the SAM and delay flowering transition. By silencing the expression of both genes, however, it would be possible to prevent the interaction of BdFD with BdFTL2 in inductive LD and, in this way, delay flowering transition. The results of the amiRNA silencing strategy resulted in a very unusual inflorescence phenotype, already observed before in other silenced lines. These abnormalities were not carried through the next generation, with all plants resembling wt controls on both the morphology and time to flower, and need further confirmation. But from the transcript immuno localization it was possible to determine that these proteins, although both expressed at the SAM, they do not share the same expression profile. In fact, BdFDL1 is expressed before flowering transition and later up-regulated, in an FD similar fashion, at the inflorescence and floral meristems. BdFDL2, on the other hand, is only expressed a few days after flowering transition had occurred, indicating that from these two peptides, BdFDL1 is likely to be FD's functional orthologue in Bd21. Still, it would be necessary to determine if BdFD1 is able to bind BdFTL2 and BdTFL1. This could be achieved by performing a yeast 2-hybrid assay between the bZIPs BdFDL1 and BdFDL2 and the PEBPs BdFTL1 and BdTFL1.

Chapter 5

Transitioning into the reproductive phase–Part II

5.1 Introduction

In the previous chapters it became clear that the search for the functional orthologues that mediate flowering transition in Brachypodium cannot rely, solely, on the analysis of protein sequence homology and phylogenetic relationship to other, well studied, genetic elements. Independent gene duplication events between species, followed by genetic specialization or subfunctionalization (Carretero-Paulet and Fares, 2012) often are the causes of the regulatory discrepancies later found in apparently conserved biological pathways. Still, it remains very important to gather valuable insight from the study of different biological systems so to build a scaffold of genetic and functional conservation between species, which will serve as the natural starting point to investigate the regulatory and physiological particularities of a given organism.

Today, technology allows us to examine the genetic shifts occurring at a particular time, within an organ or tissue of interest, through whole transcriptome microarray hybridization, and identify candidate loci with an interesting expression profile that are likely to regulate the course of a given biological process. In the second part of analysing the transition into the reproductive phase of Bd21, microarray technology has been employed in an attempt to determine the meristem identity gene, recipient of the mobile florigenic stimulus, which marks the beginning of the SAM's reproductive fate and is central to the contextualization of the flowering induction pathway. In addition to the identification of all the genetic elements displaying a significant change in their expression profile, the microarray can also provide, in an indirect way, information regarding all biological processes that are required to cease or initiate, in order to allow flowering to occur, and clues as how these are likely to be structured in the pathway hierarchy.

5.1.1 Plant development and the shoot apical meristem

The shoot apical meristem (SAM) is a self-regenerating tissue, composed of a group of pluripotent meristematic stem cells (Verdeil *et al*, 2007), that is located on the tip of the shoot axis, and is responsible for the post-embryonic development of the plant through the production of stem tissue and the differentiation of lateral organs (Haecker and Laux, 2001; Bowman and Eshed, 2000). During the vegetative growth, this group of cells continuously differentiate, on its flanks, modular units called phytomers consisting of a leaf, a node, an axilary bud, and an internode. These phytomers assemble into individual tillers that, collectively, will constitute the body mass of the plant, as it develops and grows (Figure 5.1, left). In the presence of inductive stimuli the plant enters the reproductive phase, by undergoing prominent macroscopic developmental changes: the arrest of phytomer formation, the substantial expansion of the internodes, and the emergence of the reproductive structures, collectively known as the inflorescence (Meier, 2001). The transition between the vegetative and the reproductive phases, however, is determined at the microscopic level in shoot apical meristem on the apex of the culm enclosed by several layers of leaves (Figure 5.1, right). As it progresses into floral development the SAM encompasses a series of morphological changes that typify the conversion of a shoot producing meristem into an inflorescence meristem followed by the subsequent transformation of the latter into a floral meristem, where the differentiation the floret structures will occur. This sequence of developmental steps, however, relies of the coordination of three groups of genes that regulate floral morphogenesis: 1) the meristem identity genes, which encode for transcription factors that induce the expression of floral organ identity genes; 2) the floral organ identity genes, which encode transcription factors that regulate the expression of all the downstream genetic elements involved in the specification of floral organs; and 3) the cadastral genes that define spatial boundaries to the expression of floral organ identity genes, and impede their unrestrained ectopic expression (Weigel and Meyerowitz, 1994).

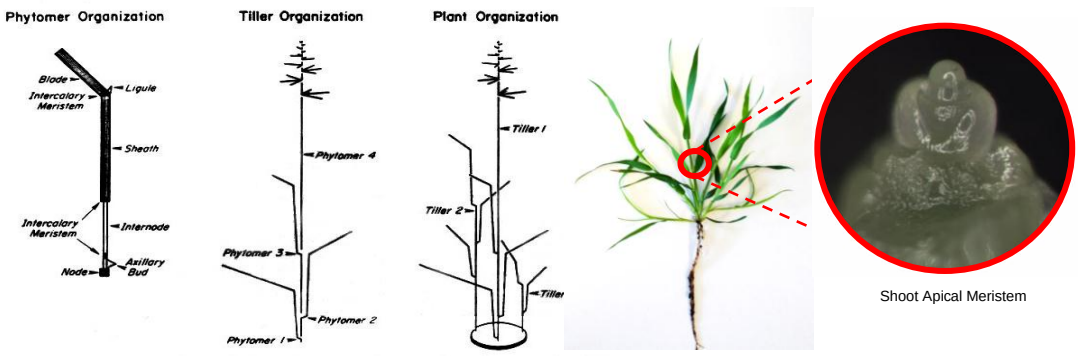


Figure 5.1: The developmental morphology of grasses originates from the successive differentiation of phytomers from the apical meristem of individual tillers (Left) (Adapted from Etter, 1951 *in* Briske, 1991); Shoot apical meristem dissected from a *Brachypodium distachyon* Bd21 tiller (Right).

5.1.1.2. The ABCDE model of floral development

In Arabidopsis, flowers are composed by four different kinds of floral organs that are displayed in concentric circles, whorls, around the centre of the vertical axis. The first outmost whorl is the calyx consisting of four green sepals that exist to protect the flower in the bud. The second inner whorl is the corolla, composed of four white petals that work to attract pollinators. The third inner whorl is defined as androecium, and it holds six pollen producing stamens (the male reproductive organs). The fourth innermost whorl is the gynoecium, and it contains the carpels (the female reproductive organs) where the ovules are produced.

The arrangement of floral organs in 4 whorls on the meristem is specified the overlapping expression of different types of floral organ identity genes, according to the ABCDE model of floral development (Figure 5.2-B) (Coen and Meyerowitz, 1991; Coen and Carpenter, 1993). The model, built on the study of homeotic mutants with misplaced floral organs, summarizes how a group of transcription factors controls the complete regulatory program involved in the formation of a particular floral structure and determines the number and position at which it will be developed. Most plant homeotic genes belong to a family of transcription factors that share a conserved peptide motif, called MADS box domain (acronym for MCM1 from *Saccharomyces cerevisiae*, AGAMOUS from *Arabidopsis thaliana*, DEFICIENS from *Antirrhinum majus*, SRF from *Homo sapiens*), which allows the proteins to bind DNA at a specific nucleotide sequence and alter gene expression. Plant homeotic proteins are classified according to their activity into five different classes: The type A activity specifies organ identity in the first and second whorls, as class A loss-of-function mutants - like *apetala1* (*ap1*) and *apetala2* (*ap2*) - display, in the first whorl, carpels instead of sepals and, in the second whorl, stamens instead of petals. The type B activity controls organ identity in the second and third whorls, since class B loss-of-function mutants - like *apetala3* (*ap3*) and *pistillata* (*pi*) - exhibit, in the second whorl, sepals instead of petals and, in the third whorl, carpels instead of stamens. The type C activity determines organ identity in the third and fourth whorls, for the reason that the class C loss-of-function mutant - *agamous* (*ag*) - possesses, in the third whorl, petals instead of stamens and, in the fourth whorl, a new flower instead of carpels – which results is an indetermined floral meristem where flowers continue to form within flowers. Class B activity is independent from Classes A and C, while Classes A and C repress each other, meaning that they have both cadastral and floral identity functions (Weigel and Meyerowitz, 1994). The class ABC loss-of-function quadruple mutant (*ap1*, *ap2*, *ap3/pi*, *ag*) produces floral meristems that give rise to green leaf-like structures in the floral verticils. The type D activity is strongly related to type C activity and determines ovule development (Liu *et al*, 2009; Chen *et al*, 2012; Colombo *et al*, 1995), and type E activity is required for the specification of all floral organs (Pelaz *et al*, 2000; Vandenbussche *et al*, 2003; Favaro *et al*, 2003; Ditta *et al*, 2004).

Monocot plants, however, have a different type of floral arrangement. They are trimerous, which means that flower organs are defined as multiples of three and, in the particular case

of cereals, they exhibit a radically different morphology of the perianth (Figure 5.2-A). The first outmost whorl consists of two green bract-like structures, possibly equivalent to sepals that, when combined, enclose and protect the inner reproductive organs - the uppermost, called palea, is thought to derive from the fusion of two independent organs; and the lowermost bract called lemma, often possesses a long bristle named awn. The second innermost whorl in cereals is equivalent to petals (Whipple *et al*, 2007). These are scale-like structures, called lodicules, which sit at the base of the verticil and are functionally designed to swell and force the aperture of the outer bracts to expose the inner reproductive organs to wind pollination (reviewed by Yoshida, 2012). The third and fourth inner whorls of cereals, consisting of the androecium and gynoecium, respectively, remain analogous to those of the conventional flower. The floral development of grasses is still very poorly understood. There are reports that mention that the rice genome harbours genetic sequences highly similar to the homeotic genes of Arabidopsis, and that Class B function appears to be conserved in maize and rice. These are good indications that the floral morphogenesis in angiosperms can be commonly summarised by the ABCDE model for floral development, firstly described for Arabidopsis and Antirrhinum (Figure 5.2-B). On the other hand, there are also reports that highlight regulatory differences in the Class C function of grasses, which appears to be subfunctionalized after gene duplication (Whipple *et al*, 2004; Yamaguchi *et al*, 2006; Yoshida and Nagato *et al*, 2011).

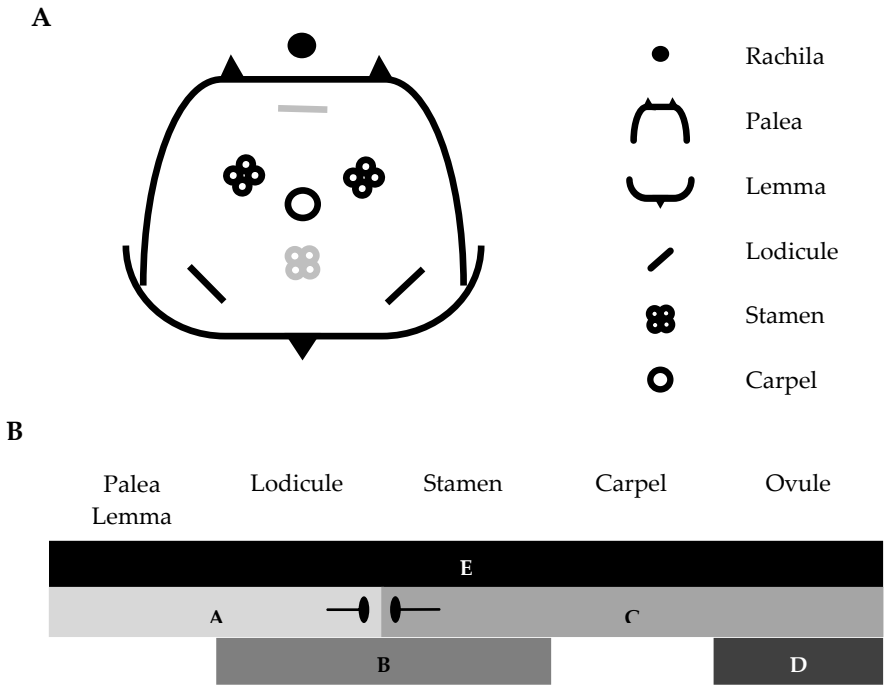


Figure 5.2: A) Schematic representation of a *Brachypodium distachyon*'s floret. First outermost whorl defined by the palea and lemma, second whorl defined by the lodicules, the thirds whorl defined by the stamens, and the fourth whorl defined by the carpel. Symbols in grey represent aborted organs. **B) The ABCDE-model of floral organ specification from Theissen (2001) and Ditta *et al* (2004) adapted to the patterning of the grass floret according to Ambrose *et al* (2000)**: class A genes specify the identity of the first outermost whorl (palea, lemma), the simultaneous activity of class A and B genes specify the second whorl (lodicules), the simultaneous activity of class B and C genes specify the third whorl (stamens), the activity of class C genes specify the fourth whorl (carpel), class E genes are essential for ovule development, and class E genes need to expressed for the specification of all floral organs.

5.2 Rational

From the analysis of the results in the previous Chapter it became evident that important flowering transition genetic makers such as LFY or AP1 do not remain functionally conserved in Bd21 plants; which means that the recipient(s) of BdFTL2's florigenic signal, at the SAM, remains unknown. Traditional methods for expression studies such as Northern blots, quantitative real time PCR (qPCR), or transgenic reporter lines are limited in the number of genes that can be monitored at a given time. Microarrays have become a widely used tool for the generation of gene expression data on a genomic scale. This large scale expression analysis monitors, simultaneously, the expression levels of tens of thousands of genetic loci, and it can be employed in functional genomics studies to identify mRNAs that may or may not be translated into proteins. It provides insight into biological processes and metabolic pathways, and can be used to investigate the spatial and/or temporal expression patterns in different plant tissues and in response to different environmental stimuli. The microarray is a collection of genetic probes representing single genes arrayed in a solid surface, such as glass, plastic or silicon chip, by covalent attachment that is based on the hybridization properties of nucleic acids under high-stringency conditions, and in a fluorophore-based way of detection. Relative intensity of the fluorophore is used to determine up-regulated and down-regulated genes in a ratio-based analysis.

The Affymetrix array designed for Brachypodium is based on the 8x genome sequence of the community standard line Bd21, and in an EST-based empirical transcriptome analysis. The array was designed to contain ~6.5 million unique, single-copy, perfect match (PM) probes, with mean probe spacing of 42 bases (Figure 5.3). On average, exons and introns are represented by 11 probes with 95% of exons or introns targeted by more than 5 probes. The array includes a total of 2,548,624 probes targeting exons and introns, 1,062 control probes, 2,050 reporter probes, and 33,886 antigenomic surrogate mismatch (MM) probes (http://www.intl-pag.org/18/abstracts/W13_PAGXVIII_095.html)

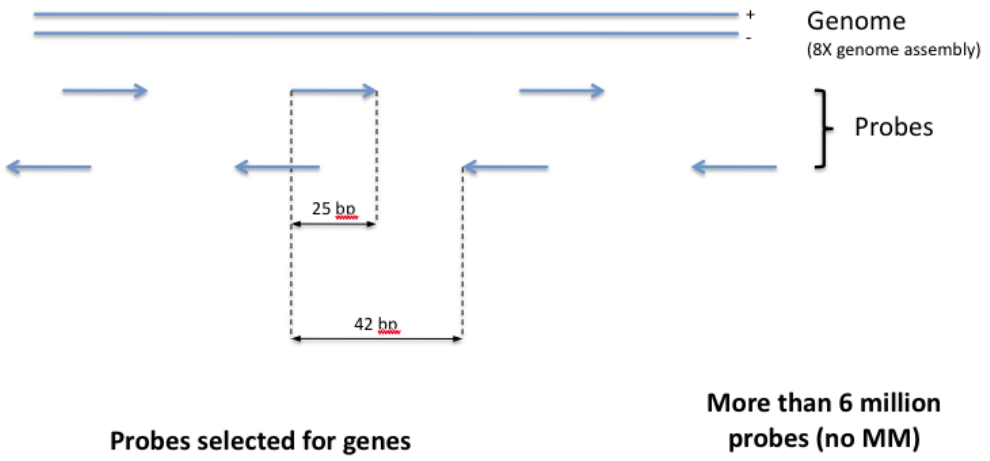


Figure 5.3: Schematic representation of Affymetrix's microarray platform for *Brachypodium distachyon* (Bd21) 8x genome assembly (Courtesy of Dr René Dreos)

5.3 Results

5.3.1 Whole transcriptome microarray analysis of the flowering SAM

In order to identify, in a broad scale analysis, the genetic mediators of Brachypodium's floral transition, it was necessary to perform a preliminary characterization the SAM's morphology, regarding flowering induction, to determine the decisive developmental stages that shape reproductive growth (inflorescence identity) and the differentiation of the floral organs (floral identity). Following this characterization, a whole transcriptome microarray analysis was set up using four independent groups of 40 Bd21 plants that were grown under non-inductive conditions of 14 h light (SD) until they had surpassed the juvenile phase (6 to 7 leaves), and later florally induced by growing them under 20 h of light (LD), until harvest. The microarray samples consisted of SAMs dissected under a binocular microscope in a time series, starting with the non-induced 0 LD plants - control; followed by the 2 LD grown plants, coincident with florigen influx at the SAM; the 7 LD grown plants that had reached the inflorescence meristem stage, and 11 LD plants at the floral meristem stage (Figure 5.4). The dissected SAMs, kept separate according to their developmental stage, were immediately frozen in dry ice and grinded to extract the corresponding RNA that was later hybridized in the microarray chip - 3 biological replicates were used for control purposes.

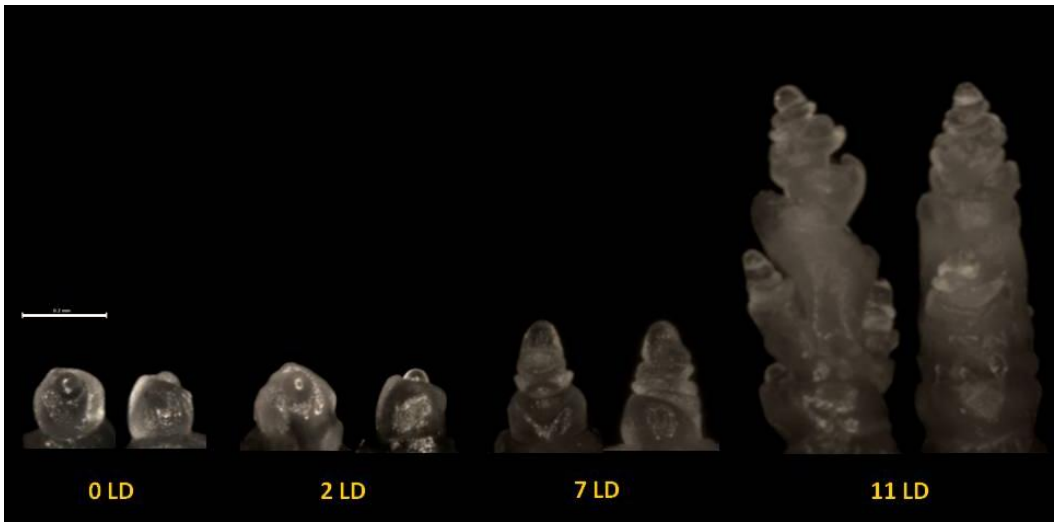


Figure 5.4: ***Brachypodium distachyon*** **(Bd21) dissected shoot apical meristems undergoing floral differentiation, frontal and lateral views**: 0 LD vegetative meristem; 2 LD transitioning meristem; 7 LD inflorescence meristem; 11 LD floral meristem. Scale bar: 0.2 mm

Only genes with a consistent expression pattern across the three replicates were considered highly significant. From those, 4065 genes displayed a significant fold change ≥1, of which 3084 transcripts were down-regulated and 3256 transcripts were up-regulated in, at least, one of the tested conditions. From this list of genes, only the ones showing a consecutively up- or down-regulation across all samples (2 LD, 7 LD, and 11 LD) or in the two latest developmental stages (7 LD and 11 LD) were analysed further (Figure 5.5).

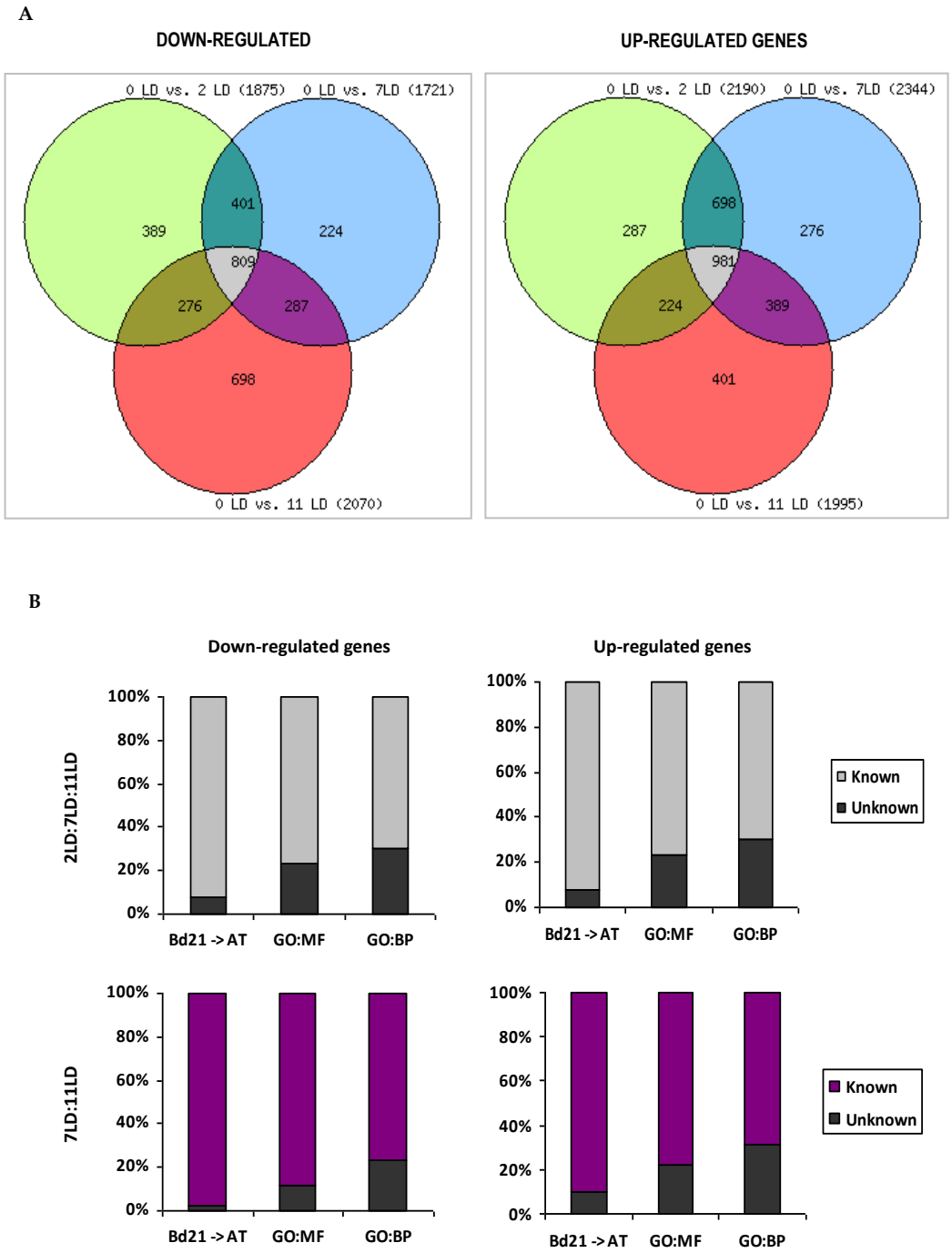


Figure 5.5: A) Venn diagram of the transcripts with ≥1 fold change in the microarray analysis: up-regulated (right), down-regulated (left). Program used for the elaboration of the Venn diagram at http://www.pangloss.com/seidel/Protocols/Venn.cgi. **B) Summary of the Cytospcape/BiNGO analysis of the genetic elements consistently up- or down-regulated after the photoperiodic induction of flowering of *Brachypodium distachyon*, for the categories 2LD:7LD:11LD and 7LD:11LD, as determined by the whole transcriptome microarray hybridization results.** Bd21 → AT represents the percentage of Bd21 loci that have a known *in silico* orthologue (p ≤ 1E-5) in *Arabidopsis thaliana*'s proteome; GO:MF represents the percentage of genes with known molecular function in the gene ontology database; GO:BP represents the percentage of genes associated with a known biological process in the gene ontology database.

To be able to functionally identify all the genetic elements in the above sub-groups of up- and down-regulated genes it was necessary to perform a homologue protein BLAST search into *Arabidopsis thaliana* proteome database (http://www.arabidopsis.org), followed by a gene ontology analysis using the Cytoscape v2.8.3 software platform (Cline *et al*, 2007) and BiNGO v2.44 plugin (Maere *et al*, 2005).

Out of the 809 genetic elements consecutively down-regulated across all samples (2 LD, 7 LD, and 11 LD), 8% have no correspondent orthologue in the Arabidopsis proteome, 23% have no known molecular function, and 30% are involved in an unknown biological process (Figure 5.5-B). Similarly, from the 981 genetic elements up-regulated at the same developmental phases, 7% have no corresponding orthologue in Arabidopsis proteome, 22% have no known molecular function, and 30% are involved in an unknown biological process (Figure 5.5-B). Regarding the genetic elements consecutively down-regulated only in the later developmental stages (7 LD, and 11 LD), out of the 287 genes analysed, 2% have no correspondent orthologue in Arabidopsis proteome, 12% have no known molecular function, and 23% are involved in an unknown biological process (Figure 5.5-B). At the same time, of the 389 transcripts up-regulated in those conditions, 10% have no correspondent orthologue in Arabidopsis proteome, 22% have no known molecular function, and 31% are involved in an unknown biological process (Figure 5.5-B). The remaining transcripts with a significant fold change in expression were identified as being involved, as well as with other biological processes, in vegetative phase change; taxis; specification of floral organ identity; senescence; secretion; pigmentation; meiosis; cellular respiration; cell adhesion; abscission; phloem or xylem histogenesis; floral meristem determinancy and floral organ formation; temperature response; aging; specification of symmetry; maintenance of meristem identity; circadian rhythm; cell fate specification and commitment; stem cell maintenance, development and differentiation; specification of axis polarity; detection and response to stimulus; cytoskeleton organization; cell communication; tropism; photosynthesis; cellular homeostasis; protein-DNA complex assembly; nucleosome assembly and organization; protein folding; meristem maintenance; chemical homeostasis; cell death; cell cycle; growth; chromatin organization; post-embryonic organ development; DNA repair; chromosome organization; cell differentiation; shoot development; defense response; organelle organization; transport; localization; signaling; reproductive process; macromolecule modification; response to stress; regulation of gene expression; developmental process; biological regulation; and metabolic process. The most representative molecular functions of the proteins involved in all these, and remaining, biological processes fall into the categories of binding, catalytic activity, transport, and transcription regulation (See Appendix).

At the top of the output list of the microarray analysis were found several members of the APETALA1 and SEPALATTA protein families, along with PISTILLATA, and AGAMOUS – all integral parts of the ABCDE model for floral development (Table 5.1 and Table 5.2). The up-regulation of these transcripts together with detectable changes in expression of other meristem related genes, such as FRUITFULL, SUPRESSOR OF OVEREXPRESSION OF CO

1, SHORT VEGETATIVE PHASE, LEUNIG, APETALA2, and CRABS CLAW validates the results obtained with the microarray hybridization - which not only genetically characterizes the SAM's flowering transition processes through a broad scale approach, but also dissects the temporal expression profile of these genetic elements, including the similar members of the same gene family. One good example, and consistent with previously presented *in situ* hybridization results, is the expression profile of AP1-like Bradi1g08430 (BdVRN1/BdFUL1) mRNA, which is only detectable after 7 LD, persisting highly expressed at 11 LD; while another AP1-like gene, Bradi1g59250, is up-regulated five days earlier, at 2 LD, right after shift into florally inductive conditions, remaining massively up-regulated throughout the following days. This data highlights the possibility that Bradi1g59250 is a more likely candidate to be the SAM's recipient of the florigen signal and to function as a meristem identity gene, perhaps regulating BdVRN1 itself. Another example are the bZIP proteins Bradi3g00300 (BdFDL1) and Bradig21820 (BdFDL2), two very different but potential FD proteins and so the probable co-partners of BdFTL2 in the up-regulation of the meristem identity genes. From the array results it is evident that each protein has a distinct temporal expression profile, indicating that it is more likely for Bradi3g00300 to be the potential BdFTL2 co-partner since its expression is initiated earlier than BdFDL2, detectable only after 7 LD. The highly significant up-regulation of an FT-like transcript at the SAM seven days after shift, coincident with the inflorescence meristem phase and with the up-regulation of several MADS-box genes including the AP1-like Bradi1g08340, is not totally unexpected as there are reports of a second FT like protein in Arabidopsis (TWIN SISTER OF FT, TSF), and in rice (RICE FLOWERING LOCUS T 1, RFT1), that is known to be involved in flowering transition by acting redundantly with FT/Hd3a (Michaels *et al*, 2004; Yamaguchi *et al*, 2005; Komiya *et al*, 2008; Komiya *et al*, 2009). However, both these proteins (TSF and RFT1) are thought to be up-regulated in the vasculature of leaves (Jang *et al*, 2009; Komiya *et al*, 2008), not in the SAM.

Consistent with previous results is also the fact that, throughout the assessment, no changes in expression was detected for LEAFY. This is consistent with the up-regulation time-line of 14 LD observed in the *in situ* hybridizations presented in Chapter 4, and once again it indicates that it's unlikely for BdLFY to be involved in the meristem's fate change from vegetative to reproductive, in Brachypodium.

The down-regulation of AFO, YAB3, and CRC - all members of the YABBY family of proteins – in the early stages of flowering is probably associated with the fact that these genes are usually involved in the specification of the abaxial cell fate in lateral organs, namely leaves, in Arabidopsis. Nonetheless, their expression is usually also required for the establishment of floral meristem identity along with LFY and AP1, as well as for the proper development of the flower, through the repression of floral homeotic genes (Golz and Hudson, 1999). Therefore, it is likely for such genes to become up-regulated at later developmental stages.

Table 5.1: List of ten of the most consistently up or down-regulated genes across all tested Bd21 SAM samples undergoing floral transition, as determined by whole transcript microarray analysis

DOWN-REGULATED TRANSCRIPTS - 2LD:7LD:11LD

Bradi4g28280	WRKY46 (WRKY DNA-binding protein 46); transcription factor
Bradi1g11090	GAI (GA INSENSITIVE); transcription factor
Bradi3g50050	AFO (ABNORMAL FLORAL ORGANS)/FIL (FILAMENTOUS FLOWER); transcription factor
Bradi3g53220	AGF1 (AT-HOOK PROTEIN OF GA FEEDBACK 1); transcription factor
Bradi3g17950	VIN3 (VERNALIZATION INSENSITIVE 3); homeodomain protein
Bradi5g16910	YAB3 (YABBY3); transcription factor
Bradi5g24360	AIL5 (AINTEGUMENTA-LIKE 5); member of the AP2 family of transcription factors.
Bradi1g72150	SVP (SHORT VEGETATIVE PHASE); transcription factor
Bradi5g24100	AP2 (APETALA 2); transcription factor
Bradi2g49420	PAP1 (PHYTOCHROME-ASSOCIATED PROTEIN 1); transcription factor

UP-REGULATED TRANSCRIPTS - 2LD:7LD:11LD

Bradi1g59250	AP1 (APETALA1); transcription factor
Bradi1g08326	SEP1 (SEPALLATA1); transcription factor
Bradi1g21980	AP1 (APETALA1); transcription factor
Bradi2g51790	ULT2 (ULTRAPETALA 2); DNA binding
Bradi1g00950	GA5 (GA REQUIRING 5); gibberellin 20-oxidase
Bradi2g24940	PI (PISTILLATA); transcription factor
Bradi3g15490	zinc finger (B-box type) family protein
Bradi1g26720	SPL4 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 4); transcription factor
Bradi5g09270	ATC (ARABIDOPSIS THALIANA CENTRORADIALIS); phosphatidylethanolamine binding
Bradi3g00300	ABF4 (ABRE BINDING FACTOR 4); transcription factor

Table 5.2: List of ten of the most consistently up or down-regulated genes in Bd21 SAM samples undergoing floral transition in the two latest stages tested, as determined by whole transcript microarray analysis

DOWN-REGULATED TRANSCRIPTS - 7LD:11LD

Bradi1g37800	ATH1 (ARABIDOPSIS THALIANA HOMEOBOX GENE 1); transcription factor
Bradi2g37800	AP2 (APETALA 2); transcription factor
Bradi4g06867	FUL (FRUITFULL); transcription factor
Bradi1g58570	Transcription factor
Bradi3g35560	AP2 domain-containing transcription factor, putative
Bradi3g48070	bHLH family protein
Bradi3g00730	SOC1 (SUPRESSOR OF OVEREXPRESSION OF CONTANS 1); transcription factor
Bradi1g72150	SVP (SHORT VEGETATIVE PHASE); transcription factor
Bradi2g49420	PAP1 (PHYTOCHROME-ASSOCIATED PROTEIN 1); transcription factor
Bradi1g69900	CRC (CRABS CLAW); transcription factor

UP-REGULATED TRANSCRIPTS - 7LD:11LD

Bradi1g08340	AP1 (APETALA1); transcription factor
Bradi2g07070	FT (FLOWERING LOCUS T); phosphatidylethanolamine binding
Bradi1g21980	AP1 (APETALA1); transcription factor
Bradi1g48520	SEP3 (SEPALLATA3); transcription factor
Bradi2g32910	AG (AGAMOUS); transcription factor
Bradi1g46340	ZFP8 (ZINC FINGER PROTEIN 8); transcription factor
Bradi3g51800	AGL6 (AGAMOUS LIKE-6); transcription factor
Bradi1g69890	SEP1 (SEPALLATA1); transcription factor
Bradi2g48690	FUL (FRUITFULL); transcription factor
Bradi2g21820	AREB3 (ABA-RESPONSIVE ELEMENT BINDING PROTEIN 3); transcription activator

5.3.2 Brachypodium's FRUITFULL2 (BdFUL2) is a meristem identity gene, and the functional orthologue of Arabidopsis APETALA1 (AP1)

Since BdVRN1/BdFUL1 failed to accelerate flowering when over-expressed in Bd21, and did not suppress the differentiation of vegetative organs, it became apparent that the downstream target of BdFTL2 had to be some other elusive genetic element, rather than the initially thought BdVRN1/BdFUL1. In order to find potential candidates that would take the place of BdVRN1/BdFUL1 as a genetic marker for the beginning of flowering fate, and to better understand the genetic "rewiring" taking place during flowering transition, a whole transcriptome microarray hybridization was performed using florally induced SAMs at different developmental stages. From that analysis one MADS box gene, from the APETALA1 group, stood out as a good candidate for being a potential meristem identity gene – Bradi1g59250 – Brachypodium's FRUITFULL2, BdFUL2 (Higgins *et al*, 2010) (Figure 5.6-A). Its high levels of expression and the fact that it was up-regulated early on (Table 5.1) and before BdVRN1/BdFUL1 - Bradi1g08340 - (Table 5.2) indicated that the early stages of flowering transition are highly dependent on its expression. The information obtained with the microarray was corroborated by means of transcript immuno-localization *in situ*, where increasing mRNA expression levels were observed along with flowering progression in the apical meristem (Figure 5.6-C). In order to determine if BdFUL2 was able to induce early flowering, and confirm if it would have a role as a meristem identity gene, it was necessary to cloned it in front of the rice actin promoter and leading intron, and constitutively over-express it in transgenic Bd21 *calli* (Alves *et al*, 2009). From the ectopic expression of BdFUL2 in meristematic callus cells, spikelets and spikes were immediately regenerated together with the simultaneous suppression of all vegetative structures (Figure 5.6-B). Both phenotypes of BdFUL2 and BdFTL2 over-expressing lines are identical, indicating that BdFUL2 is the likely recipient of the florigen signal (probably via a direct interaction with BdFTL2 in complex with a potential FD-like protein) and the functional orthologue of Arabidopsis AP1 concerning the role of meristem identity gene.

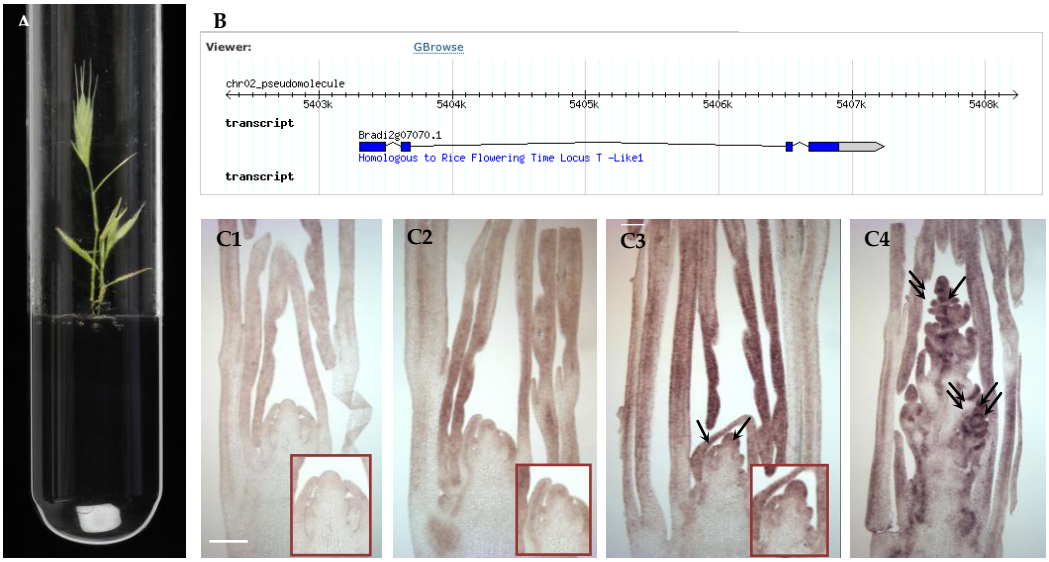
In spite of sharing a high percentage of amino acid conservation and sequence homology, the two Bd21 AP1-like proteins (BdVRN1/BdFUL1 and BdFUL2) are not functionally redundant. Possible explanations for such significant phenotypical differences displayed by the over-expressing lines could be explained by the two glutamine repeat and glutamine/alanine rich regions that are present in BdFUL2 protein and absent BdVRN1/BdFUL1 (Figure 5.6-D).


```
Bradi1g08340.1      MGRGKVQLKRIENKINRQVTFSKRRSGLLKKAHEISVLCDAEVALIIFSTKGKLYEFATD
Bradi1g59250.1      MGRGKVQLKRIENKINRQVTFSKRRNGLLKKAHEISVLCDAEVAVVVFSPKGKLYEYATD
                    *************************.******************:::** ******:***

Bradi1g08340.1      SCMDKILERYERYSYAEKVLVSTESEIQGNWCHEYRKLKAKVETIQKCQKHLMGEDLESL
Bradi1g59250.1      SSMDKILERYERYSYAEKALISAESESEGNWCHEYRKLKAKIETIQKCHKHLMGEDLDSL
                    *.****************.*:*:*** :*************:******:********:**

Bradi1g08340.1      NLKELQQLEQQLESSLKHIRSRKNQLMHESISELQRKERSLQEENKALQKELVEKQKAHT
Bradi1g59250.1      NLKELQQLEQQLESSLKHIRSRKSHLMMESISELQKKERSLQEENKALQKELVERQKAAS
                    ************************.:** *******:******************:*** :

Bradi1g08340.1      Q---------QAQWEQTHPQ-----------------TSSSSSS-MQR----EAPPTTNI
Bradi1g59250.1      RQQQQQQQQQQVQWEQHAHTQAQAHAQAQAQTQAQAQTSSSSSSFMMRDHQQARVPQQNI
                    :         *.****                     ******* * *       *  **

Bradi1g08340.1      SNR-PAAAGERT--EEAAGQAQAR-VGLPPWMVSHISG*
Bradi1g59250.1      CSYPPATMGGESSSVAAPGQGQIRIGGLPPWMLSHLNA*
                    ..  **: * .:    * **.* *  ******:**:.*
```

Figure 5.6: A) Schematic representation of Bradi1g59250 locus, BdFUL2 (MIPS). B) Phenotype of Bd21 regenerating transgenic constitutively expressing BdFUL2. C) Transcript immuno-detection of BdFUL2 in SAM (enclosed by layers of leaves) undergoing floral transition: C1 – 0LD, C2: 2LD, C3: 8LD, C4: 14LD. BdFUL2 transcript is only detectable 8 days after flowering induction in the inflorescence meristem and in the floral primordia (arrows); scale bar: 0.2 mm. **D) Protein sequence alignment using the multiple sequence alignment tool Clustal Omega v1.1.0 (EMBL-EBI). Bradi1g08340.1 (BdVRN1/BdFUL1), Bradi1g59250.1 (BdFUL2).** Highlighted in red are the polyglutamine and glutamine/alanine sequences present in BdFUL2 and absent in BdVRN1/BdFUL1.

5.3.3 The constitutive expression of BdFTL1, a SAM expressed FT-like protein, induces early flowering in Bd21

Another gene that stands out in the list of up-regulated genes in the course of flowering transition was Bradi2g07070, named BdFTL1 (Higgins *et al*, 2010). The up-regulation of a shoot apical meristem specific FT-like protein upon induction of flowering raises the questions: what is the expression domain of BdFTL1? Can BdFTL1 act redundantly with BdFTL2, i.e., trigger the flowering process in Bd21 and repress the development of vegetative structures? From the transcript *in situ* immuno-localization in meristems undergoing flowering transition it was possible to firstly confirm the microarray result, by observing that the expression profile of this PEBP increases as the flowering meristem develops (Figure 5.7-C). Additionally, BdFTL1 expression is quite localized to the base of the floral meristems at a position that may overlap with the one of FD-like protein Bradi3g00300, or even Bradi2g21820 (Figure 5.7). In order to test if BdFTL1 is capable to replace BdFTL2 in the induction of flowering it was necessary to transform *calli* from Bd21 and regenerate ectopically over-expressing BdFTL1 transgenic plants, under the rice acting promoter and leading intron. The results from the transformation clearly demonstrated that BdFTL1 has the ability to induce significantly early flowering, limit vegetative growth, and restrict inflorescence development to a single spikelet per tiller, however, when compared to BdFTL2 it is evident that it does not have the means to fully suppress the development of vegetative structures like BdFTL2 (Figure 5.7-A). The function of this gene is not clear at this point, but it is possible that it acts redundantly with BdFTL2 in a flowering induction role, with a possible FD-like partner. When compared in terms of amino acid sequence homology, the two proteins show a remarkable level of similarity. There are, however, a few amino acid substitutions (Figure 5.7-D) that may be responsible for interfering with the affinity between peptides, and preferentially stabilize or destabilize particular protein-protein interactions, and in this way differently regulate gene expression.



D

```
Bradi1g48830.1      MAGRDRDPLVVGRVVGDVLDPFVRTTNLRVSFGNRNVSNGCELKPSMVTHQPRVEVGGNE
Bradi2g07070.1      --MPRGDPLVVGRVIGDVVDPFVRRVSLRVGYASRDVANGCELRPSAIADPPRVEVGGPD
                          ********:***:***** ..***.:..*:*:*****:** ::.*******:

Bradi1g48830.1      MRTFYTLVMVDPDAPSPSDPNLREYLHWLVTDIPGTTGASFGQEVMCYESPRPSMGIHRF
Bradi2g07070.1      MRTFYTLVMVDPDAPSPSDPSLREYLHWLVTDIPATTGVSFGTEVVCYESPRPVLGIHRL
                    ********************.*************.***.*** **:******* :****:

Bradi1g48830.1      VFVLFQQLGRQTVYAPGWRQNFNTRDFAELYNLGPPVAAVYFNCQREAGSGGRRMYP*
Bradi2g07070.1      VFLLFQQLGRQTVYAPGWRQNFSTRDFAELYNLGLPVAAVYFNCQRESGTGGRRM*--

                  **:******************.**********************:*:*****
```

Figure 5.7: A) Schematic representation of Bradi2g07070 locus, BdFTL1 (MIPS). B) Phenotype of Bd21regenerating transgenic constitutively expressing BdFTL1. C) Transcript immuno-detection of BdFTL1 in SAM undergoing floral transition: C1 – 0LD, C2: 2LD, C3: 8LD, C4: 14LD. BdFTL2 transcript is only detectable 8 days after flowering induction in the inflorescence meristem and in the floral primordia (arrows); scale bar: 0.2 mm. **D) Protein sequence alignment using the multiple sequence alignment tool Clustal Omega v1.1.0 (EMBL-EBI). Bradi1g48830.1 (BdFTL2), Bradi2g07070.1 (BdFTL1).**

5.4 Discussion

In this second part of transitioning into the reproductive phase, a whole transcriptome microarray analysis was carried out with two major purposes: to gather transcriptome data from the SAM while undergoing flowering transition, and use that information to find a meristem identity gene that would be the downstream target of BdFTL2.

From the general assessment of the microarray output, one of the first conclusions that is worth pointing out is the fact that up to 10% of the genes involved in the SAM's flowering transition and early floral development have no protein sequence homology in *Arabidopsis thaliana* proteome. Although no particular analysis was performed to identify the origin of these genes it is quite possible that they are grass specific genetic elements that can only be found among members of the broad monocot lineage. Also, about 20% of the analysed genes have an unknown function and 30% are involved in an unknown biological process. These figures highlight how, in spite of the great scientific achievements of the past decades, our knowledge is still limited with regard to plants' fundamental biology. Of all the other transcripts, to which some degree of study has been dedicated to, it is possible to conclude what was already known, which is that flowering is quite a complex physiological affair that coordinates hundreds of biological processes, like cell death, chromatin organization, signalling, transport, symmetry, polarity, and, of course meristem identity.

In terms of protein similarity, the microarray experiment detected many potential orthologue proteins during the early phases of flowering development that have been functionally described in other plant systems. The most striking example concerns quite an important family of transcription factors known to be involved in flowering evocation and in floral organ specification - the MADS-box genes. At the top of the most up or down-regulated genes during flowering transition of Bd21 were found several members of this family, including a few AP1, FUL, SVP, and SEP, as well as SOC1, PI, and AG. Other protein families with homeotic functions were also differently regulated in the microarray experiment, like the YABBY family with a potential orthologue of Arabidopsis CRABS CLAW (CRC) (Yamaguchi *et al*, 2004), or the AP2/EREBP family with a potential orthologue of AP2. These evidences indicate that not only both the preparation of the array and the hybridization were successful, but also that there are good chances for the ABCDE model for flowering development to be more or less universal across Angiosperms. Nevertheless, given the morphological differences of the perianth between the conventional flower and the cereal floret, and the lack of confirmation of the biological function of all these proteins, further conclusions would be premature at this time.

With BdFUL2 (Bradi1g59250.1) being amongst the most up-regulated transcripts during flowering transition and also codifying for an AP1–like MADS-box protein, it immediately became the preferential candidate to be the elusive meristem identity gene with functional homology to Arabidopsis AP1. Its SAM expression profile was easily reconfirmed by *in situ* immuno-localization, producing a very strongly signal that is consistent with its massive

up-regulation upon flowering evocation. When constitutively expressed, BdFUL2 is a master regulator at the crossroad between fully suppressing all vegetative activity, and irreversibly committing the SAM fate to floral development. The resemblance of the constitutive expression of this gene, in regenerating callus cells, to the phenotype of the constitutive expressing lines of BdFTL2 is remarkable. There is a very strong possibility that, in addition to being a downstream target of BdFTL2, BdFUL2 is also directly up-regulated by this gene via a protein complex with a bZIP protein orthologue of Arabidopsis FD (Taoka *et al*, 2011). It would be very interesting to test this prospect in a future opportunity by performing a chromatin immuno-precipitation (ChIP) experiment targeted to BdFUL2's promoter.

Also interesting is the major difference between the flowering phenotype of the two MADS box proteins over-expressed in regenerating *calli*, BdVRN1/BdFUL1 and BdFUL2. This is particularly significant because BdVRN1/BdFUL1 has higher protein homology to AP1 than BdFUL2, and yet BdVRN1/BdFUL1 was completely unable to accelerate flowering when over-expressed in Bd21. Still, the different flowering phenotypes of both over-expressing transgenics must be associated with differences in the protein sequences; therefore, to identify the amino acid residues that are responsible for the functional differences between these two proteins, one could over-express differently truncated versions of BdFUL2's protein and correlate the loss of the early flowering phenotype with specific mutated versions of BdFUL2.

Another interesting experiment to consider in a later opportunity is the global mapping of BdFUL2 binding sites, i.e., all the genetic targets which have their expression directly regulated by BdFUL2. This could be achieved by doing a ChIP-on-chip or a ChIP-seq experiment.

In spite of the supporting evidences that BdFUL2 is a meristem identity gene and likely to be the direct recipient of the florigen stimulus, it is still necessary to examine an BdFUL2 loss of function mutant or a silenced transgenic to completely prove that without a functioning protein, flowering transition in Bd21 should be, at least, significantly delayed in photoperiodic inductive conditions. Therefore, given the lack of described mutants in Bd21, it would be pertinent to try, in a future opportunity, a gene silencing strategy, specifically targeting the degradation of this gene's mRNA, and assess its effects on the plant's flowering competency.

In the output of the microarray analysis, a gene from the FT protein family stood out as being highly up-regulated, and was object of lab experiments. From the analysis of BdFTL1 expression it was evident that this meristem specific FT-like protein can accelerate, in a significant way the transition into flowering when constitutively expressed in regenerating callus but, on the other hand, it does not have the ability to completely abolish vegetative development like BdFTL2, or BdFUL2, though it functions alongside them in the same pathway. It is unclear at this point the role of BdFTL1 in the context of flowering transition.

It is possible that, like BdFTL2, it is another "florigen" molecule possibly required to later signal the shift from inflorescence fate into floral fate. Reports from other plants systems mention the existence of FT-like proteins that are up-regulated in particular physiological conditions and that can replace FT in the induction of flowering, however, these FT-like proteins are usually expressed in leaves, and like FT, reach the SAM through the vasculature (Tsuji *et al*, 2011).

Its expression domain at the SAM was determined via *in situ* hybridization, and it overlaps with the one of the FD-like proteins presented in Chapter 4. It is, therefore, plausible that because of a shared expression domain and because of its PEPB nature, that BdFTL1 may form a complex with at least one FD-type protein, in order to influence gene expression at the SAM. During the course of this work it wasn't possible to perform a yeast 2-hybrid experiment to test the interaction affinity between the several Bd21 FT- and FD-like proteins. This, however, would be a valuable experiment to perform at a future opportunity, as it will allow the characterization of proteins according to their ability to potentially interact with other proteins, in a complex, and through this be involved in specific regulatory pathways.

Again, mutant studies or silencing inducing transgenic construct strategies would be welcomed in the future, so to define the functional scope of these genetic elements and determine appropriately the levels of interspecies conservation between flowering inducing pathways.

A final note to mention that neither BdLFY nor BdTFL1 could be found to be up-regulated in the SAM during tested time-course series. This fact is consistent with previously presented results (Chapter 4) where BdLFY and BdTFL1 expression at the SAM in the *in situ* hybridization could only be detected 14 after flowering induction

Chapter 6

Conclusion

The biology of flowering plants has been naturally evolving for more than 140 million years (Moore *et al*, 2007), and yet only twelve years have passed since scientists fully sequenced the first plant genome (The Arabidopsis genome initiative, 2000). Since then, knowledge in plant biology has advanced at an amazing rate, mainly due to daily progresses in Arabidopsis scientific research. However, in spite of all that valuable knowledge, Arabidopsis can only go so far explaining the biology of all Angiosperms. Monocots in general and temperate grasses in particular display significant morphological and, hence, genetic and physiological disparities that need to be studied in their biological context in order to be fully comprehended.

A few years ago, plant scientists from both sides of the Atlantic got together to discuss this matter and joint efforts with the ultimate goal of developing molecular and technical tools that would facilitate in-depth research into a plant system that could be used as a generic model for annual temperate grasses. Consequently, in 2009 an optimized plant transformation method applied to the temperate monocot *Brachypodium distachyon* (community standard line Bd21) was published (Alves *et al, 2009)* and a year later the international community announced its complete genome sequence (The International Brachypodium Initiative, 2010). With such resources available and with the advances made in the fields of molecular biology and computational science in the recent decades, it is now feasible to explore temperate grasses to levels of depth never before possible. However, the complexity level of fundamental biological processes is high, as the interconnected pathways regulating plant development are likely to be

heavily buffered (by chromatin remodelling and histone modification; transcription initiation, post-transcriptional modification, mRNA stability and transport; translation; and protein stability) and probably backed-up by redundancy. The gene expression profile (microarray) results described here, obtained from transitioning meristems of Brachypodium, revealed just how little we know and how complex the genetics underpinning the SAMs metamorphic transformation can be. From the about 4 000 molecular elements that allow Bd21 to control flowering time at the SAM, through the coordinated actions of photoperiod perception, signal transduction and SAM cells re-programming, a highly significant percentage is associated with unknown biological processes, and have an unknown biological function, while about 10% of the total transcripts expressed in this conditions have no orthologue in Arabidopsis' genome. And yet, although the remaining transcripts can be associated with particular molecular functions and known biological processes, the functional analysis of genes like BdCO1, BdLFY or BdVRN1/BdFUL1 demonstrated that, in order to prevent making risky assumptions, the apparently conserved regulators of flowering between plant systems mustn't be dismissed from a careful analysis in its own biological context (Table 1). A flagrant example is precisely VRN1, which has been referred in the literature as the functional orthologue of Arabidopsis AP1 (Trevaskis *et al*, 2007; Li and Dubcovsky, 2008; Higgins *et al*, 2010)), while it was demonstrated here that in Bd21 this is not the case. Instead, it is BdFUL2 that is likely to be BdFTL2's downstream target, probably through interaction with BdFDL1; while BdVRN1/FUL1 may be the target of a subsequently up-regulated potential complex, formed by BdFTL1 and BdFDL2 (Li and Dubcovsky, 2008). More definitive conclusions require the support of further experiments such as (1) chromatin immuno-precipitation (ChIP), and (2) protein complex immuno-precipitation (co-IP), as these techniques make use of antibodies to detect if (1) a specific protein is interacting directly with a particular promoter – by crosslinking DNA-protein complexes (an analysis that if coupled with high throughput sequencing of all the ChIP-DNA fragments will map the protein's promoter-binding sites across the genome), and if (2) two proteins physically cooperate in a complex – by pulling down the protein complex out of solution; and mutant studies or induced gene-silencing that simulates the effects of null mutations on the plant's physiological processes and, eventually, its phenotype. But, for now, it is possible to affirm that the expression of BdFUL2 at the SAM marks the initiation of flowering transition in Brachypodium, while simultaneously mediates the suppression of all vegetative growth. Additionally, BdFTL2 is the florigenic element that transmits the photoperiodic signal sensed in the leaves to the apex, and is the possible trigger of BdFUL2 expression. These two evidences establish the foundations for the research of flowering transition in Bd21, as it is now possible to assert genetically when the SAM's fate changes in response to a photoperiodic cue; and these are fundamental spatial/temporal references for the study of such a complex genetic network in a newly available monocot system.

Table 6.1: Summary of the genes analysed, including the performed experiments and the results obtained, as described in the previous chapters.

A. thaliana (Thale cress)	*B. distachyon* (Purple false brome)	Experiments	Results
APETALA 1 (AP1)	BdVRN1/BdFUL1 (Bradi1g08340.1)	Protein BLAST search and ClustalW alignment Constitutive expression Gene silencing via amiRNA Transcript detection via ISH (microarray)	This protein has high percentage of sequence identity. The over-expression of this gene does not accelerate flowering time under inductive LD. The silencing strategy produced T0 plants with floral defects and high levels of sterility, a phenotype not present in the T1. The expression domain determined by ISH indicates that BdVRN1/BdFUL1 is up-regulated in the inflorescence meristem and in the floral primordia, but is not involved in the flowering transition process. BdVRN1/FUL1 is not functionally conserved with AP1.
	BdFUL2 (Bradi1g59250.1)	Protein BLAST search Constitutive expression Transcript detection via ISH (microarray)	This protein has high percentage of sequence identity. The over-expression of this gene in *calli* of Bd21 suppresses vegetative growth and induces flowering in regenerating shoots. The expression domain determined by ISH indicates that BdFUL2 is up-regulated in the inflorescence meristem and in the floral primordia. BdFUL2 is functionally conserved with AP1.
CONTANS (CO)	BdCO1 (Bradi1g43670.1)	Protein BLAST search and ClustalW alignment Constitutive expression Protein immuno-detection Transcript detection via qPCR Arabidopsis mutant and wt complementation with BdCO1	This protein has high percentage of sequence identity. The expression profile in SD is characterized by high levels of mRNA at dawn and dusk. The over-expression of this gene accelerates flowering in SD and delays flowering in LD. All transgenic lines, exhibited high levels of BdCO1 mRNA in SD, but no BdFTL2 expression was detected. The protein levels are tightly regulated in the ox lines during the 24h period, but are not necessarily destabilized by the lack of light. BdCO1 can partially rescue the late flowering phenotype of At co-9 mutant, but it delays flowering in Col-0 wt. BdCO1 is not functionally conserved with CO.
FD	BdFDL1 (Bradi3g00300.1)	Protein BLAST search and ClustalW alignment Constitutive expression Gene silencing via amiRNA Transcript detection via ISH	The protein shows low levels of sequence identity outside the bZIP domain. Flowering time has not been determined for the transgenic lines, however, the ox lines exhibited high levels of biomass, floral defects and a degree of suppression of internode elongation. The silencing strategy produced T0 plants with floral defects and high levels of sterility, a phenotype not present in the T1. The expression domain determined by ISH indicates that BdFDL1 is up-regulated in the vegetative meristem and all the floral SAM analysed. BdFDL2 is up-regulated in the inflorescence meristem and in the floral primordia. From these results BdFDL1 is the most likely candidate to be functionally conserved with FD.
	BdFDL2 (Bradi2g21820.1)	Protein BLAST search and ClustalW alignment Constitutive expression Gene silencing via amiRNA Transcript detection via ISH	
FLOWERING LOCUS T (FT)	BdFTL2 (Bradi1g48830.1)	Protein BLAST search and ClustalW alignment Constitutive expression Gene silencing via	This protein has high percentage of sequence identity. The expressions levels of this transcript were highly up-regulated under LD in the vasculature of leaves of Bd21. The over-

		amiRNA Transcript detection via ISH and qPCR	expression of this gene in *calli* of Bd21 suppressed all vegetative growth and induced flowering in regenerating shoots. The silencing strategy produced T0 plants with floral defects and high levels of sterility, a phenotype not present in the T1. BdFTL2 is functionally conserved with FT.
	BdFTL1 (Bradi2g07070.1)	Protein BLAST search and ClustalW alignment Constitutive expression Transcript detection via qPCR and ISH (microarray)	This protein has high percentage of sequence identity. The over-expression of this gene in *calli* of Bd21 significantly suppresses vegetative growth and induces flowering in regenerating shoots. The expression domain determined by ISH indicates that BdFTL1 is up-regulated in the inflorescence meristem and in the floral primordia. The relative expression levels of this transcript in the leaves of plants florally induced by LD were low. BdFTL1 is not FT's orthologue, although it is florigenic when ectopically expressed.
GA INSENSITIVE (GAI)	BdGAI (Bradi1g11090.1)	Protein BLAST search and ClustalW alignment Constitutive expression of the native protein and of a truncated DELLA domain peptide GA application (microarray)	This protein has high percentage of sequence identity. The ox of the native protein does not produces a phenotype, however, the ox of the truncated DELLA domain protein produces severely dwarfed plants, insensitive to GA application. The BdFTL2 levels in these dwarf plants are significantly lower than wt under inductive conditions.
LEAFY (LFY)	BdLFY (Bradi5g20340.1)	Protein BLAST search and ClustalW alignment Constitutive expression Gene silencing via amiRNA Transcript detection via ISH	This protein has high percentage of sequence identity. The over-expression of this gene had no effect on flowering time under inductive LD. The silencing strategy produced T0 plants with floral defects and high levels of sterility, a phenotype not present in the T1. The expression domain determined by ISH indicates that LFY is not involved in the flowering transition process, but is up-regulated in the floral primordia. BdLFY is not functionally conserved with LFY.
PHYTOCHROME INTERACTING FACTOR 4 (PIF4)	BdPIF1 (Bradi2g11100.1) BdPIF7 (Bradi3g33170.1)	Protein BLAST search and ClustalW alignment Constitutive expression GA treatment	The protein shows low levels of sequence identity outside the APB and bHLH domains. The over-expression induces early flowering in both SD and LD. The application of GA to the ox lines does not accelerate flowering, but promotes severe internode elongation. BdPIF1 and BdPFI7 show functional conservation on the experiments performed.
TERMINAL FLOWER 1 (TFL1)	BdTFL1 (Bradi4g42400.1)	Protein BLAST search and ClustalW alignment Constitutive expression Gene silencing via amiRNA Transcript detection via ISH Protein detection via a protein fusion with GUS reporter	This protein has high percentage of sequence identity. The over-expression of this gene delayed flowering under inductive LD. The silencing strategy produced T0 plants with floral defects and high levels of sterility, a phenotype not present in the T1. The expression domain determined by ISH indicates that TFL1 is not involved in the flowering transition process, but it is likely to prevent the SAM's determinacy. The protein is also found in the vasculature across the plant. BdTFL1 is functionally conserved with TFL1.

Chapter 7

Experimental procedures

The present chapter intends to assemble all experimental procedures used in the course of this research work. In general terms, it includes all plant handling practices such as seed sterilization, sowing and growth conditions; general molecular biology protocols such as DNA and RNA extraction, cDNA synthesis, polymerase chain reaction, agarose gel electrophoresis, production of competent cells of *Escherichia coli* and *Agrobacterium tumefaciens* for genetic transformation, molecular cloning of GATEWAY® recombination compatible, and pGEM®-T Easy vectors, plasmid purification, restriction digest analysis, plasmid sequencing reactions; gene expression analysis techniques such as quantitative real time polymerase chain reaction, microarray hybridization, and non-radioactive transcript's *in situ* immuno-histochemical detection; optical, and scanning electron microscopy preparations; protein immuno-chemistry; plant tissue culture; and plant transformation technology via *Agrobacterium tumefaciens*. All experimental procedures presented in the course of this chapter are comprised of an outline of the technique's theoretical background, the most common applications, and a detailed step-by-step protocol. Whenever possible, there will also be a schematic representation of the protocol and/or the demonstration of the technical principle behind the procedure.

7.1 Plant work

7.1.1 Plant growth

Protocol

Dry seeds were soaked in sterile water for about 2 h to re-hydrate. Lemmas were removed to aid sterilization, and facilitate seed germination. Seeds were immersed for 30 s in 70% ethanol (v/v), and sterilized in 10% sodium hypochlorite (v/v) with a drop of Tween® 20, for 4 min. Sterilized seeds were rinsed three times with sterile water, and stratified in a Petri dish between two layers of damped sterile filter paper for 2 d, at 4 °C, in the dark. Following stratification, seeds were transferred into a 25 °C incubator, first in the dark to stimulate germination, and later with light to promote photosynthesis. Seedlings were sowed into a compost mix (300 litres of JI Nº2 compost (J Arthur Bowers); 300 litres of F2 compost (Scotts Levington); 50 litres of Grit; 1kg of Osmocote®Exact® 3-4 month (16+9+12+Mg0)), on a 40 cells tray (2 cm x 2 cm each cell) and grown under controlled conditions of 22 °C, 80% relative humidity and, unless specified differently, a 20 h light/4 h dark photoperiod, covered by a propagation lid. Plantlets were later transferred into bigger pots as their development progressed.

7.2 Nucleic acid manipulation

7.2.1 Plant nucleic acids extraction

7.2.1.1 Genomic DNA

The genomic DNA extraction protocol used it's based on the Nuclei Isolation Kit: Nuclei PURE Prep (Sigma®) that makes use of a dense sucrose cushion for the purification of un-disrupted nuclei. This method allows the recovery of a nuclei enriched suspension, free of cytoplasmic contaminants, from which highly pure DNA can be obtained *a posteriori* via a cetyltrimethylammonium bromide (CTAB) extraction, followed by phenol:chloroform:isoamylic acid purification, and isopropanol precipitation. The CTAB DNA extraction is a routine laboratory practice that is based on the employment of a cationic surfactant (CTAB) to disrupt the cellular membranes and free intramembranous contents into solution. Proteins and RNA are usually removed by proteases and RNases present in the extraction buffer, and the DNA's physical integrity guaranteed by chelating agents, like EDTA, that sequester cations/cofactors, essential for the DNases' activity. DNA-bound proteins, like histones, are removed through a Tris-buffered phenol:chloroform:isoamylic acid liquid–liquid extraction. This is based on the principle that when in a biphasic mixture, the DNA will remain in the aqueous phase, while proteins will be separated into the organic phase. Highly pure DNA is, finally, recovered through an isopropanol precipitation, and re-suspended in DNase free water.

Protocol

The protocol initiated with the purification of nuclei from about 1.5 g of plant tissue that was frozen in liquid nitrogen upon harvest, and ground to a fine powder with a mortar and pestle. The pulverized plant tissue was, then, re-suspended with 25 ml of Extraction buffer I (0.4 M sucrose; 10 mM Tris-HCl, pH 8; 10 mM $MgCl_2$; 5 mM β-mercaptoethanol; 0.1 mM phenylmethylsulfonylfluoride [PMSF]; 1x Complete protease inhibitor [Roche]), and filtered through two layers of Miracloth. The filtrate was centrifuged at 4 000 rpm, 4 °C, for 15 min. The supernatant was discarded and the pellet re-suspended in 1 ml of Extraction buffer II (0.25 M sucrose; 10 mM Tris-HCl, pH 8; 10 mM $MgCl_2$; 1% Triton® X-100; 5 mM β-mercaptoethanol; 0.1 mM phenylmethylsulfonylfluoride [PMSF]; 1x Complete protease inhibitor [Roche]), followed by a 3 min incubation on ice, and a centrifugation step at 14 000 rpm, for 10 min, at 4 °C. Following nuclei isolation, the crude pellet was re-suspended in Extraction buffer III (0.14 M d-sorbitol; 0.22 M Tris-HCl, pH 8; 0.022 M ethylenediaminetetraacetic acid [EDTA], pH 8; 0.8 M NaCl; 0.8% cetyltrimethylammonium bromide [CTAB]; 1% n-lauroylsarcosine), and incubated at 65 °C, with intermittent mixing, for 30 min. DNA was purified by adding an equal volume of phenol:chloroform:isoamylic acid, mixing well and incubating at room temperature for 5 min. The mixture was centrifuged at 12 000 rpm for 10 min, and the upper phase pipetted into a fresh tube. An equal volume of chloroform was added to the recovered upper phase, mixed well and

incubated at room temperature for 5 min. The mixture was, once more, centrifuged at 12 000 rpm for 10 min, and the upper phase pipetted into a fresh tube. Pure DNA was precipitated by adding an equal volume of isopropanol, followed by spinning at 10 000 rpm for 10 min. The pellet was washed with 1 ml of 70% ethanol (v/v), spun for 5 min at 10 000 rpm, and air dried. The DNA pellet was re-suspended in 25 µl of water, for 30 min at 37 °C, followed by quantification, quality check, and storing at -20 °C.

7.2.1.2 Total RNA

The total RNA purification procedure using RNeasy technology (Figure 7.1) combines the use of a high-salt buffer system, containing chaotropic-denaturating agents and ethanol, with the selective binding properties of a silica-based membrane to ensure the purification of intact RNA. Biological samples are lysed in guanidine-thiocyanate and β-mercaptoethanol to promote, the release of the RNA from proteins, such as ribosomes, and the inactivation of RNases that may degrade the sample. Ethanol is added to the lysate, to maximize RNA adsorption to the silica membrane, and contaminants are efficiently removed through washing steps. High quality RNA, suitable to be employed in sensitive quantitative gene expression analysis is eluted with RNase-free water, through a brief centrifugation step (QIAGEN RNeasy® Mini kit).

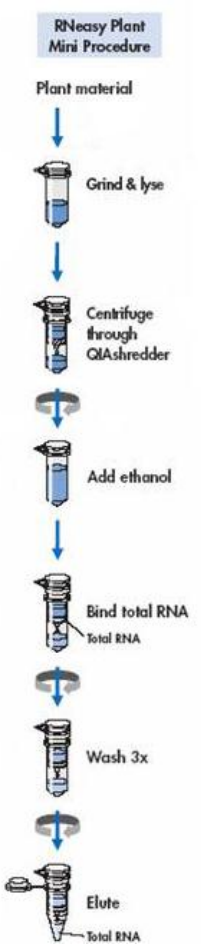


Figure 7.1: RNA purification procedure by QIAGEN RNeasy® Mini kit (modified from QIAGEN RNeasy® Mini kit).

Protocol

The RNA extraction was performed according to the protocol provided on the QIAGEN RNeasy® Mini kit, for "Purification of Total RNA from Plant Cells and Tissues and Filamentous Fungi". Due to the extreme RNA susceptibility to degradation, the RNA extraction was conducted with nuclease-free labware. Tissue samples weighing no more than 100 mg were frozen in liquid nitrogen upon harvest, and ground to a fine powder in a SPEX SamplePrep Geno/Grinder 2010®, with the help of 3 mm (diameter) metallic ball bearings. The pulverized plant tissue was, re-suspended with 450 µl of RLT buffer plus 1% β-mercaptoethanol (v/v), and vortexed vigorously. Tissue lysis was aided by incubating the mixture at 56 °C for 3 min, following which, the lysate was transferred into a QIAshredder spin column, and centrifuged for 2 min at 14 000 rpm. The supernatant of the flow-through was carefully transferred into another microcentrifuge tube, mixed gently with 0.5 volumes of 100% ethanol (v/v), transferred into an RNeasy spin column, and centrifuged for 15 s at 14 000 rpm. The flow-through was discarded, and 350 µl of buffer RW1 were added to initiate the "On-Column DNase Digestion with the RNase-Free DNase Set". The column was washed for 15 s at 14 000 rpm, and 80 µl of DNase I incubation mix were directly loaded onto the membrane. The DNase digestion[1] was performed for 1 h at room temperature, following which, the column was washed with another 350 µl of buffer RW1 and spun for 15 s for 14 000 rpm. After the DNase digestion, the column was washed, one more time, with 500 µl of buffer RPE to precipitate the RNA, and centrifuged for 2 min at 14 000 rpm. The flow-through was discarded and the column dried by centrifugation, at 14 000 rpm for 1 min. The total RNA was eluted in 50 µl of RNase-free water, after spinning the column for 1 min at 14 000 rpm. The quality of the eluate was assessed in 1% (w/v) agarose gel electrophoresis; the concentration was determined with a NanoDrop™ 1000 spectrophotometer.

[1] The original protocol from QIAGEN RNeasy® Mini kit, for "Purification of Total RNA from Plant Cells and Tissues and Filamentous Fungi" indicated that the DNase digestion should be performed for at room temperature, for 15 min. The digestion time was increased to 1 h to guaranty that no DNA remained in the sample.

7.2.2 Plant nucleic acids synthesis

7.2.2.1 Complementary DNA (cDNA) synthesis via reverse transcription of total messenger RNA (mRNA)

Complementary DNA (cDNA) synthesis via reverse transcription of total messenger RNA (mRNA) is a routine technique, employed in molecular biology laboratories, that capitalizes on the ability of RNA-dependent DNA polymerases (reverse transcriptases) to synthesize a single-stranded DNA (TGAC) molecule, from a fully matured eukaryotic mRNA template (ACUG) (Figure 7.2). In the eukaryotic cell, the post transcriptional mRNA processing comprises, apart from intron splicing and other modifications, the addition of a poly-A tail to the 3' end of the mRNA molecule. This feature is commonly exploited during the reverse transcription of mRNA, by the use of oligo (dT) primers, to initiate the synthesis of the new DNA strand. During this process, the reverse transcriptase will generate complementary DNA molecules composed exclusively by the exons that can be cloned into bacteria, for the purposes of protein expression studies, the production of genetic probes, or for the creation of a cDNA library.

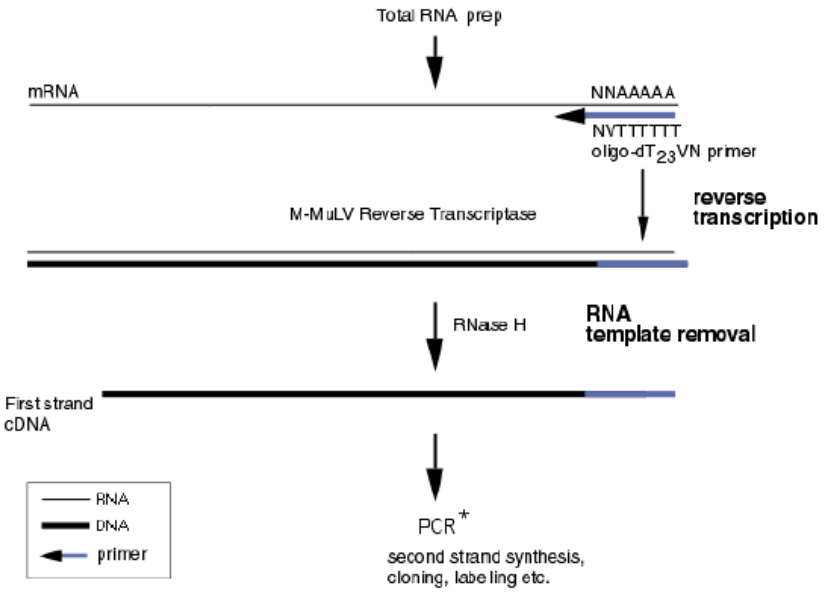


Figure 7.2: Schematic representation of the synthesis of the first strand cDNA (modified from http://www.neb.com/nebecomm/productfiles/674/images/E6500_fig1_v1_000029.gif

Protocol

The reverse transcription reaction was set up, using nuclease-free labware, on ice, according to the technical instructions of the First Strand cDNA Synthesis Kit #K1611: 0.1-5 μg of total RNA, 1 μl of oligo $(dT)_{18}$ primer, and up to 11 μl of nuclease-free water. These components were heated at 65 °C for 5 min, to remove secondary structures on the template, chilled on

ice and mixed with 4 µl of 5x reaction buffer, 1 µl of RiboLock™ RNase inhibitor (20 U/µl), 2 µl of 10 mM dNTPs mix, and 2 µl of M-MuLV Reverse Transcriptase (20 U/µl); in a final volume of 20 µl per reaction. The synthesis reaction was performed for 1 h at 45 °C, and inactivated by a 5 min incubation at 70 °C. The product of the first strand cDNA synthesis was used directly, as a template, for PCR applications.

7.2.2.2 Polymerase chain reaction (PCR)

The DNA amplification method through the means of a polymerase chain reaction is, probably, the most elementary and yet ground-braking tool in molecular biology. It was optimized by Dr. Kary Mullis in the 1980s and it exploits on the ability of DNA polymerases to synthesize new strands of DNA, complementary to the template one wishes to amplify (Saiki *et al*, 1988). It's a quite simple and elegant procedure that involves the separation of both strands of DNA by subjecting it to high temperatures (melting), the annealing of oligonucleotides (primers) specific to the region to be amplified, and the incorporation of new and complementary deoxynucleotides (A, T, C, and G) to the 3'-OH group of the primer sequence, by action of a DNA polymerase. These 3 steps (1 cycle) are repeated 35 times in an exponential amplification, yielding billions of amplicons (Figure 7.3). Due to the nature of the technique, it is essential that the enzyme is thermo-stable, and able to sustain normal activity under temperatures near the boiling point (Saiki *et al*, 1988). Particularly important when it comes to cloning genetic sequences, is the use of an enzyme that reproduces DNA with high fidelity.

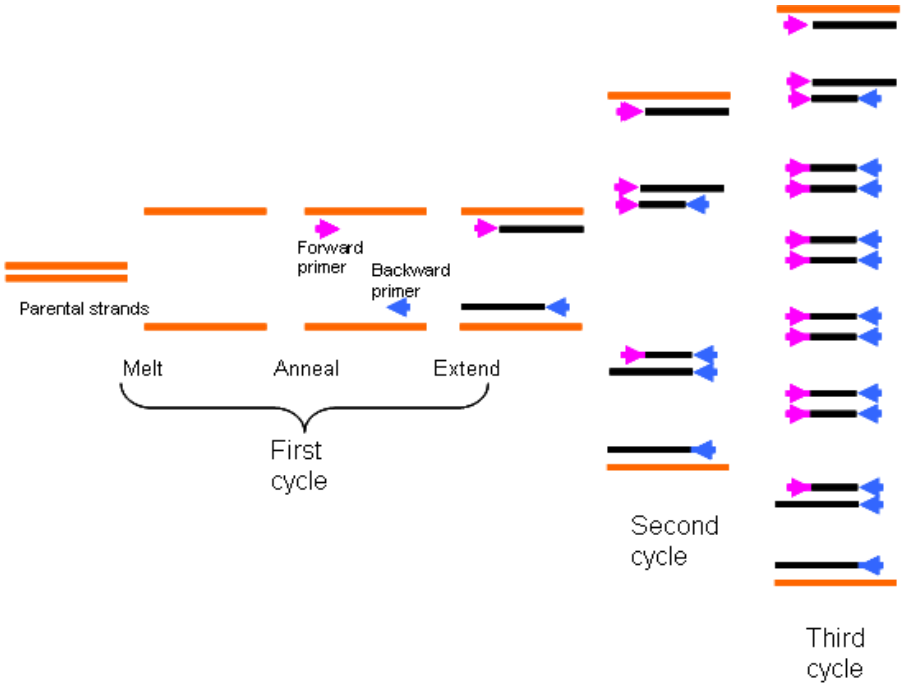


Figure 7.3: Polymerase chain reaction (adapted from http://www.xn--krinfo-wxa.hu/drupal/en/node/8912).

Protocol

The standard PCR reaction was composed of 4 µl of 5x Phusion HF buffer, 2 µl of 2 mM deoxynucleotide triphosphates (dNTPs) mix, 2 µl of 5 mM Forward primer, 2 µl of 5 mM Reverse primer, 1 µl of template DNA, 0.6 µl of DMSO, 10 µl DNase free water, 0.2 µl of Phusion® High-Fidelity DNA Polymerase; for a total reaction volume of ~ 20 µl. The standard reaction was set up to run in a thermal cycler with heated lid, with the following conditions: step 1 – 98 °C for 3 min; step 2 – 98 °C; step 3 – x °C (with x being 3 °C below primers' melting temperature (Tm)) for 20 s; step 4 – 72 °C for 30 s/kb of amplicon; step 5 to 35 - repeat step 2 to 4; step 36 – 72 °C for 10 min; step 37 – end.

7.2.3 Agarose gel electrophoresis

Agarose is a galactose polymer extracted from some species of red algae that is used, as a gelatinous molecular sieve, in electrical-mobility assays. DNA is negatively charged due to the phosphate groups it possesses in its backbone chains, and this feature is conveniently exploited to separate differently sized molecules by electrophoresis, as when an electric current is applied, the DNA will migrate towards de positively charged electrode across the pores in the agarose gel, in a manner that is dependent of its size and shape (supercoiled/linear). The resolution of the separation will depend on the percentage of agarose used to make the gel. Highly concentrated gels will separate smaller DNA fragments more effectively than larger fragments. The electrophoresis has to be accompanied by the use of a DNA marker, which is a commercially available mixture of differently sized DNA fragments, and a reference on the electrophoretic mobility. Ethidium bromide is a molecular dye, with high affinity to nucleic acids that is usually added to gels to allow DNA to be visualized under U.V. light.

Protocol

Gels were prepared by dissolving 1 g of agarose in 100 ml of 1xTAE buffer (50xTAE buffer, 1l: 242 g Tris base; 57.1 ml glacial acetic acid; 100 ml 0.5 M EDTA, pH 8.0). The mixture boiled in microwave for about 1 min, until fully dissolved. Before pouring the gel in the appropriate gel cast, with the necessary comb to form the wells, 6 µl of ethidium bromide were added to the agarose solution. The gels were poured and left to solidify for ~30 min. The gel was placed inside the electrophoresis chamber and submerged in 1xTAE buffer. DNA samples were loaded, into the wells of the gel, mixed with half volume of bromophenol blue loading dye (Bromophenol blue loading dye, 10 ml: 25 mg Bromophenol blue; 5 ml glycerol), and next to 3 µl of 2-log DNA ladder (N3200, New England BioLabs® *inc*.) for size reference. Electric current was set at 100 V, for 30 min.

7.2.4 DNA purification from agarose gels

The DNA extraction and purification from agarose gels was performed by using the QIAGEN QIAquick® Gel Extraction Kit, following the protocol provided. Its procedure is, like the QIAGEN RNeasy® Mini Kit, based on a silica membrane that adsorbs DNA in the presence of a highly concentrated salt buffer, while remaining solutes pass through the membrane. This enables the efficient removal of impurities such as agarose, primers, nucleotides, enzymes, salts, etc… that may interfere with subsequent reactions, such as cloning or enzymatic digestion. The binding ability of the silica membrane is, however, pH dependent. The optimal pH binding conditions remain below 7.5, after which the membrane's adsorption competence drops significantly. This can be overcome by adding a small volume of 3 M sodium acetate, pH 5.0, to the DNA solution, prior to the binding step. The colour of the solubilization buffer will indicate if the pH level is optimal by displaying the yellow colour. Once purified, the DNA is eluted in a low-salt buffer or water, ready to be handled in other downstream procedures (Figure 7.4).

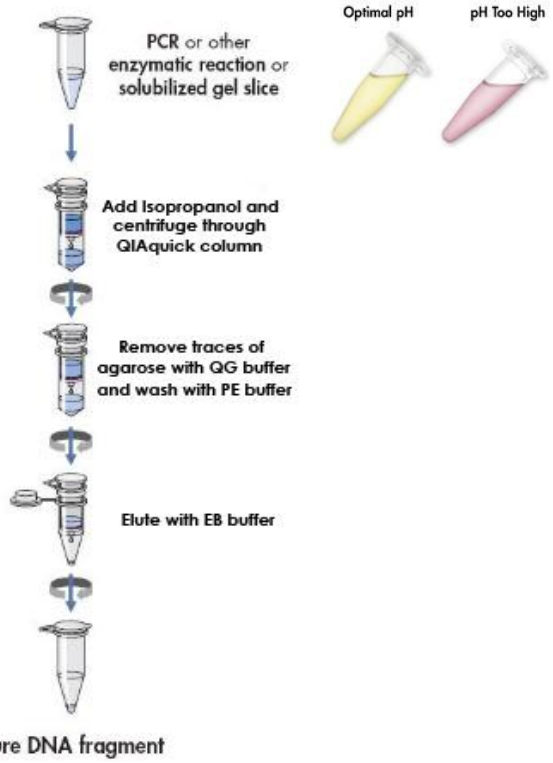


Figure 7.4: QIAquick Gel Extraction Kit Protocol (Adapted from http://www.qiagen.com/products/dnacleanup/gelpcrsicleanupsystems/qiaquickgelextractionkit.aspx)

Protocol

The gel slice containing the DNA for purification (1 volume) was incubated with 3 volumes of QG solubilization buffer, at 50 °C, until the gel was fully dissolved. The optimum pH for DNA binding to the column was assessed by the colour of the buffer. When colour of the buffer turned orange or violet, the pH was adjusted by adding 10 µl of 3 M sodium acetate,

pH 5.0, and mixing well (Figure 7.4). One volume of isopropanol was added to increase the recovery of DNA fragments <500 bp and >4 kb, and this solution was poured into the column provided in the kit. The column containing the silica membrane was spun for 1 min, at 14 000 rpm, to allow the adsorption of the DNA and the elimination of unwanted solutes. The flow-through was discarded, and the membrane washed for 1 min at 14 000 rpm, with 500 µl of buffer QG to remove any trace of agarose. This was followed by another 1 min at 14 000 rpm washing step with 750 µl of buffer PE. The flow–through was discarded and the column spun for an additional minute at 14 000 rpm to remove any residual PE buffer off the membrane. The column was placed in a clean collection tube for DNA elution in 30 µl of pre-warmed EB buffer, by a quick centrifugation step.

7.3 Cloning *Escherichia coli*

7.3.1 GATEWAY® recombination cloning technology

The GATEWAY® technology, developed by Invitrogen, is a commercially available cloning kit that capitalizes on the site-specific recombination features of the bacteriophage lambda – the DNA recombination sequences (att sites), and the recombination proteins that mediate the reaction (clonase™ enzyme mix) – to easily clone and transfer heterologous DNA sequences from one entry vector, such as the pENTR™ Directional TOPO® Cloning plasmids, into multiple destination vectors, while maintaining the orientation, and without losing frame. The recombination will occur between the att1 and att2 sites (Figure 7.5), assuring that DNA fragments of interest, flanked by these two sequences, are exchanged without additional technical hitches (Invitrogen).

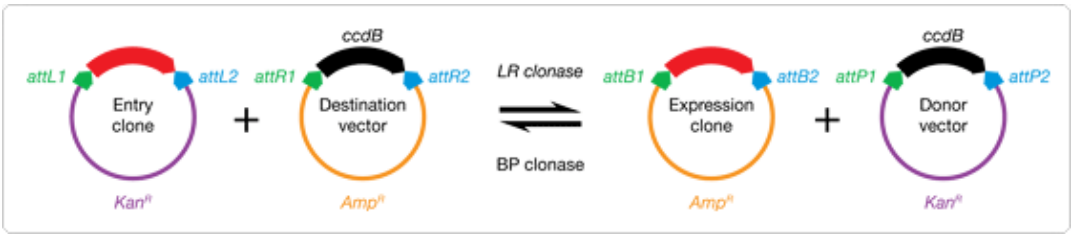


Figure 7.5: Schematic representation of the Gateway® technology (adapted from http://www.Invitrogen.com).

The pENTR™/D-TOPO® entry plasmid is compatible with direct blunt end ligation of purified PCR products. However, for directional TOPO® cloning it's imperative to add a CACC sequence to the 5' end of the forward primer (Figure 7.6) prior to amplification.

The expression of the ccdB gene in the donor vector, after recombination, inhibits the growth of most *E. coli* strains, such as the ones supplied in the kit. This feature improves

greatly the efficient recovery of the expected clones. Conversely, due to the toxicity of this protein, all destination vectors that possess the ccdB gene must be propagated in resistant *E. coli* strains like DB3.1™ (reviewed by Invitrogen).

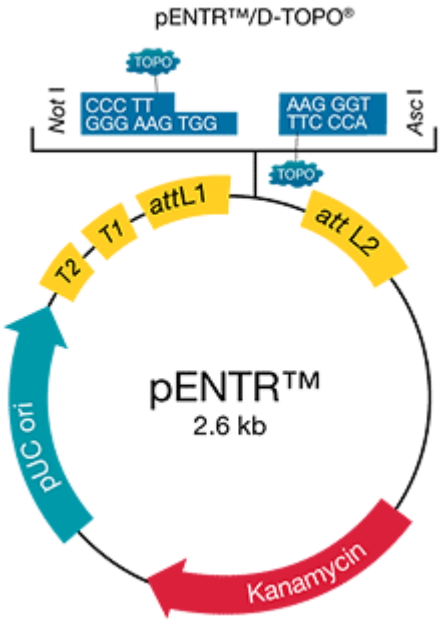


Figure 7.6: Schematic representation of pENTR™/D-TOPO® plasmid (adapted from Invitrogen)

7.3.1.1 pENTR™ Directional TOPO® Cloning

Protocol

The genes of interest were amplified from Brachypodium's genomic DNA or cDNA templates, by PCR using appropriate oligonucleotides and Phusion® High-Fidelity DNA Polymerase; The PCR products were, then, separated by electrophoresis in a 1-1.2% (w/v) agarose gel, and gel extracted using a QIAGEN QIAquick® Gel Extraction Kit. The TOPO® cloning reactions were setup according to the pENTR™ Directional TOPO® Cloning kit instructions; by adding up to 4 µl PCR product to 1 µl salt solution and 1 µl pENTR TOPO® vector; and incubated at room temperature for 30 min. The pENTR TOPO® vector incubated on ice for 30 min with 10 µl One Shot® TOP10 competent cells, followed by heat-shock transformation at 42 °C for 45 s. Heat-shocked cells recovered in 200 µl SOC medium, for 1 h, at 37 °C, and 200 rpm, and were later plated in solid LB medium supplemented with 100 µg/ml of kanamycin. Transformed cells formed visible colonies after 24 h at 37 °C. Independent clones were later sequenced using M13 Forward and M13 Reverse sequencing primers.

7.3.1.2 GATEWAY LR clonase reaction

Protocol

A mix of 0.4 µl of the pENTR plasmid, 0.4 µl destination plasmid, 0.3 µl TE buffer, and 0.3 µl clonase mix incubated at room temperature for at least 1 h. The recombination reactions were stopped by incubating at 37 °C for 10 min with 0.5 µl proteinase K. One Shot® TOP10 competent cells were transformed by heat-shocking at 42 °C for 45 s, after incubating on ice for 30 min. Heat-shocked cells recovered in 200 µl SOC medium, for 1 h, at 37 °C, and 200 rpm, and were later plated in solid LB medium supplemented with 100 µg/ml of Spectinomycin. Transformed cells formed visible colonies after 24 h at 37 °C. Independent clones were later sequenced using appropriate sequencing primers.

7.3.2 pGEM®-T Easy vector system cloning

The pGEM®-T Easy vector has been developed by Promega, as a linearized high copy number cloning plasmid with 3' terminal thymidine overhangs in both ends of the insertion site, T7 and SP6 RNA polymerase promoters flanking the gene insertion, and with blue-white screening (insertional inactivation of the alpha-peptide coding region β-galactosidase) of transformants.

Protocol

The genes of interest were amplified from Brachypodium's cDNA template, by PCR using appropriate oligonucleotides and Phusion® High-Fidelity DNA Polymerase; The PCR products were, then, separated by electrophoresis in a 1-1.2% (w/v) agarose gel, and gel extracted using a QIAGEN QIAquick® Gel Extraction Kit. The pGEM®-T Easy vector system is not compatible with blunt end cloning, therefore, an intercalary TA overhangs reaction was setup by mixing 8 µl PCR product, 1 µl Taq buffer, 1 µl dNTPs, and 0.1 µl Taq polymerase (NEB); the TA reaction incubated for 20 min at 72 °C. 3 µl TA-overhangs PCR product were mixed with 1 µl pGEM®-T Easy, 1 µl T4 DNA ligase, 5 µl 2X Rapid Ligation Buffer and incubated overnight at 4 °C. 50 µl of Rubidium Chloride competent cells were mixed with 10 µl pGEM®-T Easy ligation mix and transformed by heat-shocking at 42 °C for 45 s, after incubating on ice for 30 min. Heat-shocked cells recovered in 200 µl SOC medium, for 1 h, at 37 °C, and 200 rpm, and were later plated with 40 µl 2% (w/v) X-GAL (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) and 40 µl 10 mM IPTG (Isopropyl-β-D-thiogalactoside) on solid LB medium supplemented with 100 µg/ml of Carbenicilin. Transformed cells formed visible (white) colonies after 24 h at 37 °C. Independent clones were later sequenced using T7 Forward and SP6 Reverse sequencing primers.

7.3.3 *Escherichia coli* competent cells preparation

Artificial competence refers to the ability to chemically permeabilize bacterial cells to foreign DNA, by exposing it to divalent cations solutions under cold conditions. It is thought that the under such conditions the cell wall is weakened, and becomes sensitive to transformation by heat-chock.

Protocol

E. coli cells of the strain DH5α were streaked out onto solid LB medium, from a glycerol stock, and incubated overnight at 37 °C. A single colony was picked and inoculated into 10 ml of liquid LB medium for an overnight culture, at 37 °C. This small culture was upscaled to 2x 500 ml of liquid Psi broth, also at 37 °C, until the $O.D._{600\ nm}$=0.5. Cells were spun at 5000 rpm for 5 min, at 4°C, and pellets re-suspended in 2x 200 ml of ice-cold Tf buffer I. Cells incubated for 15 min at 4 °C, and were centrifuged once more at 5000 rpm, for 5 min, at 4°C, and re-suspended in 2x 20 ml of Tf buffer II. Cells incubated on ice for 15 min, before being divided in 50 µl aliquots, frozen in liquid nitrogen and stored at -80°C.

Genotypes

DH5α F^-, Φ80lacZΔM15, Δ(lacZYA-argF), U169, recA1, endA1, hsdR17, (rK^-, mK^+), phoA, supE44, λ^-, thi-1, gyrA96, relA1

TOP10 F^-, mcrA, Δ(mrr-hsdRMS-mcrBC), Φ80lacZΔM15, ΔlacX74, recA1, araD139, Δ(ara leu), 7697, galU, galK, rpsL, (StrR), endA1, nupG

DB3.1 F^-, gyrA462, endA1, Δ(sr1-recA), mcrB, mrr, hsdS20(rB^-, mB^-), supE44, ara-14, galK2, lacY1, proA2, rpsL20(SmR), xyl-5, λ^-, leu, mtl1

7.3.4 Plasmid purification from bacterial cells

Plasmid purification from bacterial cells is a routine molecular biology technique that is based on the alkaline lysis protocol developed by Birnboim and Doly in 1979. It is based on the rupture of the bacterial cells, through the use of an alkaline buffer; the precipitation of cellular debris along with chromosomal DNA; and the recovery of highly pure plasmid DNA. Commercial kits for plasmid purification usually provide columns with DNA-binding silica membranes that help retaining the plasmid DNA while remaining solubles are washed away. The plasmid DNA can, then, be precipitated with ethanol, and eluted with the appropriate solvent (Figure 7.7).

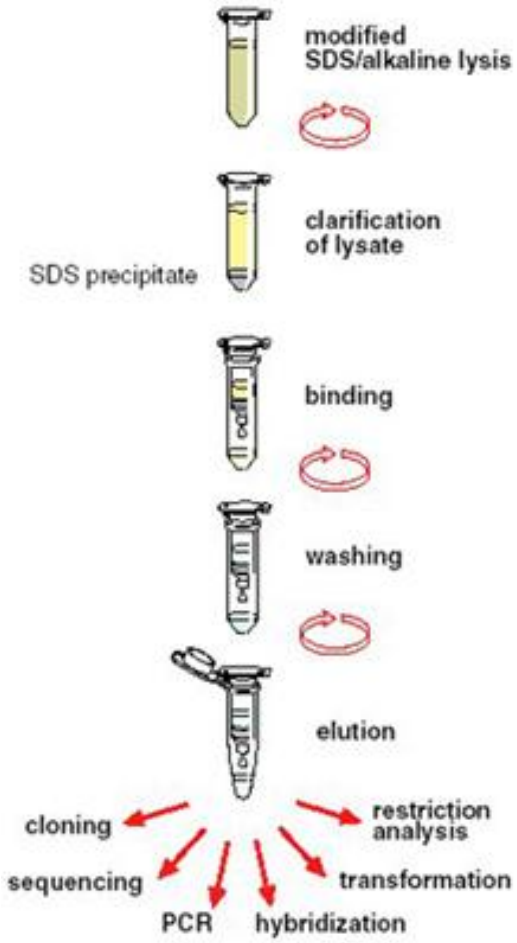


Figure 7.7: Schematic representation of the plasmid purification protocol of Macherey-Nagel NucleoSpin® Plasmid kit (adapted from www.mn-net.com)

Protocol

The plasmid purifications were performed by using a Macherey-Nagel NucleoSpin® Plasmid kit. The protocol used respected the standard technical instructions provided in the kit. For each independent clone, 4 ml of bacterial (overnight grown) culture was used. The cells were spun for 1 min at 14 000 rpm, and re-suspended by adding 250 µl of buffer A1. Cell lysis occurred by adding 250 µl of buffer A2 and incubating at room temperature for 5 min. The lysate was neutralised by adding 300 µl of buffer A3, and the cell debris were removed by spinning the samples for 10 min, at 14 000 rpm, at room temperature. The supernatant was loaded onto a column (provided) and the plasmid bound to the silica membrane by short-spinning at 14 000 rpm. The membrane was washed with 500 µl of pre-warmed (50 °C) buffer AW, to improve enzymatic digestion and DNA sequencing output, followed by another wash of 600 µl of buffer A4, to remove contaminants. The membrane was dried by spinning for 2 min at 14 000 rpm, and the plasmid eluted in 2 volumes of 50 µl of pre-warmed (50 °C) buffer AE. Plasmid concentration was assessed spectrophotometrically in a NanoDrop™ 1000 Spectrophotometer.

7.3.5 Plasmid digestion with restriction enzyme

Restriction enzymes are endonucleases that are able to recognize specific nucleotide sequences (restriction sites) within dsDNA, and chop the phosphate bonds in both strands of the double helix, fragmenting it. Their biological activity is frequently employed in molecular biology labs to cut different DNA molecules while cloning, and in the creation of restriction maps, among other uses.

Protocol

There are hundreds of restriction enzymes, each with its own specificities and requirements for optimum activity. The standard plasmid restriction reaction was set-up by digesting 2 µl of plasmid DNA, with 1 µl of restriction buffer (enzyme specific), 7 µl water, and 0.1 µl of restriction enzyme; for a total reaction volume of 10 µl. Reactions were incubated for 2 h at the optimum temperature for the chosen enzyme. Some enzymes also required the addition of BSA in order to work efficiently. In those cases, 0.1 µl of BSA was added to the reaction.

7.3.5.1 DNA clean-up

In cloning procedures, whenever it was essential to perform a double restriction digestion with two enzymes with incompatible restriction buffers, a sequential digest was performed. However, in order to do so it was necessary to digest the DNA with one of the enzymes first, followed by the purification of the DNA (Figure 7.8), and the setup the next restriction reaction.

Protocol

The DNA clean-up was performed using QIAGEN MinElute PCR purification Kit, according to the supplier recommendations. 3.5 volumes of PBI buffer were added to the DNA solution to be cleaned, poured into a supplied column, and spun for 1 min at 14 000 rpm. The flow-through was discarded, and the column washed with 750 µl of PE buffer. The column was spun, again, for 1 min at 14 000 rpm, and the flow-through discarded. In order to remove any residual PE buffer from the silica membrane, the column was once more spun at 14 000 rpm, this time for 2 min. The DNA was eluted in pre-warmed (50 °C) 30 µl of EB buffer, through a brief spin at full speed.

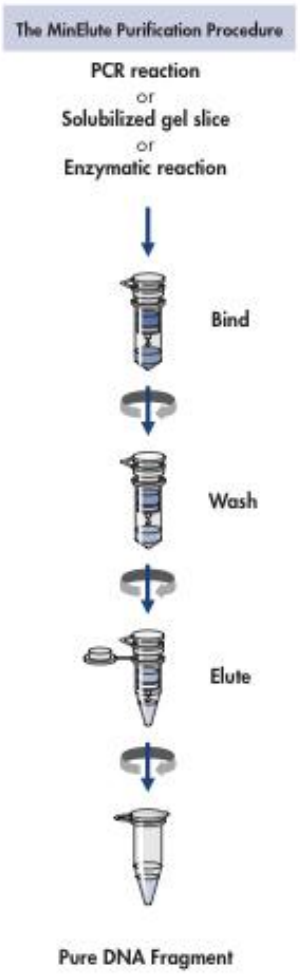


Figure 7.8: Schematic representation of the DNA cleanup protocol of QIAGEN MinElute PCR purification Kit (adapted from QIAGEN MinElute PCR purification Kit handbook)

7.3.6 Sequencing reactions

DNA sequencing is an essential molecular tool in biological research. The process, developed by Frederick Sanger in 1977, combines the principle of PCR with the separation of the reaction product by electrophoresis; however, instead of using a normal mixture of deoxyribonucleotides (dNTPs) as the building blocks of the newly synthesized chain, it uses a combination of normal dNTPs with a smaller proportion of dideoxyribonucleotides (ddNTPs). These ddNTPs lack both 2′-hydroxyl and 3′-hydroxyl groups (-OH) in their deoxyribose, which impedes the formation of phosphodiester bonds with other nucleotides; once a ddNTP is randomly incorporated into the elongating strand, the DNA synthesis comes to a stop. Since the incorporation of this type of nucleotide is random, and its incorporation implies the termination of the strand elongation, by the end of the PCR reaction all kinds of differently sized fragments would have been produced, each with a distinctive ddNTP at the 3′ end. These fragments are separated according to their size, by capillary electrophoresis, and because the reaction is set-up with differently labelled fluorescent-ddNTPs (each emitting at a different wavelength), the sequence can be determined by the automated sequence analysers, which are able to detect the fluorescence of the ddNTP in each DNA strand produced (Figure 7.9).

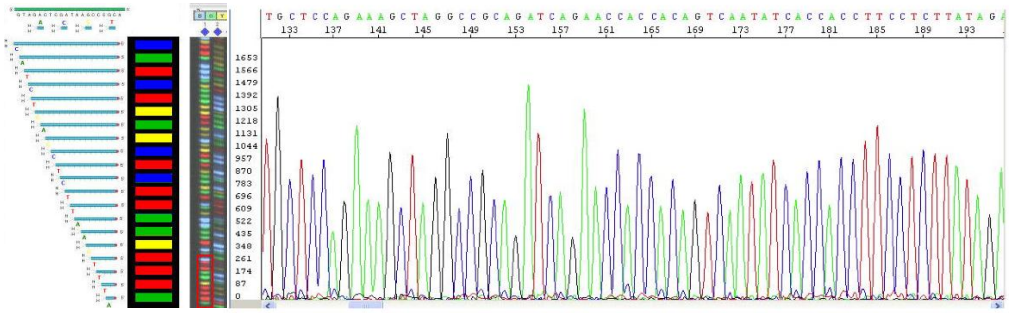


Figure 7.9: Sequencing gel [left] **and chromatogram** [right]

(Gel, modified from www.mun.ca/biology/scarr/377_gel_file.html);
(Chromatogram, adapted http://medsci.udel.edu/cores/bcl/forms/BCL_Sequencing_Troubleshooting_Guide.pdf)

Protocol

The standard sequencing reaction for a plasmid preparation was setup by mixing 2 µl plasmid DNA, 2 µl of 10 µM forward sequencing primer, 1 µl Big Dye® Terminator v3.1 Cycle Sequencing Ready Reaction Mix (Applied Biosystems), and 1 µl 5x Big Dye® Terminator v3.1 Cycle Sequencing Buffer (Applied Biosystems). The same mix was prepared for the reverse sequencing primer. The PCR sequencing program was as follows: 96 °C for 1 min; 96 °C for 10 s; 50 °C for 5 s; 60 °C for 4 min; 25 cycles; 4 °C for 10 min; 10 °C for ever. Samples were submitted to The Genome Analysis Centre (www.tgac.ac.uk/), which performed the readings and provided the sequencing results.

7.4 Cloning *Agrobacterium tumefaciens*

7.4.1 *Agrobacterium tumefaciens* transformation

Agrobacterium cells were transformed by electroporation, or electropermeabilization, a process which involves the application of an electric current to the bacterial suspension, causing the cellular membranes of the bacteria to become permeabilized to DNA molecules in solution (Neumann *et al*, 1982). Suspensions with high salt concentrations can cause an electric discharge (arcing), when electroporated, leading to the loss of cells. This phenomenon can be avoided with the resuspension of bacterial cells in salt-free solutions before aliquoting; the elution of the plasmid in water, after purification; or, when eluted in buffer, the dilution of the plasmid solution prior to use.

Protocol

A thawed 50 µl aliquot of Agrobacterium cells was mixed with 1 µl plasmid solution, and electroporated in a Bio-Rad Gene Pulser™ set to: capacitance of 25 µF, capacitance extender of 125 µF, Resistance of 400 Ω and electric tension of 2.5 kV. Electrical discharge, due to high salt concentration, was avoided by diluting the plasmid solutions 40x. Electroporated cells recovered at 28 °C for 4 h in liquid YEP medium, and were later plated on solid LB medium supplemented with 50 µg/ml Rifampicin, 100 µg/ml Carbenicilin, and 100 µg/ ml Spectinomycin. Transformed cells formed visible colonies after 48 h at 28 °C.

7.4.2 *Agrobacterium tumefaciens* electro-competent cells preparation

The disarmed AGL1 strain is characterized by possessing the C58, RecA chromosomal background, the hyper-virulent pTiBo542-ΔT-DNA plasmid harbouring additional Vir genes from *Agrobacterium* strain A281 (Jin SG, Komari T, Gordon MP *et al*, 1987), and the resistance to the antibiotics rifampicin, and carbenicilin. (Lazo *et al*, 1991).

Protocol

Cells from a glycerol stock were streaked out onto solid LB medium supplemented with 50 µg/ml rifampicin, and 100 µg/ml carbenicilin, and incubated for 48 h at 28 °C. A single colony was picked into 2 ml of liquid LB medium with antibiotics and incubated for 48 h at 28°C. This small culture was upscaled to 2x 500 ml of liquid LB medium plus antibiotics for 48 h at 28°C. Cells were spun at 5000 rpm for 10 min, at 4°C, when the $O.D._{600\ nm}$=0.5. Pellets were re-suspended in 2x 500 ml of 10% glycerol (2x). Cells were centrifuged once more at 5000 rpm for 10 min at 4°C and re-suspended in 2 ml of 10% glycerol. Cells were, then, divided in 50 µl aliquots, frozen in liquid nitrogen and stored at -80°C.

7.5 Agrobacterium-mediated transformation of *Brachypodium distachyon*

7.5.1 *Agrobacterium tumefaciens* – Nature´s genetic engineer

Rhizobium radiobacter or *Agrobacterium tumefaciens* (Young *et al*, 2001; Smith and Townsend 1907) is an alpha proteobacterium of the family *Rhizobiaceae*, with a 5.67 Mb genome consisting of a circular chromosome, a linear chromosome, and two plasmids. It can be described as an obligatory aerobic, non-sporing, rod shaped, gram negative bacterium, surrounded by a small number of peritricious flagella. *Agrobacterium tumefaciens* is not an obligate pathogen, as it can grow vigorously as a saprophyte or colonize plants without causing any symptoms, however, this ubiquitous soil borne bacteria is generally known as a parasitic pathogen responsible for Crown Gall disease, a common plant neoplasia, affecting over 600 types of woody and herbaceous plants, mostly dicotyledonous species, members of the *Rosaceae* family. Infected plants can be identified by the appearance of tumours or galls of varying size and shape on the lower stem and main roots (Smith and Townsend 1907) (Figure 7.10).

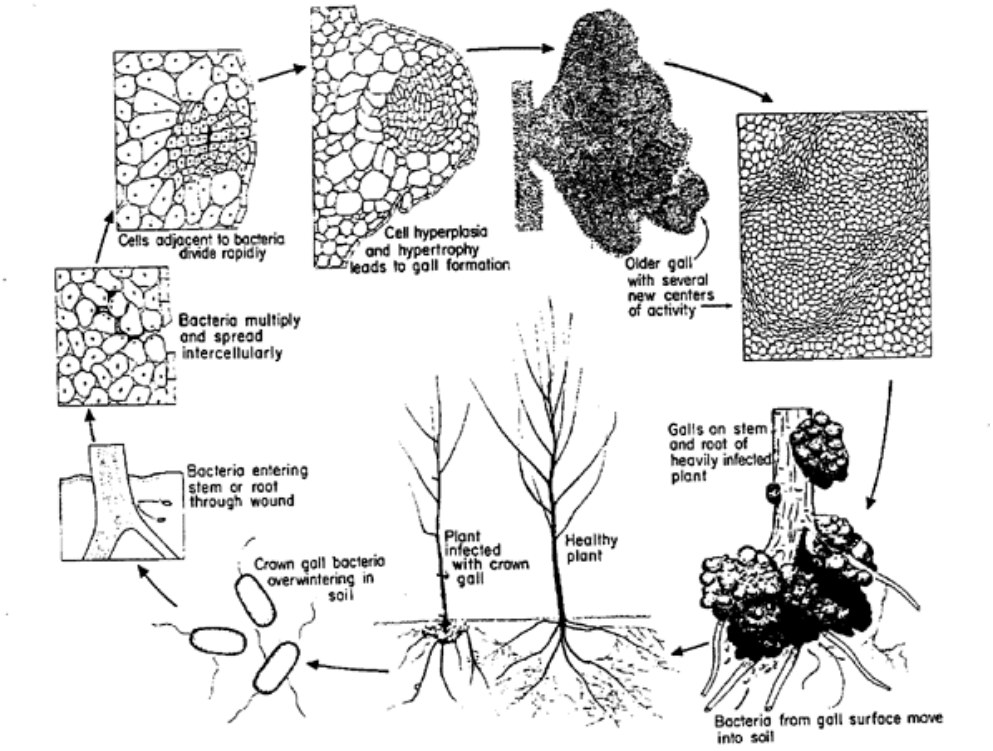


Figure 7.10: Schematic representation of the infectious cycle of *Agrobacterium tumefaciens* (Adapted from www.hortnet.co.nz)

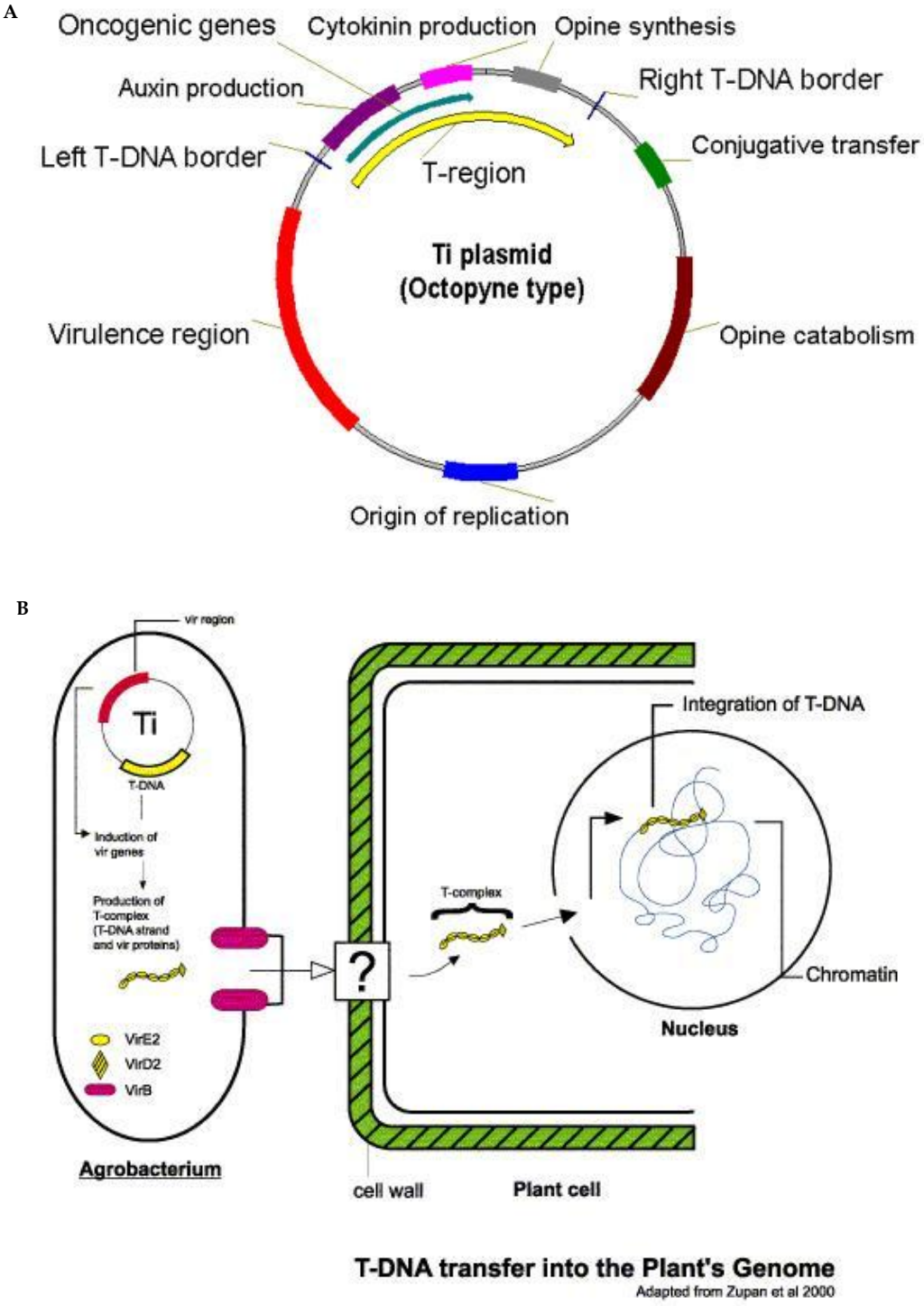


Figure 7.11: A) Schematic representation of the tumour inducing (Ti) plasmid from *Agrobacterium tumefaciens*. B) Schematic representation of the T-DNA transfer between the *Agrobacterium tumefaciens* cell into the host-cell (adapted from www.patentlens.net/daisy/AgroTran/g1/843.html)

In natural conditions, the motile cells of *A. tumefaciens* are attracted by the chemical signals produced by wounded plants, in a Chemotaxis-response. The chromosomal-encoded adhesion (chv) genes are activated, and their expression products mediate the first steps of bacterial attachment to the host surface. Phenolic compounds, produced by the plant's wounded tissue, such as acetosyringone, activate the virulence (Vir) genes on the 200 kb tumour inducing (Ti) plasmid. The collective function of the Vir genes consists in the excision, from the Ti plasmid, of a specific ssDNA fragment of 20 kb and its transfer into the plant cell genome. Known as transferred DNA (T-DNA), and delimited by the left and right borders, this small portion of genetic material harbours in its sequence two sets of genes: the oncogenic (Onc) genes responsible for perturbing the plant's cell cycle leading to the formation of a gall; and the opine biosynthetic genes. Opines, like nopaline or octopine, are a group of specific oligosaccharide amino acid derivatives that are metabolized purely by the infecting bacteria, as a nitrogen and carbon source. In essence, the purpose of the infection is the diversion of the host normal metabolism, under the direction of the T-DNA, into the production of nutrients that can later be catabolised by the bacteria, by calling on opine-utilizing (Noc) genes on the Ti plasmid. In naturally occurring bacterial infections these genes alter the development and metabolism of the infected cells, but without this Ti plasmid, the bacterium is described as being non virulent and won't be able to cause disease on the plant (reviewed by Gelvin, 2003) (Figure 7.11).

7.5.1.1 From pathogen to research tool

The molecular basis for the genetic transformation process has been the subject of numerous studies over the past several decades. There are many problems associated with current methods of transgenic plant production, most notably the low transformation frequency encountered, or the failure of transgenic plants to grow at all. The use of *Agrobacterium tumefaciens* in transgenic plant production has arisen from the need to find an effective vector system to successfully integrate the gene of interest into the correct area of the plant genome. The natural behaviour of the Ti plasmid makes it well suited to the role of plant vector. By modifying the T-DNA region of the Ti plasmid, through the use of restriction enzymes and ligases, and utilising the Vir gene functions resident in modified *Agrobacterium* strains, it has been possible to generate synthetic vectors which are designed to stable integrate manipulated DNA into the genome of plants. Although in nature, *Agrobacterium* transforms mainly dicotyledonous plants, under controlled culture conditions it has been shown to possess a broader host range which includes monocot plants, gymnosperms, yeast and other fungi, and even human cells. If the bacterial infection of plant cells takes place, any genetic material between the left and right T-DNA border sequences can be inserted into the plant chromosomes. Since many plants can be regenerated from single cultured cells, transgenic plants can be established directly from cells into which recombinant DNA has been introduced in culture. The growth of such cells on selective medium is an indication that transformation has taken place. These cells can be induced to differentiate shoots and roots, and with time, to develop into fully mature plants (reviewed by Gelvin, 2003).

Strains of *A. tumefaciens* that are useful for plant transformation are defined by their chromosomal background and resident Ti plasmid. The C58 chromosomal background (from the first fully sequenced pathovar) (Wood *et al*, 2001; Goodner *et al*, 2001) has proved to be popular for plant transformation and now harbours several kinds of wild-type and disarmed Ti plasmids, including strains that are effective at transforming cereals (reviewed by Gelvin, 2003).

7.5.2 Transformation vectors

To be able to carry out genetic transformation of a wide range of plant species by capitalizing on a natural system to introduce DNA into the nuclear genome of plants, it's always essential to develop vectors that will carry the gene of interest and perform the cell's genetic modification. All Agrobacterium-mediated transformation vectors must possess either a broad host range replication origin (*ori*) that will permit plasmid maintenance in a wide range of gram-negative bacteria including *E. coli* and *Agrobacterium* or, alternatively, two separate replication origins for plasmid maintenance in each of the bacterial species concerned. Other essential DNA sequences called molecular markers, such as antibiotic resistance (suitable for selection in both *E. coli* and *A. tumefaciens*) or reporter genes must also be present in the vector. These molecular markers will enable the determination, as soon as possible, of which cells were successfully transformed and are expressing the inserted gene of interest (Hellens *et al*, 2000). Hygromycin B, is an aminoglycosidic antibiotic produced by *Streptomyces hygroscopicus* that effectively kills bacteria, fungi and higher eukaryotic cells by inhibiting protein synthesis. It's particular toxic for plant cells such as those of cereals, and so the Hygromycin B phosphotransferase gene (Hph), has been a very popular choice of a molecular marker when designing vectors for genetic transformation of crops (www.hygromycin.com/).

The Gateway "over-expression" vector with the OsAct1 promoter obtained from the Leibniz Institute for Plant Genetics and Crop Plant Research (Himmelbach *et al*, 2007) encodes for Hygromycin resistance for plant transformation, and spectinomycin resistance for bacteria transformation.

7.5.3 Genetic transformation of *Brachypodium distachyon*, Bd21

The genetic transformation of *Brachypodium distachyon* community standard line Bd21 (Alves *et al*, 2009) is based on the protocols developed for the genetic transformation of other cereal crops, such as rice. Its key features are the production of compact and embryogenic callus (CEC), from immature embryos of healthy 7-8 week old donor-plants, prior to transformation; the desiccation of CEC after inoculation with *Agrobacterium tumefaciens*; and the selection of transgenic cells in culture media with the antibiotic Hygromycin B. This protocol enables the harvest of dried transgenic seeds ~30 weeks after sowing.

Protocol

7.5.3.1 Production of CEC from immature embryos

Tillers from 7- to 9-week-old Bd21 plants were collected when the immature seeds were swollen but still green. Immature seeds with a soft endosperm were selected, and were sterilized as described previously. Immature embryos up to and including 0.3 mm in length were dissected from seeds using fine forceps and a stereomicroscope under sterile conditions (laminar flow hood). The immature embryos (10–20 per plate) were cultured, scutellum facing up, onto MSB3 + Cu0.6 solid medium, for 3 weeks, at 25 °C, in the dark. The shoots were excised under sterile conditions, as they elongate during the first 2–3 d of culture. At week 3 (i.e., 3 weeks after dissection), the CEC with a creamy colour and pearly surface were fragmented in 1–3 pieces. Those pieces of CEC were transferred onto fresh MSB3 + Cu0.6 solid medium (16–20 *calli* per plate) for another 2 weeks at 25 °C in the dark. All non-CEC tissue was discarded. At week 5 (i.e., 5 weeks after dissection), the CEC was again multiplied and transferred onto fresh MSB3 + Cu0.6 solid medium (16–20 calli per plate) for another week at 25 °C in the dark. At week 6 (i.e., 6 weeks after dissection – the day of transformation), the CEC were split into small pieces, and about 100 CEC pieces per transformation were pooled onto fresh MSB3 + Cu0.6 solid medium before inoculation with Agrobacterium.

7.5.3.2 Agrobacterium-mediated transformation of CEC

The AGL1 strain of *A. tumefaciens* carrying the plasmid of interest was inoculated into 1 ml of LB + S50 liquid medium for an overnight culture at 28 °C and 200 rpm. From the liquid culture, 200 µl were plated onto solid MG/L + S50 + AS30 and cultured (upside down) for 2 days at 28 °C in the dark. Approximately half a plate of Agrobacterium film was scooped with an L-shaped sterile glass Pasteur pipette into 10 ml of MSB + AS45 liquid medium. The suspension was vigorously shaked to efficiently disperse the Agrobacterium cells at 220 rpm for 45 min, in a 28 °C incubator. CEC plates were flooded with 13 ml of Agrobacterium suspension OD_{600}=1, and left for a 5 min inoculation in a laminar flow hood at room temperature. The bacterial suspension was completely pipetted out from the CEC plates, and the handpicked CEC were transferred onto a filter paper with 750 µl of MSB + AS45

medium. Plates were sealed with cling film, and incubated for 2 days at 25 °C in the dark.

7.5.3.3 Selection and regeneration of transgenic plants

After 2 days of co-culture, the CECs were transferred onto selective MSB3 + Cu0.6 + H50 + T225 solid medium (20 *calli* per plate) for 6 weeks at 25 °C in the dark. After every 2 weeks of selective period, the medium was refreshed, but in keeping with the same selective pressure. Six weeks after transformation, all hygromycin-resistant *calli* were transferred onto the MSR26 + H50 + T225 regeneration medium (12–16 *calli* per plate) for 2–3 weeks at 25 °C under 16-h photoperiod. After that period, all developing shoots were transferred into tubes containing MSR63 + Ch7 + H50 + T225 germination medium. These were kept in germination media for 2–3 weeks at 25 °C under 16-h photoperiod, or until they developed strong and healthy roots in Hygromycin B. Fully rooted transgenic plantlets were transferred to soil, and kept at 22 °C with a 20-h photoperiod. A propagator lid was used to ensure the adaptation of transgenic plantlets to low-humidity conditions. When the seeds were fully mature, plants were dried for 2–4 weeks before harvesting.

7.6 Expression analysis

7.6.1 Gene expression profiling - Microarray technology

The gene expression profiling (microarray) technology is a relatively recent biological assay based on the principle of hybridization between two DNA strands, i.e., the formation of stable hydrogen bonds under high-stringency conditions, between complementary nucleotides of a sample to be tested [targets], and the tens of thousands of oligonucleotides attached to the solid support of a DNA chip [probes] (Figure 7.12). The hybridization levels between samples can be quantified through the detection of the chemically-labelled targets, which correlates with the abundance of a particular target in the samples analysed. This technique is employed in wide gene-expression analysis between distinct biological or physiological conditions, through the simultaneous measurement of changes in the expression levels of a vast number of genes (Schena and Shalon *et al*, 1995)).

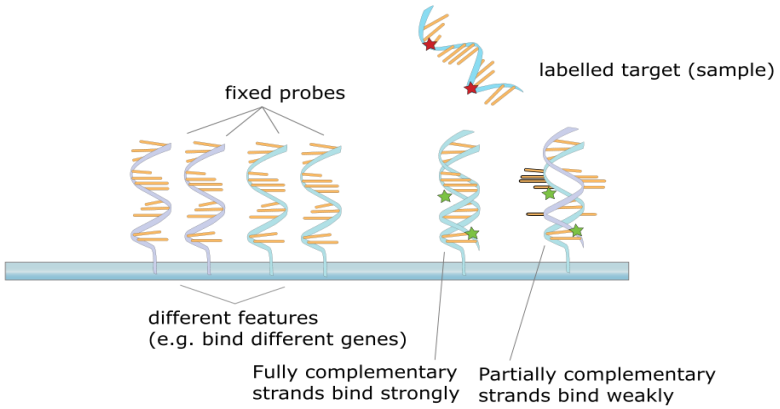


Figure 7.12: Schematic representation of the microarray principle (adapted from Wikipedia)

The Ambion® WT Expression Kit together with the Affymetrix® GeneChip® Whole Transcript (WT) Expression arrays, generate biotinylated sense-strand cDNA targets from total RNA through a reverse transcription method that specifically primes non-ribosomal RNA, including both poly(A) and non-poly(A) mRNA, in a complete and unbiased coverage of the transcriptome. The ssDNA synthesis, with random primers and an unnatural uracil base (dUTP), produces a substrate that can be fragmented by the combined activities of a Uracil DNA Glycosylase (UDG) – an enzyme which specifically removes the uracil residue from the ssDNA molecule – and a apurinic/apyrimidic endonuclease 1 (APE1) – a phosphodiesterase that cuts the phosphodiester bond of the DNA backbone, where the (uracil) base is missing. After fragmentation, the DNA molecules are terminally biotinylated (labelled), via a terminal deoxynucleotidyl transferase (TdT), before being hybridized in the array (Ambion® WT Expression Kit & Affymetrix® GeneChip® Whole Transcript (WT) Expression arrays).

Protocol

7.6.1.1 Whole transcript expression – sense-strand cDNA synthesis

For the preparation of total RNA page 130. The synthesis of the first-strand of cDNA was set up with 4 µl of First-Strand Buffer Mix, 1 µl of First-Strand Enzyme Mix, and 5 µl of RNA (100 ng). This mix incubated for 1 h at 25 °C, followed by another incubation of 1 h, at 42 °C, and a 2 min step at 4 °C. After the synthesis of the first-strand of cDNA, the samples were spun briefly at full speed to collect the cDNA at the bottom of the tube, and placed on ice while the Second-Strand Master Mix was prepared. This mix was composed of 32.5 µl of Nuclease-free Water, 12.5 µl of Second-Strand Buffer Mix, and 5 µl Second-Strand Enzyme Mix. The Second-Strand Master Mix was mixed with the first-strand cDNA samples, incubated for 1 h at 16 °C, followed by an incubation step of 10 min at 65 °C, and rested for 2 min at 4 °C, while the *in vitro* Transcription Mix was prepared. This mix was composed of 24 µl of IVT Buffer mix, and 6 µl of IVT Enzyme mix. The second-strand reaction product was briefly spun at full speed to collect the cDNA at the bottom of the tube, and mixed with the IVT Master Mix. The IVT reaction incubated for 16 h at 40 °C, and then overnight at 4 °C. The cRNA was purified by adding 60 µl of cRNA Binding Mix (10 µl Nucleic Acid Binding Beads, and 50 µl Nucleic Acid Binding Buffer Concentrate) to each sample. The suspension was mixed by pipetting up and down, and transferred into a U-bottom plate where 60 µl of 100% isopropanol were added. The cRNA and beads incubated at room temperature, with gentle shaking for 2 min so the cRNA could be bound to the beads. The beads were captured by placing the tubes in a magnetic stand, and the supernatant was discarded. The tubes were removed from the magnetic stand and the beads were washed with 100 µl of Nucleic Acid Wash Solution, for 1 min with moderate shaking. The beads were again captured in a magnetic stand so the supernatant could be discarded; and washed with another 100 µl of Nucleic Acid Wash Solution. The supernatant was, again discarded, and the plate was shaken vigorously for 1 min to evaporate any residual ethanol from the beads. The purified cRNA was eluted from the beads by adding 40 µl of pre-heated (55 °C) Elution Solution and by incubating without shaking for 2 min. The beads were afterwards vigorously shaken for 3 min to fully disperse them, and magnetically captured so the supernatant, containing the eluted cRNA, could be recovered. The 2nd-cycle cDNA synthesis was performed by adding 22 µl of cRNA (10 µg) and 2 µl of Random Primers. This mix incubated for 5 min at 70 °C, followed by 5 min at 25 °C, and 2 min at 4 °C. The solution was incubated in ice while the 2nd-cycle cDNA Master Mix was prepared. This master mix was composed of 8 µl of 2nd-Cycle Buffer Mix, and 8 µl of 2nd-Cycle Enzyme Mix, and it was pipetted into the cRNA/Random Primer sample. The reaction took place for 10 min at 25 °C, then 90 min at 42 °C, followed by 10 min at 70 °C, and for at least 2 min at 4 °C, before being hydrolyzed by RNase H. For the hydrolysis, 2 µl of RNase H were added to the 2nd-Cycle cDNA product, mixed gently and incubated for 45 min at 37 °C, followed by 5 min at 95 °C, and then 2 min at 4 °C.

The cDNA was purified by mixing it with 10 µl of Nucleic acid Binding Beads, 50 µl of Nucleic Acid Binding Buffer Concentrate, and 18 µl of nuclease-free water. The suspension was transferred into a U-bottom plate, mixed with 120 µl of 100% ethanol, and gently shaken for 2 min so the cDNA could bind the beads. The beads were captured in a magnetic stand for ~5 min, and the supernatant was discarded. The beads were washed, twice, with 100 µl of Nucleic Acid Washing Solution, and gentle shaking for 1 min; followed by a vigorous shaking for 1 min to evaporate any residual ethanol from the beads. The purified cDNA was eluted from the Nucleic Acid Binding Beads by adding 30 µl of pre-warmed (55 °C) Elution Solution, and incubating for 2 min at room temperature, without shaking. The beads were dispersed by vigorously shaking for 3 min and captured in a magnetic stand. The solution containing the cDNA was recovered into a multiwall plate where it was fragmented and labelled, according to the Affymetrix GeneChip® WT Terminal Labelling Kit (Figure 7.13).

7.6.1.2 Whole transcript fragmentation and terminal labelling

The whole transcript fragmentation reaction was setup by diluting 5.5 µg of ssDNA with 31.2 µl of RNase-free water; and mixing it with a Fragmentation Master Mix, composed of 10 µl of RNase-free water, 4.8 µl of 10x cDNA Fragmentation Buffer, 1 µl of 10U/µl UDG, and 1 µl 1 000 U/µl APE 1. The reaction incubated for 60 min at 37 °C, followed by 2 min at 93 °C, and 2 min at 4 °C. The labelling reaction was setup by mixing 45 µl of Fragmented ssDNA, 12 µl of 5x TdT Buffer, 2 µl TdT, and 1 µl of 5 mM DNA Labelling Reagent. This mix incubated for 60 min at 37 °C, followed by 10 min at 70 °C, and then 2 min at 4 °C.

7.6.1.3 Transcript hybridization

The hybridization cocktail was prepared according to the GeneChip® Hybridization, Wash and Stain Kit instructions, i.e., 25 ng/µl of Fragmented and Labelled DNA Target, 50 pM of Control Oligonucleotide B2 (3 nM), 1.5 pM of 20x Eukaryotic Hybridization Control *bioB*, 5 pM of 20x Eukaryotic Hybridization Control *bioC*), 25 pM of 20x Eukaryotic Hybridization Control *bioD*), 100 pM of 20x Eukaryotic Hybridization Control *cre*, 1x of 2x Hybridization Mix, and 7% of DMSO. This mix was heated for 5 min at 99 °C, cooled to 45 °C for 5 min, and spun for 1 min at 14 000 rpm. The GeneChip ST Array was equilibrated at room temperature and injected; after which it incubated in an oven for 17 h, at 45 °C, and shaken at 60 rpm. A WT Hybridization Mix was prepared by adding (per array) 24 µl of 5x WT Hyb Add 1, 1.2 µl of Control Oligo B2 (3 nM), 6 µl of 20x Eukaryotic Hybridization Controls (*bioB*, *bioC*, *bioD*, *cre*), and 8 µl 15x WT Hyb Add 4. This was mixed with 32.8 µl of Fragmented and Labelled DNA, and 48 µl of 2.5x WT Hyb Add 6. The hybridization cocktail was denatured for 5 min at 99 °C, followed by a 45 °C incubation for another 5 min, before a 1 min centrifugation at 5 000 rpm to remove any insoluble product from the hybridization mixture. 90 µl of this supernatant were placed into the hybridization tray. To process the Gene 1.1 ST Array plates on the GeneTitan® Instrument, 105 µl of the Stain 1 were aliquoted into the GeneTitan Stain Tray; and, after removing the static electricity, a

stain tray cover was placed on top of it. This procedure was repeated for Stain Tray 2 and 3, with Stain 2 and 3, respectively. The forth scan tray cover provided in the kit was used to cover the HT Scan Tray P/N 500860 with 150 µl of Array Holding Buffer. All trays were loaded into the GeneTitan Instrument, following the instructions provided in the Affymetrix GeneChip Command Console 2.0 User Guide.

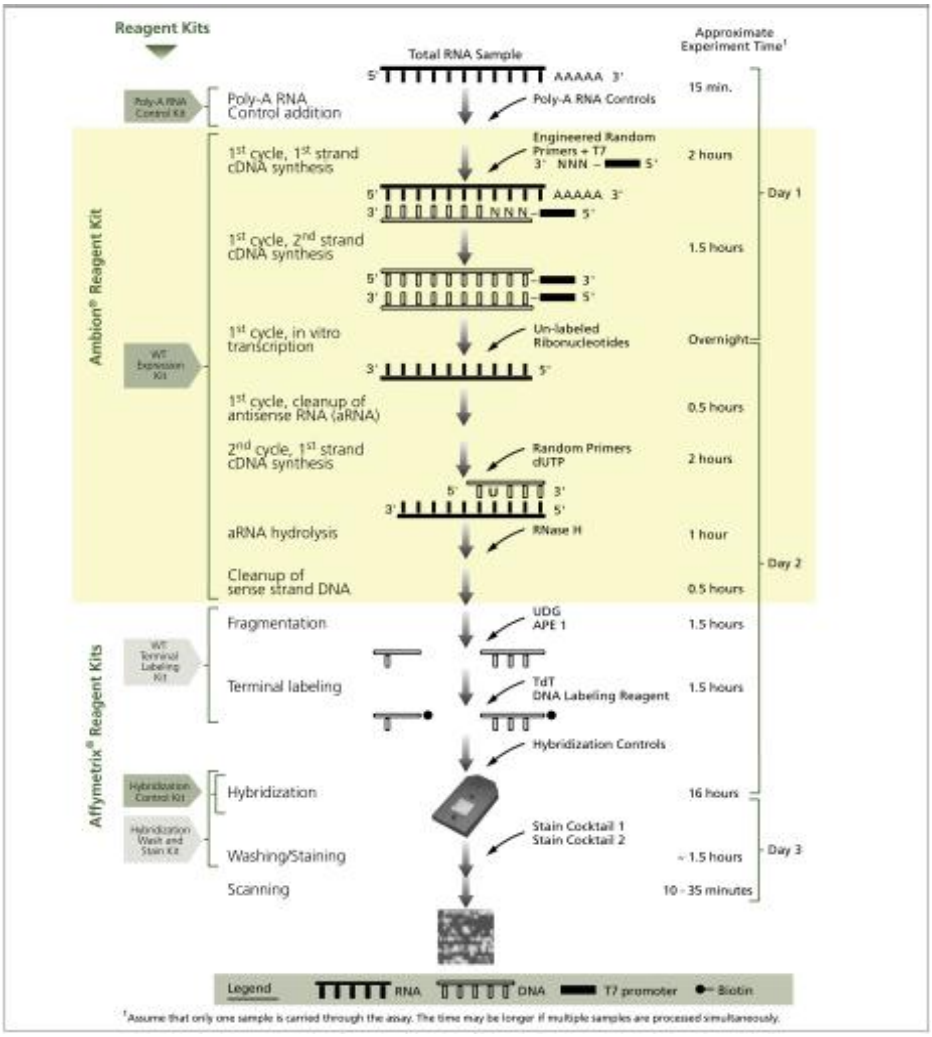


Figure 7.13: Schematic representation for generating whole transcript sense target (adapted from GeneChip® WT Terminal Labeling and Hybridization User Manual for use with the Ambion® WT Expression Kit)

7.6.2 Transcript's *in situ* immuno-histochemical detection

Transcript immuno-histochemistry (*in situ* hybridization) is a routine technical procedure that detects the precise localization of a specific mRNA within a well preserved heterogeneous histological section, through the bond of a labelled complementary strand of RNA (riboprobe). This technique is usually employed to detect the presence of a particular transcript in a histological preparation, and to characterize its expression pattern across a range of tissue samples. It's particularly useful when studying gene function and expression dynamics, during organ development or between distinct physiological conditions. The success of the procedure, however, depends in finding the correct balance between permeabilizing the sample to ease the access of the probe to the target mRNA, and the preservation of the tissue's integrity and cellular contents at the time of the harvest. Tissue's preservation from postharvest decay is achieved, primarily, by chemically destroying opportunistic microorganisms that will cause putrefaction, and secondly by ceasing all on-going biochemical reactions that could lead to autolysis – in a process called fixation. Chemical fixatives, like formaldehyde, act mainly by creating irreversible covalent bonds between proteins (cross-link), resulting in the arrest of all enzymatic activity, impeding gene expression, metabolism, and autolysis. In addition, this process also affixes soluble proteins to the cytoskeleton, which enhances the cell's mechanical strength, and contributes to the maintaining of the overall cell's structure, including its constituents. Other fixatives, like ethanol and acetic acid, are also used in the preservation of the sample's tissue due to their ability to precipitate proteins. However, to better preserve the tissue's morphology, these substances should be used together, as ethanol is known to promote significant shrinkage and hardening of the tissue, and acetic acid counteracts this effect by causing swelling. Once fixed, the tissue must be embedded in a hard matrix, like paraffin wax, that will allow the sample to be cut in micrometer-thin sections. The paraffin wax, however, is immiscible with water and will not infiltrate the tissue unless this has been fully dehydrated. The dehydration steps are usually done with a series of increasingly concentrated ethanol dips, and finalized with a hydrophobic clearing solution, like xylene, to remove the ethanol. After infiltration, the samples are cast into paraffin hardened blocks, ready to be sectioned. Anti-sense RNA labelled probes are obtained by cloning the correspondent cDNA sequence into a vector containing phage transcription promoters, such as T3, T7, or SP6, and by using it as a template for an *in vitro* transcription reaction, along with the incorporation of uridine triphosphate nucleotides conjugated with digoxigenin (DIG) – a steroid extracted from plants from the genus Digitalis (foxgloves). The detection of the riboprobe, at the site of hybridization, is achieved colorimetrically with anti-digoxigenin antibodies conjugated with alkaline phosphatase. In the presence of the colorless substrates 5-Bromo-4-chloro-3-indolyl phosphate (BCIP), and Nitro blue tetrazolium (NBT), the alkaline phosphatase will oxidize BCIP to indigo, by releasing a phosphate group, at the same time NBT will be reduced to diformazan, resulting in the production of a black-purple precipitate easily detectable under a light microscope (reviewed by Eisel *et al*, 2002 and Wilk *et al*, 2010)(Figure 7.14).

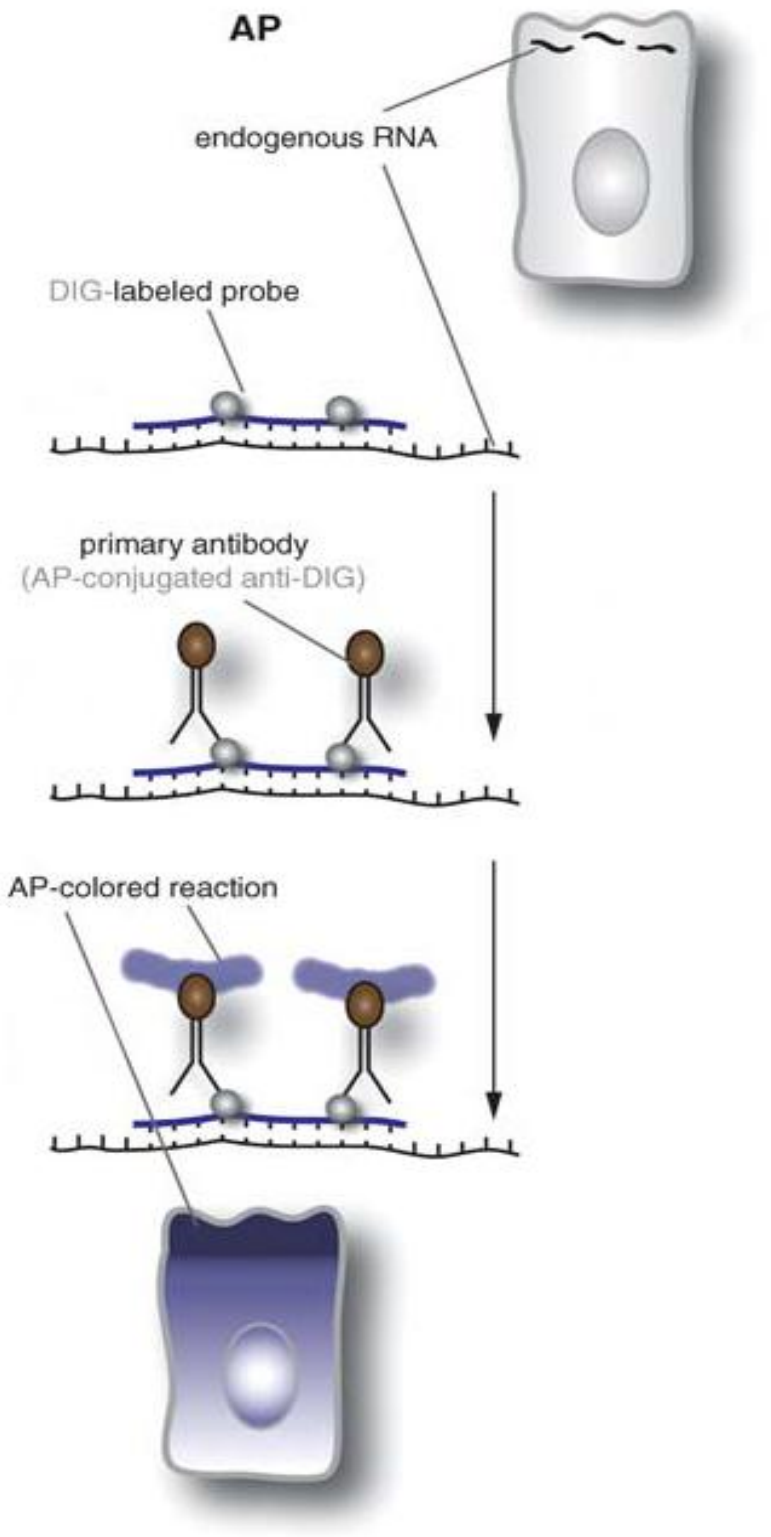


Figure 7.14: Alkaline phosphatase-mediated transcript immuno-localization (adapted from Wilk *et al,* 2010)

To maximize the hybridization of the riboprobe to its complementary mRNA, while preventing any false interactions causing unspecific binding it is necessary to take into consideration certain parameters that will influence the nucleic acid hybridization. As mentioned before, transcript histochemical detection implies that a riboprobe of interest will be used to detect the presence of a complentary mRNA sequence within a tissue sample. The nucleation rate is the limiting step in the hybridization reaction, and it defines how quickly the first few base pairs are formed. This parameter will be dependent, of course, on the amount of riboprobe available for hybridization, on the ability of the riboprobe to enter the cells, on secondary structures the nucleic acids may have and that may interfere with an efficient hybridization, and on the stability of the duplex. The application of dextran sulphate (a polyanionic derivative of dextran) and of Denhardt's solution (a mixture of high-molecular weight polymers) in the hybridization buffer significantly accelerates the nucleation rate by artificially increasing the riboprobe concentration in solution. To ensure that the probe can reach its mRNA target inside the cells it is necessary to permeabilize the samples by digesting with proteinase K. However, the concentration of proteinase K is critical; and the digestion time must be tightly controlled: sufficient to allow riboprobe penetration, but not so long that will destroy the morphology of the tissue. Additionally, if the size of the riboprobe to test goes beyond 500 bp, one can reduce its size to ~200 bp fragments, through alkaline hydrolysis, with carbonate buffer. The secondary structures on nucleic acids can be eliminated by adding formamide and dithiothreitol (DTT). These are organic solvents that will reduce the melting temperature of nucleic acids allowing hybridization to be carried out at lower temperatures. To reduce unspecific interactions between the riboprobe and endogenous nucleic sequences one usually adds EDTA, which is a chelator that removes cations from the hybridization solution. Free mono and divalent cations interact with the phosphate groups of the nucleic acids stabilizing the electrostatic bonds formed between random duplexes. In addition to EDTA, the samples are usually washed with a saline sodium citrate (SSC) buffer at high temperature. The combination between low salt concentration of both the hybridization and washing buffers, and the high temperature of incubation, increases the stringency and reduces the background (reviewed by Eisel *et al*, 2002 and Wilk *et al*, 2010).

Protocol

7.6.2.1 Probe Synthesis

7.6.2.1.1 Cloning the template

Total RNA was extracted from plantlets, grown in LD, with 5 to 6 leaves, and used as a template to synthesize cDNA. The cDNA sequences of genes of interest were amplified, with appropriate primers, by PCR, separated in a 1% agarose gel (w/v) via electrophoresis, gel purified, and cloned into the pGEM-T easy vector. Individual clones were analysed by enzymatic digestion in a 1% agarose gel (w/v), and sequenced; to exclude possible point mutations, and to assess the insert's orientation in the plasmid.

The template for the riboprobe synthesis, was amplified from the correspondent, fully sequenced, pGEM-T easy clone by PCR using SP6/T7 primers (see 7.9), separated in a 1% agarose gel (w/v) through electrophoresis, gel purified, and eluted in 30 μl of DEPC-treated water.

7.6.2.1.2 Reverse-transcription reaction

Each reverse-transcription reaction was set up according to the instructions in the DIG RNA labelling kit (SP6/T7) from Roche [Cat. Nº 11 175 025 910]: 2 μl 10x transcription buffer (8), 1 μl RNase inhibitor (10), 2 μl 10x NTP mix, 2 μl (40 Units) enzyme, and 200 ng template cDNA, in a final volume of 20 μl per reaction. Each reverse-transcription/labelling reaction incubated at 37 °C for 2 h. Template cDNA was removed by incubating each sample for 15 min at 37 °C with 0.25 μl (2 Units) of RNase-free DNase. The polymerase reaction was stopped by keeping samples in ice, and by adding 1 μl of 0.5 M EDTA. The labelled RNA was, then, precipitated at –70 °C for 30 min after adding 2.5 μl of 4M LiCl and 75 μl of ice-cold 100% EtOH (v/v). Samples were centrifuged at 4 °C for 30 min, at 13000 rpm, and the pellet washed with 100 μl of ice-cold 80% EtOH (v/v). Samples were, again, centrifuged for 5 min at 4 °C, 13000 rpm, and pellet vacuum-dried for 15 min, before being re-suspended in 100 μl of DEPC-treated water for 30 min at 37 °C.

7.6.2.1.3 Probe hydrolysis

Probes with length above 450 bp were hydrolyzed with 100 μl of carbonate buffer, at 60 °C during a time that was proportional to the size of each probe. The reactions were then neutralized with 20 μl of 10x neutralization buffer and precipitated overnight at -20 °C after adding 1 μl of glycogen, 1 μl of 1M $MgCl_2$, and 600 μl of ice-cold 100% EtOH. Samples were centrifuge at 13000 rpm, for 30 minutes, at 4 °C; the pellet washed with 100 μl of ice-cold 80% EtOH, and vacuum-dried for 15 min, before being re-suspended in 50 μl of DEPC-treated water.

A 5 µl aliquot was loaded on a 1% agarose gel (w/v) with DNA size marker to confirm hydrolysis, and the remaining volume was diluted in 450 µl of hybridization buffer, plus 1 µl of RNAsin® to avoid any long term degradation, and stored at –20 °C.

7.6.2.2 Plant tissue fixation and embedding

Plant samples to probe were harvested and immediately transferred into the fixative FAA solution. The fixation and wax embedding were performed automatically, in a Sakura Tissue-Tek® VIP® Processor, with the following program:

Step	Reagent	Duration	Temperature
1	FAA-fixative	4 h	RT
2	70% Ethanol	1 h	RT
3	90% Ethanol	1 h	RT
4	90% Ethanol	1 h	RT
5	99,8% Ethanol + eosin	1 h	RT
6	99,8% Ethanol	1 h	RT
7	100 % Ethanol	1 h	RT
8	Xylene	1 h	RT
9	Xylene	1 h	RT
10	Xylene	1 h 15 min	RT
11	Histowax™ LT	1 h	62 °C
12	Histowax™ LT	1 h	62 °C
13	Histowax™ LT	3 h	62 °C

Samples were immediately placed at 65 °C inside a Sakura Tissue-Tek® TEC™ Paraffin Embedding Station, where they were cast into paraffin using stainless steel base moulds and plastic processing cassettes. Plant tissue sections of 8 µm were cut in a Leica RM2255 microtome**,** placed on a 40 °C Barnstead Electrothermal Paraffin Section Mounting water-bath to remove compressions, and attached in pre-cleaned Polysine™ microscope slides overnight at 42 °C, on a Barnstead Electrothermal Slide Drying Bench.

7.6.2.3 *In situ* Hybridization

7.6.2.3.1 Sample de-waxing and permeabilization

Slides containing the plant sections were treated twice, for 10 min, with Histo-clear (R A Lamb, cat. C78-G, Thermo scientific) to remove paraffin, and washed twice for 2 min in 100% EtOH. Following the de-waxing step, the samples were rehydrated for 1 minute in a

series of EtOH dilutions (95%, 90%, 80%, 60%_0.75% saline, 30%_0.75% saline), followed by 2 min in 0.75% saline, and 2 min in 1x PBS.

Slides incubated for 30 minutes in a freshly prepared 1μg/ml Proteinase K solution. The digestion was stopped by keeping the slides for 2 min in a 2 mg/ml Glycine-PBS buffer, and slides were washed twice for 2 min in 1x PBS buffer. Samples were once more fixed in freshly prepared fixative, FAA, for 2 to 5 min, and washed, twice, in 1x PBS buffer for 5 min.

This was followed by another 30 s dehydration step, with an increasing EtOH series (30%_0.75% saline, 60%_0.75% saline, 80%, 90%, 95%), after a 2 min immersion in 0.75% (w/v) NaCl.

Following the dehydration step, samples were left to air-dry at room temperature for 30 min to 1 h, before proceeding with the hybridization.

7.6.2.3.2 Probe hybridization

For each slide, 5 μl Probe, 5 μl H_2O, 10 μl Formamide were mixed and kept in ice, before adding 80 μl of Hybridization buffer. The final solution was vortexed after 2 min at 80 °C, pipetted onto the slide, and spread across the tissue sample with a glass cover slip. The samples and probes incubated overnight, in a hermetic humidified box with fresh soaking solution, at 55 °C.

7.6.2.3.3 Immunological Detection

Slides were dipped into 55 °C pre-warmed 2x SSC to float off cover slips, and washed four times, in 0.2x SSC at 55°C for 30 min, with gentle agitation. This was followed by two brief 5 min incubations at 37 °C in 0.2x SSC, and a 5 min immersion in 1X PBS.

The samples on each slide were blocked with 2 ml of 1x blocking solution, for 45 minutes. This was followed by, another, 30 min incubation with 2 ml of 1x blocking solution_0.3% Triton, to reduce the background, and a wash of 45 min with 2 ml of BSA washing solution.

The antibody (Anti-Digoxigenin-AP, Fab fragments from sheep; cat. 110932274910, Roche) was applied as a 1:1250 dilution in BSA wash solution, 150 μl per slide. The samples were covered with a glass cover slip and left at room temperature, inside a hermetic humidified box, for 1 h 30 m.

Slides were washed four times for 30 min, with 2 ml of BSA wash solution, and twice for 15 min with TNM-50, with slow shaking. This was followed by the application of 150 μl of a 1:50 dilution of NBT-BCIP solution (cat. 11681451001, Roche), in TNM-50 buffer, in each slide. Slides were, then, covered with glass cover slips, and placed inside a light protected

humidified box, and left overnight at room temperature. The colorimetric reaction was stopped by rinsing, twice, for 5 min with 1x TE buffer, and results analysed in a Fully Automated Upright Leica Microscope DM6000, with a LAS AF 2.4.1 software.

7.6.3 Protein immunoblot (Western blot)

The protein immunoblot is an analytic technique employed in the detection and quantification of the relative amounts of a specific protein of interest from a crude protein extract. The protocol usually involves cell lysis for protein extraction, protein denaturation and separation by polyacrylamide gel electrophoresis, protein transfer onto a membrane (usually nitrocellulose) and protein immuno-detection using specific antibodies (Burnette, 1981) (Figure 7.15).

The protein separation step makes use of polyacrylamide gels and buffers loaded with sodium dodecyl sulphate (SDS). SDS is a strong negatively charged surfactant that is able to disrupt non-covalent bonds in the proteins, promoting their denaturation and thus allowing their separation by electrophoresis, as the proteins will migrate towards de positively charged electrode across the pores in the polyacrylamide gel, according to their molecular weight. The resolution of the separation will depend on the percentage of polyacrylamide used to make the gel. The bigger the percentage, the smaller the pores. The electrophoresis has to be accompanied by the use of a protein marker, which is a commercially available mixture of differently known sized proteins, and is used a reference on the electrophoretic mobility (Burnette, 1981).

Once the proteins have been separated by electrophoresis, they are electroblotted onto a nitrocellulose or polyvinylidene difluoride membrane. This method uses an electric current to force the proteins out of the gel and onto the membrane; which will bind all proteins with the same affinity and in keeping of their relative position in the gel. The binding of the proteins to the surface of the membrane is a critical step, as it will facilitate the access to the antibody later used in the immuno-detection. The presence of protein on the surface of the membrane can be attested by incubating the electroblotted membrane with a Ponceau S dye solution, without compromising the end result.

To prevent the binding of the antibody to the membrane, it is necessary to block it before antibody application with a diluted solution of protein, usually 5% (w/v) skimmed milk in Tris-buffered saline and a bit of Tween®20. This blocking step will reduce the “background noise, and will guaranty a more efficient and sharper detection of the target epitope.

The immunologic detection is usually achieved by an antibody that is attached to a reporter enzyme able to perform either a colorimetric or chemiluminescent reaction, in the presence of the appropriate substrate (Figure 7.15). The horseradish peroxidase (HRP) is a 44 kDa glycoprotein found in the Brassicaceae *Armoracia rusticana* P.G. Gaertn., B. Mey. & Scherb,

that is commonly conjugated with commercially available antibodies. This enzyme is able to catalyse the conversion of luminol to 3-aminophthalate; using hydrogen peroxide as an oxidizing agent. The reaction is accompanied by emission of light at 428 nm; which can be detected through photography (reviewed by Western blot handbook and troubleshooting guide – thermo scientific).

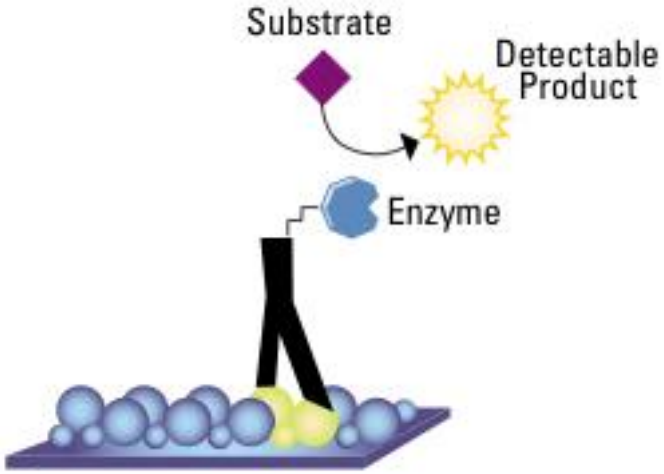


Figure 7.15: Schematic representation of the immunological detection of a protein bound to a membrane, by an antibody conjugated to a reporter enzyme. (modified from Western blot handbook and troubleshooting guide – thermo scientific)

Protocol

Total protein content was extracted from liquid nitrogen-frozen grinded tissue by adding equal volume sample of 2x Laemmli buffer (Laemmli *et al*, 1970). Samples were centrifuged for 1 min at room temperature and incubated for 3 min at 95 °C. This was followed by another centrifuge step at maximum speed for 10 min at 4 °C, and recover of the supernatant into a fresh microcentrifuge tube kept on ice. Samples were loaded into previously prepared polyacrylamide gels (Sambrook and Russel, 2001) and ran in a SDS-PAGE at constant amperage of 30 mA/gel, for 1-2 h at 4°C; with 10 µl of a pre-stained protein ladder (P7708, New England BioLabs® inc.) for reference. The proteins were electroblotted into a Amersham Hybond™ ECL™ nitrocellulose membrane at 80v for 1h30, and blocked in 50 ml 1x Blocking buffer for 2 h at room temperature. The antibody was diluted in 10 ml of 1x blocking buffer (1:5000) and applied for 1 h at room temperature. The membrane was, later, washed for 10 min (3x) at room temperature with 1X TBST. When a non-conjugated antibody was used, a secondary antibody was later applied, and washed from the membrane, as previously described. The SuperSignal® West Pico Chemiluminescent substrate solution was applied in 1:1 ratio (luminol/enhancer solution: stable peroxide solution) in a 5 ml final volume and incubated for 5 min at room temperature. The excess substrate solution was removed from the membrane, and the signal

was detected by exposing a Fuji super RX medical x-ray film to the membrane. The film was subsequently detected in a Konika SRX 101A Automatic film processor.

7.6.4 Quantitative real time polymerase chain reaction (qPCR)

The quantitative real time polymerase chain reaction (Higuchi *et al*, 1992) is an analytical methodology based on the principle of PCR that is used in the detection and quantification of nucleic acids during the exponential phase of amplification. It makes use of a molecular dye with high affinity to double stranded nucleic acids, such as SYBR® Green I that, when in a dsDNA complex, emits green light (λ_{max} = 520 nm) detectable by the optical system of a LightCycler®. As the PCR progresses and more amplicons are synthesized, the fluorescence emission also increases, as more SYBR® Green I molecules will be intercalated between the two DNA strands (Figure 7.16-A). The LightCycler® software converts the sample's fluorescence, detected by the optical system, into units of a variable quantity plotted in relation to the number of qPCR cycles (Figure 7.16-B). The correspondent DNA quantity can, then, be extrapolated and compared to amount of an appropriate reference gene expressed in the same biological context. (http://www.appliedbiosystems.com/absite/us/en/home/applications-technologies/real-time-pcr/taqman-and-sybr-green-chemistries.html)

As this technique is often employed in gene expression assays and in the authentication of microarray results, it is necessary to precede it with the synthesis of cDNA via a reverse transcription reaction. However, given the high sensitivity of this technique, the choice of SYBR® Green I as molecular dye does not come without disadvantages. The employment of multiple binding dyes such as this one will ease the amplicon detection, but it comes with an increased risk of generating false positives, as it binds to any dsDNA sequence in an unspecific way; as well as ssDNA and RNA, although, with lower affinity. Additionally, for the same amplification efficiency, the signal intensity will be in the proportion to the length of the amplicon, i.e., longer amplification products will generate a more intensive signal than smaller amplicons. Therefore, to minimize these shortcomings it is important to efficiently remove all contaminating DNA from the RNA sample prior to cDNA synthesis, and to carefully design primers that span an intron sequence, do not form primer-dimmers, and that produce equally sized amplicons between the gene in analysis and the reference gene. (http://www.appliedbiosystems.com/absite/us/en/home/applications-technologies/real-time-pcr/taqman-and-sybr-green-chemistries.html) Another consideration one should keep in mind when setting up a qPCR reaction is the use of white-opaque plates. This small detail is of great importance, because opaque plasticware reflects the fluorescence of each reaction in the direction the optical detector, as opposed to transparent plates that allow the fluorescence to be scattered in all directions (PrimerDesign™).

Protocol

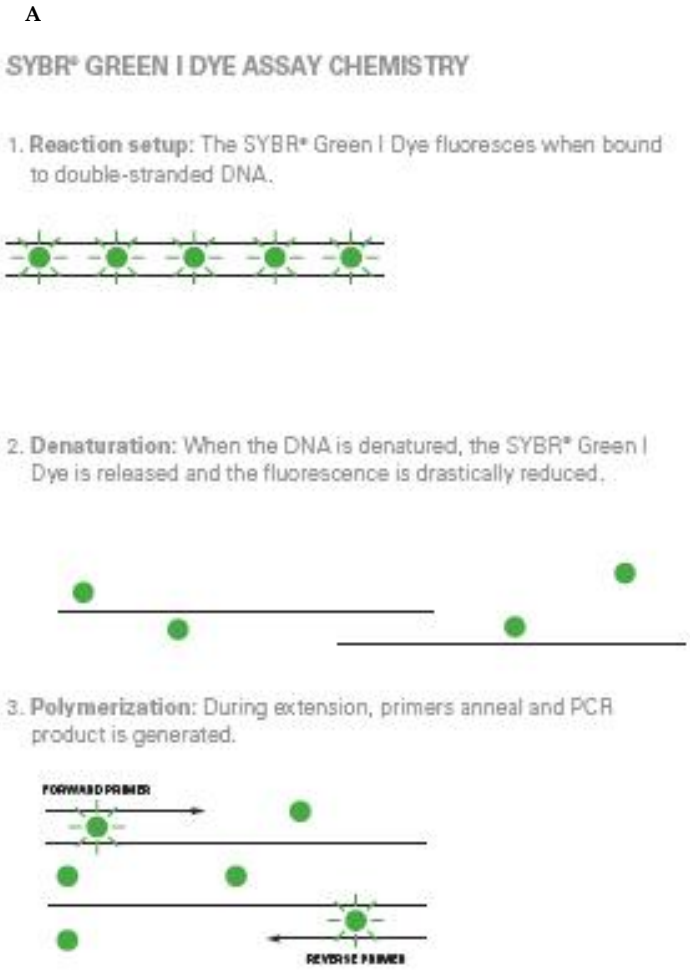
Each qPCR experiment was set-up with three biological replicates of different developmental stages – each replicate composed by cDNA from dissected shoot apical

meristems of 40 individual plants – and two technical replicates to account for pipetting errors. The cDNA samples were diluted with autoclaved water to final volume of 200 µl prior to use. A 1x master mix for each oligonucleotide pair was prepared, as follows: 5 µl 2x SYBR® Green I (cat. 04887352001, Roche), 1 µl 5 µM (each) oligonucleotide mix, and 2 µl water. Samples were loaded into 384 welled plates (cat. 04729749001, Roche) in a final reaction volume of 10 µl per well (2 µl of cDNA plus 8 µl of 1x master mix). A transparent and adhesive film (cat. 04729757001, Roche) was carefully overlaid onto the plate in order to seal the wells. The plate was spun, and loaded into a Roche LightCycler® 480 II. The fluorescence emitted by each sample was detected by the LightCycler's optical detection system and converted by the software (version 1.5) into a graphic representation. The quantification cycle (Cq) – also referred as the threshold cycle (Ct) (Figure 7.16-B) – is defined as the first cycle that gives signal above the background, and it was calculated by the software for all samples. These values were exported into an excel sheet where the average values between replicates were estimated. Relative expression levels (REL) between each sample (s) and the gene of reference (r) were determined using the following mathematical formula:

$$REL=2^{[AverageCq(r)-AverageCq(s)]}$$

The reference gene used was *Brachypodium distachyon´*s *ubiquitin-conjugating enzyme 18* (Hong and Seo *et al*, 2008).

A



B

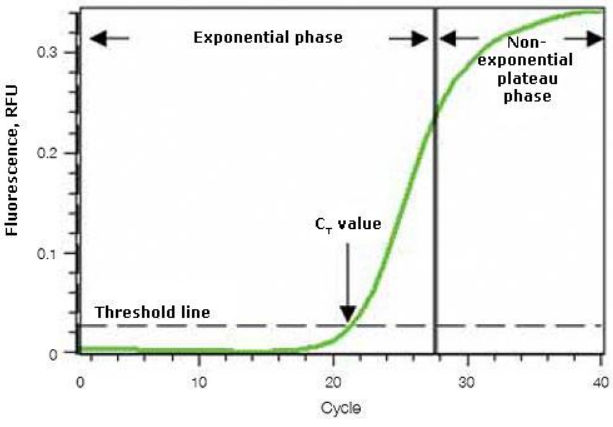


Figure 7.16: A) SYBR GREEN I dye assay chemistry for real time quantitative PCR (adapted from http://www.appliedbiosystems.com/absite/us/en/home/applications-technologies/real-time-pcr/taqman-and-sybr-green-chemistries.html); **B) Fluorescence units plotted against the number of cycles, and determination of the threshold cycle, Ct** (adapted from http://www3.bio-rad.com/gexp/images/support/amp_central/ac303_1.jpg).

7.7 Media, buffers, and solutions recipes

2,4-Dichlorophenoxyacetic acid, 2,4-D (Fluka 31518)

Acetosyringone (Sigma D134406)

Acrylamide/Bis-acrylamide [30% solution] (Sigma A3699) *Store at 4°C*

Agar (Sigma A5306)

Ammonium nitrate, NH_4NO_3 (Sigma A3795)

Ammonium persulphate (APS) [10%] (Sigma A3678)

Anti-DIG antibody (Roche 11 093 274 910) *Store at 4°C*

Anti-DIG antibody [1:1250], 4 ml: 3.2 µl Anti-DIG antibody, 4 ml BSA wash solution

Bacteriological peptone, Bacto peptone (Fluka P0556)

Biotin (Sigma B4639)

Blocking buffer [1x], 100 ml: 5g dried milk, 100 ml 1x TBST

Blocking reagent (Roche 11 096 176 001)

Blocking solution stock [10x], 100 ml: 10 g Blocking reagent, 100 ml Maleic acid buffer, pH 7.5 *Heat in microwave to dissolve. Autoclave. Store aliquots at -20°C.*

Blocking solution-0.3%Triton [1x], 60 ml: 180 µl Triton™-X100, 60 ml 1x Blocking solution

Boric acid H_3BO_3 (Sigma B6768)

Bovine Serum Albumin, BSA (Sigma A3059) *Store at 4°C*

Bromophenol blue (Sigma B0126)

BSA wash solution, 300 ml: 3 g BSA, 900 µl Triton-X™100, 30 ml 1M Tris-HCl (pH 7.5), 9 ml 5M NaCl. *Store at 4°C*

Butanol/Isopropanol (Sigma B7906/Sigma I9516)

Calcium chloride dihydrate, $CaCl_2.2H_2O$ (Sigma C7902)

Carbonate buffer, 100 ml: 1.27 g Na_2CO_3, 0.67 g $NaHCO_3$; pH 10.2. *Store aliquots at -20ºC.*

Charcoal (Sigma C9157)

Cobalt(II) chloride, $CoCl_2$ (Sigma 409332)

Cobalt(II) chloride, $CoCl_2.6H_2O$ (Sigma 60818)

Coomassie brilliant blue (Sigma B7920)

Coomassie brilliant blue, 100 ml: 0.1 g Coomassie brilliant blue, 45 ml MeOH, 10 ml acetic acid

Copper(II) sulfate pentahydrate, $CuSO_4.5H_2O$ (Sigma C3036)

Denhardt's [50x] (Sigma D2532), 100 ml: 1 g Ficoll 400, 1 g Polyvinylpyrrolidone, 1 g BSA *Filter. Store aliquots at -20°C.*

DEPC treated water, 1 l: 1 ml DEPC, 1 l water. *Stir at RT, o/n to dissolve. Autoclave.*

Dextransulphate (Amersham 17-0340-01)

Dextransulphate [50%], 100 ml: 50 g Dextransulphate. *Heat to 80ºC to dissolve. Store aliquots at -20ºC.*

Diethyl pyrocarbonate, DEPC (Sigma D5758)

DIG RNA labelling kit (Roche 11 175 025 910) *Store at -20ºC*

Dimethyl sulfoxide, DMSO (Sigma D4540)

EDTA [0,5M], 5 ml: 0.93 g EDTA; pH 8.0

EDTA [0,5M], 500 ml: 93 g EDTA; pH 8.0

Ethanol absolute, EtOH [100%] (Sigma 24194)

Ethylenediaminetetraacetic acid, EDTA (Sigma E4382)

EtOH [30%]+ NaCl [0,75%], 2 l: 600 ml EtOH, 60 ml 5M NaCl

EtOH [60%]+ 0.75% NaCl [0,75%], 2 l: 1.2 l EtOH, 60 ml 5M NaCl

FAA, 500 ml: 250 ml EtOH, 25 ml acetic acid, 50 ml 37% formaldehyde.

Fe-EDTA (Sigma E9884)

Formaldehyde [37%] (Sigma F1268)

Formamide/Hybridization (Invitrogen 15515026) *Store at 4ºC*

Formamide/Soaking solution (Fluka 47671)

Glacial Acetic acid (Sigma A6283)

Glucose (Fluka G0350500)

Glycerol (Sigma G5516)

Glycine (Fluka 50050)

Glycine (Sigma G5417)

Glycine [2%], 500 ml: 10 g Glycine. *Store at 4ºC.*

Glycogen (Roche 10 901 393 001) *Store at -20ºC*

G-TE, 2 ml: 1 ml Glycerol, 1 ml 1xTE; pH 8.0

Histoclear (Vogel ND-HS-2001)

Hygromycin B (Sigma H0654)

Kinetin (Sigma K3378)

Laemmli buffer [2x], 100 ml (Sigma S3401): 25 ml of 0.5 M Tris-HCl pH 6.8, 20 ml glycerol, 40 ml 10% SDS, 5 ml 0.1 Bromophenol blue, 5 ml β-mercaptoethanol

L-cysteine (Sigma C7352)

LiCl [4M], 5 ml: 0.847 g LiCl. *Store at 4ºC.*

Lithium chloride, LiCl (Sigma L9650)

Loading dye [10x]: 50% glycerol (v/v) 0.25% bromophenol blue (w/v)

Luria-Bertani medium, 1 l: 1% (w/v) tryptone, 0.5 % (w/v) yeast extract, 1% NaCl; pH 7.0.

Magnesium chloride, $MgCl_2$ (Sigma M4880)

Magnesium sulfate heptahydrate, $MgSO_4.7H_2O$ (Sigma 63138)

Magnesium sulphate, $MgSO_4$ (Sigma M2643)

Maleic Acid (Sigma M0375)

Maleic acid buffer, 1 l: 11.6 g Maleic acid, 8.8 g NaCl; pH7.5

Manganese chloride (Sigma M5005)

Manganese(II) sulfate hydrate, $MnSO_4.4H_2O$ (Sigma 229784)

Mannitol (Sigma M1902)

Methanol, MeOH (Sigma 494437)

MG/L medium, 1 l: 0.5% (w/v) tryptone, 0.25 % (w/v) yeast extract, 0.5% (w/v) NaCl, 1% (w/v) mannitol, 0.2% (w/v) sodium glutamate, 0.05% (w/v) KH_2PO_4, 0.02% (w/v) $MgSO_4.7H_2O$, 1.5% (w/v) agar; pH 7.0.

$MgCl_2$ [1M], 1 l: 95.2 g $MgCl_2$ anhydrous

$MgCl_2$ [1M], 5 ml: 0.476 g $MgCl_2$ anhydrous

Monoclonal ANTI-FLAG® M2-Peroxidase (HRP) antibody produced in mouse (Sigma A8592)

Myo-inositol (Sigma I7508)

NaCl [0,75%], 1 l: 30 ml 5M NaCl

NaCl [5M], 200 ml: 58.4 g NaCl

Nail polish

Na-phosphate buffer [1M], 100 ml: 46.3ml 1M Na_2HPO_4, 53.7 ml 1M NaH_2PO_4; pH 6.8

NBP/BCIP Solution: 18.75 mg/ml nitro blue tetrazolium chloride and 9.4 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate, toluidine-salt in 67 % (DMSO) (v/v)

NBT-BCIP stock solution (Roche 11 681 451 001) *Store at 4ºC*

Neutralization buffer, 100 ml: 24.6 g NaOAc, 1 ml Acetic Acid; pH 6.0

Nicotinic acid (Sigma N0761)

PBS [10x], 1 l: 75.92 g NaCl, 3.6 g NaH_2PO_4 anhydrous, 9.94 g Na_2HPO_4 anhydrous; pH 7.0

PBS-G [1x], 500 ml: 50 ml 2% Glycine, 450 ml 1xPBS. *Store at 4°C.*

Phytagel™ (Sigma P8169)

Polyvinyldifluoride membrane, PVDF (Life technologies LC2005)

Ponceau S (Sigma P3504)

Ponceau S staining solution, 1 l: 1g Ponceau S, 50 ml acetic acid. *Store at 4 ºC*

Potassium acetate (Sigma P5708)

Potassium chloride, KCl (Sigma P5405)

Potassium hydroxide, KOH (Sigma P5958)

Potassium iodide, KI (Sigma P8166)

Potassium nitrate, KNO_3 (Sigma P8291)

Potassium phosphate monobasic, KH_2PO_4 (Sigma P5655)

Probe hybridization solution, 2.5 ml: 125 µl probe, 125 µl water, 250 µl Formamide, 2 ml hybridization buffer. Store at 4 °C.

Proteinase K [20 mg/ml], 1 ml: 20 mg Proteinase K, 1 ml Proteinase K buffer. *Store at -20ºC.*

Proteinase K buffer, 1 l: 100 ml 1M Tris-HCl (pH7.5), 100 ml 0.5M EDTA

Proteinase K digestion buffer, 500 ml: 25 µl of Proteinase K (20 mg/ml), 500 ml Proteinase K buffer.

Proteinase K, PCR grade (Roche 03 115 844 001)

Psi Broth, 1l: 2% (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) $MgSO_4$; pH 7.6.

Pyridoxine-HCl, Vitamin B6 (Sigma V-018)

Rifampicin (Sigma R7382)

RNaseZap (Sigma: R2020)

Rubidium chloride, RbCl (Sigma 83979)

Running buffer [1x], 1 l: 50 ml 10% SDS, 100 ml 10x SDS-PAGE stock

SDS-PAGE stock [10x], 1 l: 29 g Tris base, 145 g Glycine

Soaking solution, 35 ml: 17.5 ml Formamide, 3.5 ml 20x SSC

SOC medium, 1 l: 2% (w/v) tryptone, 0.5 % (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose; pH 7.0. Note: Glucose added after autoclaving.

Sodium Acetate Anhydrous, NaOAc (Sigma W302406)

Sodium bicarbonate, $NaHCO_3$ (Sigma S5761)

Sodium carbonate, Na_2CO_3 (Sigma 451614)

Sodium chloride, NaCl (Sigma S5886)

Sodium Citrate, (Fluka PHR1416)

Sodium dodecylsulphate (SDS) [10%] (Sigma L3771)

Sodium glutamate, L-Glutamic acid (Sigma RES5063G-A7)

Sodium molybdate dihydrate, Na_2MoO_4 (Sigma M1651)

Sodium phosphate dibasic, Na_2HPO_4 (Sigma S5136)

Sodium phosphate monobasic, NaH_2PO_4 (Sigma S5011)

SSC [20x], 1 l: 175.3 g NaCl, 88.2 g Sodium-citrate; pH 7.0

Streptomycin sulphate (Sigma S9137)

Sucrose (Sigma S7903)

Supersignal™ West Pico Chemiluminescent substrate (Thermo scientific 34079)

TBST [10x], 1 l: 100 ml 1 M Tris-HCl pH 7.5, 87.7 g NaCl, 10 ml Tween® 20

TE [10x], 1 l: 100 ml 1M Tris-HCl, pH 8.0; 40 ml 0.5M EDTA, pH 8.0

Tetrametylethylenediamine, TEMED (Sigma T9281)

Tf buffer I, 400 ml: 30 mM potassium acetate, 100 mM rubidium chloride, 50 mM manganese chloride, 15% (v/v) glycerol; pH 5.8.

Tf buffer II, 100 ml: 10 mM MOPS, 75 mM calcium chloride, 10 mM rubidium chloride, 15% (v/v) glycerol; pH 6.5.

Thiamine-HCl, Vitamin B1 (Sigma V-014)

Timentin, Ticarcillin/Clavulanate [15/1] (Duchefa T0190-10)

TNM-50, 1 l: 100 ml 1M Tris-HCl (pH 9.5), 20 ml 5M NaCl, 50 ml 1M $MgCl_2$

Transfer buffer [1x], 1 l: 200 ml MeOH, 50 ml 10% SDS, 100 ml 10x SDS-PAGE stock

TRIS hydrochloride, Tris-HCL (Sigma RES3098T-B7)

Tris-HCl [0,5M] (pH 6.8)

Tris-HCl [1,5M] (pH 8.8)

Tris-HCl [1M], 1 l: 121 g Tris, 55 ml HCl; pH 7.5

Tris-HCl [1M], 500 ml: 60.5 g Tris, 17 ml HCl; pH 8.0

Tris-HCl [1M], 500 ml: 60.5 g Tris, 3.5 ml HCl; pH 9.5

Triton™-X100 (Sigma T8787)

tRNA [50 mg/ml] (Roche 10 109 495 001) *Store at -20ºC*

Tryptone (Fluka T7293)

Tween® 20 (Sigma P9416)

Washing solution, 100 ml: 45 ml MeOH, 10 ml acetic acid

Yeast extract (Sigma Y4250)

YEP medium: 1% yeast extract (w/v) 1% bacto peptone (w/v) 0.5% NaCl (w/v); pH 7.0.

Zinc sulfate heptahydrate, $ZnSO_4.7H_2O$ (Sigma Z1001)

Brachypodium distachyon Agrobacterium-mediated transformation

All media for bacteria or plant culture are autoclaved (90 min total cycle, including 15 min at 120 °C) unless stated otherwise.

250 mg/l $CuSO_4$ stock solution, 50 ml: 12.5 mg of $CuSO_4.5H_2O$ in 50 ml of deionized water. Store at 4 °C in the dark.

1 mg/ml $CuSO_4$ stock solution, 50 ml: 50 mg of $CuSO_4.5H_2O$ in 50 ml of distilled water. Store at 4 °C in the dark.

280 mg/l $CoCl_2$ stock solution, 50 ml: 14 mg of $CoCl_2.6H_2O$ in 50 ml of distilled water. Store at 4 °C in the dark.

10x Macro MS salts stock solution, 1 l: 19 g of KNO_3, 16.5 g of NH_4NO_3, 4.4 g of $CaCl_2.2H_2O$, 3.7 g of $MgSO_4.7H_2O$ (if anhydrous, use only 1.8 g), 1.7 g of KH_2PO_4 in 1 litre of deionized. Store at 4 °C in the dark.

100x Micro MS salts stock solution, 1 l: 0.62 g of H_3BO_3 , 2.23 g of $MnSO_4.4H_2O$, 0.86 g of $ZnSO_4.7H_2O$, 0.083 g of KI, 0.025 g of $Na_2MoO_4.2H_2O$, 10 ml of $CuSO_4$ stock solution (250 mg/l), 9 ml of $CoCl_2$ stock solution (280 mg/l) in 1 litre of deionized water. Store at 4 °C in the dark.

100x M5 vitamins stock solution, 1 l: Dissolve 0.04 g of nicotinic acid, 0.05 g of thiamine-HCl, 4 g of cysteine, 0.2 g of glycine, 0.04 g of pyridoxine-HCl in 1 litre of deionized water; pH 5.8 with KOH, and filter-sterilize. Store at -20 °C in the dark.

100x B5 vitamins stock solution, 1 l: 100 mg of nicotinic acid, 1 g of thiamine-HCl, 100 mg of pyridoxine HCl, 10 g of myo-inositol in 1 litre of deionized water; pH 5.8 with KOH, and filter-sterilize. Store at -20 °C in the dark.

1 mg/ml 2,4-D stock solution, 100 ml: 100 mg of 2,4-D, 250–500 µl of 1 M KOH in 100 ml deionized water. Store at -20 °C in the dark.

100x Fe-EDTA stock solution, 1 l: 4 g of $C_{10}H_{12}FeN_2NaO_8$ in 1 litre of deionized water. Store at 4 °C in the dark.

30 mg/ml acetosyringone stock solution, 20 ml: 600 mg of 3′,5′- dimethoxy-4′-hydroxyacetophenone in 20 ml of dimethyl sulfoxide. Store at -20 °C in the dark.

320 mg/ml timentin stock solution, 10 ml: 3.2 g of timentin in 10 ml of sterile deionized water. Store at -20 °C in the dark up to 2 months.

1 mg/ml biotin stock solution, 10 ml: 10 mg of biotin, 200–300 µl of 1 M KOH in 10 ml of deionized water. Filter- sterilize. Store at 4 °C in the dark.

0.1 mg/ml kinetin stock solution, 45 ml: 5 ml of kinetin (1 mg/ml) in 45 ml of sterile deionized water. Store at 4 °C in the dark.

100 mg/ml streptomycin sulphate stock solution, 10 ml: 1 g of streptomycin sulphate in 10 ml of sterile deionized water and filter-sterilize. Store at -20 °C.

25 mg/ml rifampicin stock solution, 40 ml: 1 g of rifampicin in 40 ml of DMSO. Store at -20 °C.

MG/L + S50 + AS30 solid medium for Agrobacterium culture, 1 l: 5 g of tryptone, 2.5 g of yeast extract, 5.2 g of NaCl, 10 g of mannitol, 2.32 g of L- glutamic acid sodium salt, 0.5 g of KH_2PO_4 and 0.2 g of $MgSO_4.7H_2O$, 10 g of Micro agar in 1 litre of deionized water; pH 7.2 with KOH and autoclave. After sterilization: 2 ml of biotin (1 mg/ml), 500 ml of streptomycin (100 mg/ml) and 1 ml of acetosyringone (30 mg/ml). Store at 4 °C in the dark.

MSB + AS45 liquid medium for Agrobacterium suspension, 1 l: 100 ml of Macro MS salts stock solution (10x), 10 ml of Micro MS salts stock solution (100x), 10 ml of Fe-EDTA stock solution (100x), 30 g of sucrose, 30 g of glucose in 1 litre of deionized water; pH 5.5, autoclave. After sterilization: 300 ml of acetosyringone (30 mg/ml) per 200 ml aliquot, before use. Store at 4 °C, up to 3 months.

MSB3 + Cu0.6 solid medium for callus culture, 1 l: 100 ml of Macro MS salts stock solution (10x), 10 ml of Micro MS salts stock solution (100x), 10 ml of Fe-EDTA stock solution (100x), 30 g of sucrose, 2.5 ml of 2,4-D (1 mg/ml), 600 µl $CuSO_4$ (1 mg/ml), 3.5 g of Phytagel in 990 ml of deionized water; pH 5.8 with KOH. After sterilization: 10 ml of filter-sterilized M5 vitamins stock solution (100x). Store at 4 °C in the dark.

MSB3 + Cu0.6 + H50 + T225 solid medium for callus culture, 1 l: 100 ml of Macro MS salts stock solution (10x), 10 ml of Micro MS salts stock solution (100x), 10 ml of Fe-EDTA stock solution (100x), 30 g of sucrose, 2.5 ml of 2,4-D (1 mg/ml), 600 µl $CuSO_4$ (1 mg/ml), 3.5 g of Phytagel in 990 ml of deionized water; pH 5.8 with KOH. After sterilization: 10 ml of filter-sterilized M5 vitamins stock solution (100x), 1 ml hygromycin B (50 mg/ml), 700 ml of timentin (320 mg/ml). Store at 4 °C in the dark.

MSR26 + Cu0.6 + H50 + T225 medium for plant regeneration, 1 l: 100 ml of Macro MS salts stock solution (10x), 10 ml of Micro MS salts stock solution (100x), 10 ml of Fe-EDTA stock solution (100x), 600 µl $CuSO_4$ (1 mg/ml), 30 g of sucrose, 3.5 g of Phytagel in 990 ml of

deionized water; pH 5.8 with KOH. After sterilizing: 2 ml of kinetin (0.1 mg/ml), 10 ml of M5 vitamins stock solution (100x), 1 ml of hygromycin B (50 mg/ml), 700 ml of timentin (320 mg/ml). Store at 4 °C in the dark.

MSR63 + Ch7 + H50 + T225 medium for plantlet germination, 1 l: 40 ml of Macro MS salts stock solution (10x), 40 ml of Micro MS salts stock solution (100x), 10 ml of Fe-EDTA stock solution (100x) 10 g of sucrose, 6 g of agar, 2 g of phytagel, 7 g of charcoal in 990 ml of deionized water; pH 5.8 with KOH. After sterilizing: 10 ml of vitamin B5 stock solution (100x), 1 ml of hygromycin B (50 mg/ml), 700 ml of timentin (320 mg/ml). Pipette 10 ml of medium into sterilized glass tubes. Store at 4 °C in the dark.

7.8 Oligos

7.8.1 pENTR cloning for constitutive expression

Bradi1g08340.1	S	CACCATGGGGCGCGGGAAGGTG
	AS-1	TTTATAGTCTCCTAGGCCCTTATCATCGTCGTCCTTATAATCGCCGCTGATGTGGCTCAC
Bradi1g11090.1	S	CACCATGAAGCGCGAGTACCAAGACGGC
	AS	TCACGGCGCAGCCATGCG
Bradi1g43670.1	S	CACCATGAATTATAATTTCAACAGCTCCG
	AS-1	TTTATAGTCTCCTAGGCCCTTATCATCGTCGTCCTTATAATCGAACCATGGAACGGTGCT
Bradi1g48830.1	S	CACCATGGCCGGGAGGGACAGG
	AS	TCAGGGGTACATCCTCCTGCC
Bradi1g59250.1	S	CACCATGGGGCGCGGGAAGGT
	AS-1	TTTATAGTCTCCTAGGCCCTTATCATCGTCGTCCTTATAATCAGCGTTGAGGTGGCTCAGC
Bradi2g07070.1	AS	TCACATTCTTCTCCCTCCAGTTCC
	S	CACCATGCCGCGGGGGGATC
Bradi2g21820.1	S-1	GGCGCTGCATTGTCGACAATGATTCAGGCAATGTCGT
	AS	TCAAACAGGGGAAGAGCTTG
Bradi3g00300.1	S	CACCATGGAGGAGGAGGTGATCTGGAA
	AS	TCATGGTGCAGGGGCGGA
Bradi4g42400.1	S	CACCATGTCTAGGTCTGTGGAGCCTCTTATTG
	AS	TCAGCGCCTCCTGGCAGC
Bradi5g20340.1	S	CACCATGGATCCCAACGACGCCT
	AS	CTAGAACATGGGCGGCGG
3xflag C' (2nd PCR)	AS-2	TTACTTGTCGTCATCATCCTTGTAATCTTTATCATCGTCGTCTTTATAGTCTCCTAGGCC
N′ HA tag (2nd PCR)	S-2	CACCATGGAGTACCCATACGACGTACCAGATTACGCTGGCGCTGCATTGTCGACA

7.8.2 pENTR cloning for gene silencing via amiRNA

Each amiRNA was designed to target solely the corresponding gene, no off-targets allowed.

Bradi1g08340.1	I miR-s	S	agTTTTGTCCATACATGAGTCGCcaggagattcagtttga
	II miR-a	AS	tgGCGACTCATGTATGGACAAAActgctgctgctacagcc
	III miR*s	S	ctGCGACACATCTATGGACAAAAttcctgctgctaggctg
	IV miR*a	AS	aaTTTTGTCCATAGATGTGTCGCagagaggcaaaagtgaa
Bradi1g48830.1	I miR-s	S	agTAACCAGTGTAGATACTGCCGcaggagattcagtttga
	II miR-a	AS	tgCGGCAGTATCTACACTGGTTActgctgctgctacagcc
	III miR*s	S	ctCGGCACTATGTACACTGGTTAttcctgctgctaggctg
	IV miR*a	AS	aaTAACCAGTGTACATAGTGCCGagagaggcaaaagtgaa
Bradi2g21820.1	I miR-s	S	agTCGATTCCAAAATATCACCTCcaggagattcagtttga
	II miR-a	AS	tgGAGGTGATATTTTGGAATCGActgctgctgctacagcc
	III miR*s	S	ctGAGGTCATAATTTGGAATCGAttcctgctgctaggctg
	IV miR*a	AS	aaTCGATTCCAAATTATGACCTCagagaggcaaaagtgaa
Bradi3g00300.1	I miR-s	S	agTCAATCGAGCGTTTAGGTCTTcaggagattcagtttga
	II miR-a	AS	tgAAGACCTAAACGCTCGATTGActgctgctgctacagcc
	III miR*s	S	ctAAGACGTAATCGCTCGATTGAttcctgctgctaggctg
	IV miR*a	AS	aaTCAATCGAGCGATTACGTCTTagagaggcaaaagtgaa
Bradi4g42400.1	I miR-s	S	agTCATAGCTTATTACCTGCCGTcaggagattcagtttga
	II miR-a	AS	tgACGGCAGGTAATAAGCTATGActgctgctgctacagcc
	III miR-s	S	ctACGGCTGGTTATAAGCTATGAttcctgctgctaggctg
	IV miR*a	AS	aaTCATAGCTTATAACCAGCCGTagagaggcaaaagtgaa
Bradi5g20340.1	I miR-s	S	agTGAACAGATAATCAAGCCCGTcaggagattcagtttga
	II miR-a	AS	tgACGGGCTTGATTATCTGTTCActgctgctgctacagcc
	III miR*s	S	ctACGGGGTTGTTTATCTGTTCAttcctgctgctaggctg
	IV miR*a	AS	aaTGAACAGATAAACAACCCCGTagagaggcaaaagtgaa

7.8.3 pENTR cloning for reporter studies

Bradi4g42400.1	S	CACCCTCTCCTTTTTTTTAGTGCCTGTGG
	AS	GCGCCTCCTGGCAGCAGTT

7.8.4 pGEM cloning of full cDNA (ISH probes)

All probes produced from the reverse transcription of the full cDNA sequence of each gene.

Bradi1g08340.1	S	CACCATGGGGCGCGGGCCGGTGCA
	AS	TTAGCCGCTGATGTGGCTCACC
Bradi1g48830.1	S	CACCATGGCCGGGAGGGACAGG
	AS	TCAGGGGTACATCCTCCTGCC
Bradi1g59250.1	S	GAGGCAGAAGGCAGCCAG
	AS	TCAAGCGTTGAGGTGGCT
Bradi2g07070.1	S	ATGTGAATTTTCTCTCCTCTCTATATATGA
	AS	CGAATCATCGGTTACATAAGACTC
Bradi2g21820.1	S	CACCATGATTCAGGCAATGTCGTC
	AS	TCAAACAGGGGAAGAGCTTG
Bradi3g00300.1	S	CACCATGGAGGAGGAGGTGATCTGGAA
	AS	TCATGGTGCAGGGGCGGA
Bradi4g42400.1	S	CACCATGTCTAGGTCTGTGGAGCCTCTTATTG
	AS	TCAGCGCCTCCTGGCAGC
Bradi5g20340.1	S	CACCATGGATCCCAACGACGCCT
	AS	CTAGAACATGGGCGGCGG

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Appendix

A.1 Microarray frequency analysis for biological process

A.1.1 Transcripts simultaneously down-regulated in 2LD:7LD:11LD samples

Table A.1: Frequency analysis of the microarray results according to biological process, of the simultaneously down-regulated transcripts in 2LD, 7 LD and 11 LD samples, using gene ontology information; performed using the Cytoscape v2.8.3 software platform (Cline *et al*, 2007) and BiNGO v2.44 plugin (Maere *et al*, 2005)

DOWN-REGULATED GENES (2LD:7LD:11LD)

GO-ID	BIOLOGICAL PROCESS	p-val	Freq
9394	2'-deoxymetabolic processribonucleotide	2,04E-01	0.1%
10158	Abaxial cell fate specification	7,64E-02	0.3%
9687	Abscisic acid metabolic process	5,05E-01	0.1%
9838	Abscission	5,20E-01	0.1%
15800	Acidic amino acid transport	2,24E-01	0.1%
10541	Acropetal auxin transport	1,66E-01	0.1%
30036	Actin cytoskeleton organization	4,07E-01	0.3%
7015	Actin filament organization	5,59E-01	0.1%
30029	Actin filament-based process	5,75E-01	0.3%
2253	Activation of immune response	2,70E-01	0.3%
2218	Activation of innate immune response	2,70E-01	0.3%
9943	Adaxial/abaxial axis specification	1,79E-03	0.8%
9955	Adaxial/abaxial pattern formation	3,93E-03	0.8%
48830	Adventitius root development	2,24E-01	0.1%
10618	Aerenchyma formation	2,04E-01	0.1%
7568	Aging	7,18E-01	0.3%
46165	Alcohol biosynthetic process	3,27E-01	0.3%

46164	Alcohol catabolic process	6,28E-01	0.3%
6066	Alcohol metabolic process	6,81E-01	0.8%
46185	Aldehyde catabolic process	2,81E-01	0.1%
19400	Alditol metabolic process	5,72E-01	0.1%
43450	Alkene biosynthetic process	2,53E-01	0.3%
9309	Amine biosynthetic process	9,40E-01	0.3%
9310	Amine catabolic process	6,97E-01	0.1%
9308	Amine metabolic process	9,34E-01	1.0%
15837	Amine transport	1,62E-02	1.2%
6865	Amino acid transport	1,50E-02	1.2%
48532	Anatomical structure arrangement	7,88E-03	1.0%
48856	Anatomical structure development	1,24E-05	12.0%
48646	Anatomical structure formation involved in morphogenesis	2,57E-01	0.8%
60249	Anatomical structure homeostasis	4,03E-01	0.1%
9653	Anatomical structure morphogenesis	2,67E-03	4.9%
48466	Androecium development	6,87E-02	0.8%
6820	Anion transport	3,88E-01	0.5%
48653	Anther development	1,71E-01	0.5%
48654	Anther morphogenesis	2,24E-01	0.1%
43481	Anthocyanin accumulation in tissues in response to UV light	2,24E-01	0.1%
43288	Apocarotenoid metabolic process	5,05E-01	0.1%
6915	Apoptosis	3,50E-01	0.8%
15801	Aromatic amino acid transport	1,07E-01	0.1%
19438	Aromatic compound biosynthetic process	8,87E-01	0.5%
15700	Arsenite transport	2,04E-01	0.1%
9067	Aspartate family amino acid biosynthetic process	3,57E-01	0.3%
9066	Aspartate family amino acid metabolic process	2,13E-01	0.7%
15810	Aspartate transport	1,07E-01	0.1%
8356	Asymmetric cell division	3,99E-04	0.7%
51313	Attachment of spindle microtubules to chromosome	1,66E-01	0.1%
8608	Attachment of spindle microtubules to kinetochore	1,66E-01	0.1%
51455	Attachment of spindle microtubules to kinetochore involved in homologous chromosome segregation	1,66E-01	0.1%
51316	Attachment of spindle microtubules to kinetochore involved in meioticchromosome segregation	1,66E-01	0.1%
9851	Auxin biosynthetic process	2,24E-01	0.3%
10252	Auxin homeostasis	2,04E-01	0.3%
9734	Auxin mediated signaling pathway	6,58E-01	0.1%
9850	Auxin metabolic process	2,21E-02	0.8%
9926	Auxin polar transport	4,85E-01	0.3%
60918	Auxin transport	5,01E-01	0.3%
9798	Axis specification	7,28E-03	0.8%
6284	Base-excision repair	2,17E-01	0.3%
15802	Basic amino acid transport	2,57E-01	0.1%
10540	Basipetal auxin transport	3,09E-01	0.1%
7610	Behavior	3,69E-01	0.1%
19484	Beta-alanine catabolic process	1,07E-01	0.1%
19482	Beta-alanine metabolic process	1,07E-01	0.1%
22610	Biological adhesion	4,59E-01	0.1%
65007	Biological regulation	1,83E-09	25.6%

9058	Biosynthetic process	1,00E+00	7.4%
16132	Brassinosteroid biosynthetic process	1,07E-01	0.3%
10268	Brassinosteroid homeostasis	2,81E-01	0.1%
9742	Brassinosteroid mediated signaling pathway	1,48E-02	0.8%
16131	Brassinosteroid metabolic process	1,71E-01	0.3%
55074	Calcium ion homeostasis	6,01E-01	0.1%
10120	Camalexin biosynthetic process	3,27E-01	0.1%
52317	Camalexin metabolic process	3,27E-01	0.1%
16051	Carbohydrate biosynthetic process	7,66E-01	0.8%
16052	Carbohydrate catabolic process	6,47E-01	0.5%
5975	Carbohydrate metabolic process	8,95E-01	2.8%
46394	Carboxylic acid biosynthetic process	9,24E-01	0.8%
46395	Carboxylic acid catabolic process	8,89E-01	0.1%
19752	Carboxylic acid metabolic process	9,07E-01	2.1%
46942	Carboxylic acid transport	7,63E-03	1.4%
16116	Carotenoid metabolic process	6,25E-01	0.1%
48440	Carpel development	1,76E-01	0.7%
9056	Catabolic process	3,15E-01	3.7%
55080	Cation homeostasis	1,66E-01	0.8%
6812	Cation transport	7,66E-01	1.0%
7155	Cell adhesion	4,59E-01	0.1%
7154	Cell communication	8,37E-01	0.7%
7049	Cell cycle	9,07E-02	1.5%
75	Cell cycle checkpoint	3,09E-01	0.1%
33205	Cell cycle cytokinesis	2,38E-01	0.3%
22403	Cell cycle phase	2,59E-01	0.7%
22402	Cell cycle process	3,07E-02	1.5%
8219	Cell death	2,22E-01	1.5%
48468	Cell development	4,59E-01	1.0%
30154	Cell differentiation	3,05E-02	2.8%
51301	Cell division	7,88E-03	1.4%
45165	Cell fate commitment	1,64E-01	0.5%
1708	Cell fate specification	9,60E-02	0.5%
16049	Cell growth	2,22E-01	1.7%
902	Cell morphogenesis	2,17E-01	1.7%
904	Cell morphogenesis involved in differentiation	8,65E-01	0.3%
8283	Cell proliferation	1,71E-01	0.5%
8037	Cell recognition	6,01E-01	0.1%
45454	Cell redox homeostasis	8,89E-01	0.1%
7166	Cell surface receptor linked signaling pathway	3,80E-04	2.3%
9932	Cell tip growth	7,36E-01	0.3%
16998	Cell wall macromolecule catabolic process	2,38E-01	0.3%
44036	Cell wall macromolecule metabolic process	4,18E-01	0.3%
42545	Cell wall modification	5,43E-01	0.7%
42547	Cell wall modification involved in multidimensional cell growth	3,04E-01	0.3%
71555	Cell wall organization	7,06E-01	0.7%
71554	Cell wall organization or biogenesis	7,42E-01	1.0%
16337	Cell-cell adhesion	2,24E-01	0.1%
7267	Cell-cell signaling	7,52E-01	0.1%

6081	Cellular aldehyde metabolic process	5,36E-01	0.1%
43449	Cellular alkene metabolic process	2,53E-01	0.3%
44106	Cellular amine metabolic process	9,41E-01	0.8%
6519	Cellular amino acid and derivative metabolic process	9,72E-01	1.2%
8652	Cellular amino acid biosynthetic process	9,16E-01	0.3%
9063	Cellular amino acid catabolic process	6,78E-01	0.1%
42398	Cellular amino acid derivative biosynthetic process	7,32E-01	0.7%
42219	Cellular amino acid derivative catabolic process	5,59E-01	0.1%
6575	Cellular amino acid derivative metabolic process	7,75E-01	0.8%
6520	Cellular amino acid metabolic process	9,16E-01	0.8%
6725	Cellular aromatic compound metabolic process	8,32E-01	1.0%
44249	Cellular biosynthetic process	1,00E+00	6.7%
6874	Cellular calcium ion homeostasis	6,01E-01	0.1%
34637	Cellular carbohydrate biosynthetic process	8,76E-01	0.5%
44275	Cellular carbohydrate catabolic process	8,07E-01	0.3%
44262	Cellular carbohydrate metabolic process	9,41E-01	1.2%
44248	Cellular catabolic process	3,75E-01	2.8%
30003	Cellular cation homeostasis	2,23E-01	0.7%
55082	Cellular chemical homeostasis	2,78E-01	0.7%
22607	Cellular component assembly	1,99E-01	1.9%
10927	Cellular component assembly involved in morphogenesis	5,36E-01	0.1%
44085	Cellular component biogenesis	6,01E-01	2.1%
32989	Cellular component morphogenesis	2,66E-01	1.7%
16043	Cellular component organization	3,84E-02	6.5%
48869	Cellular developmental process	5,20E-03	4.2%
30005	Cellular di-,tri-valent inorganic cation homeostasis	7,75E-01	0.1%
6073	Cellular glucan metabolic process	9,55E-01	0.1%
19725	Cellular homeostasis	4,76E-01	0.8%
34754	Cellular hormone metabolic process	3,57E-01	0.3%
6873	Cellular ion homeostasis	2,70E-01	0.7%
42180	Cellular ketone metabolic process	9,16E-01	2.1%
44255	Cellular lipid metabolic process	9,24E-01	1.2%
51641	Cellular localization	8,86E-01	1.4%
34622	Cellular macromolecular complex assembly	1,42E-01	1.4%
34621	Cellular macromolecular complex subunit organization	1,76E-01	1.4%
34645	Cellular macromolecule biosynthetic process	1,00E+00	3.8%
44265	Cellular macromolecule catabolic process	2,37E-01	1.9%
70727	Cellular macromolecule localization	7,37E-01	1.0%
44260	Cellular macromolecule metabolic process	5,21E-02	20.3%
6944	Cellular membrane fusion	6,58E-01	0.1%
16044	Cellular membrane organization	9,07E-01	0.1%
44237	Cellular metabolic process	1,70E-01	27.2%
6875	Cellular metal ion homeostasis	1,07E-01	0.7%
30004	Cellular monovalent inorganic cation homeostasis	3,09E-01	0.1%
44271	Cellular nitrogen compound biosynthetic process	9,41E-01	1.0%
44270	Cellular nitrogen compound catabolic process	2,53E-01	0.3%
34641	Cellular nitrogen compound metabolic process	1,89E-02	9.9%
44264	Cellular polysaccharide metabolic process	9,78E-01	0.1%
30007	Cellular potassium ion homeostasis	2,57E-01	0.1%

9987	Cellular process	1,27E-02	39.2%
44257	Cellular protein catabolic process	2,04E-01	1.9%
43623	Cellular protein complex assemby	9,16E-01	0.1%
34613	Cellular protein localization	6,78E-01	1.0%
44267	Cellular protein metabolic process	3,88E-01	13.4%
45333	Cellular respiration	8,46E-01	0.1%
71214	Cellular response to abiotic stimulus	8,46E-01	0.1%
71365	Cellular response to auxin stimulus	6,78E-01	0.1%
71367	Cellular response to brassinosteroid stimulus	1,48E-02	0.8%
70887	Cellular response to chemical stimulus	1,13E-02	3.5%
71368	Cellular response to cytokinin stimulus	4,03E-01	0.3%
71359	Cellular response to dsRNA	2,70E-01	0.3%
71495	Cellular response to endogenous stimulus	9,54E-03	2.8%
71369	Cellular response to ethylene stimulus	7,75E-01	0.1%
71496	Cellular response to external stimulus	9,72E-01	0.1%
31668	Cellular response to extracellular stimulus	9,72E-01	0.1%
71370	Cellular response to gibberellin stimulus	3,04E-01	0.3%
32870	Cellular response to hormone stimulus	1,22E-01	1.9%
71395	Cellular response to jasmonic acid stimulus	6,14E-03	1.0%
71482	Cellular response to light stimulus	7,75E-01	0.1%
43562	Cellular response to nitrogen levels	4,90E-01	0.1%
6995	Cellular response to nitrogen starvation	3,27E-01	0.1%
31669	Cellular response to nutrient levels	9,59E-01	0.1%
71310	Cellular response to organic substance	7,88E-03	3.3%
34599	Cellular response to oxidative stress	4,90E-01	0.1%
71478	Cellular response to radiation	7,75E-01	0.1%
71489	Cellular response to red or far-red ligh	7,43E-01	0.1%
71446	Cellular response to salicylic acid stimulus	2,89E-01	0.3%
9267	Cellular response to starvation	9,43E-01	0.1%
71383	Cellular response to steroid hormone stimulus	1,48E-02	0.8%
51716	Cellular response to stimulus	1,75E-04	7.0%
33554	Cellular response to stress	6,30E-03	3.7%
71466	Cellular response to xenobiotic stimulus	2,24E-01	0.1%
48878	Chemical homeostasis	5,91E-02	1.4%
6935	Chemotaxis	3,27E-01	0.1%
15996	Chlorophyll catabolic process	3,50E-01	0.1%
15994	Chlorophyll metabolic process	4,03E-01	0.3%
10020	Chloroplast fission	1,60E-01	0.3%
9658	Chloroplast organization	2,17E-01	0.7%
10478	Chlororespiration	1,07E-01	0.1%
31497	Chromatin assembly	7,67E-03	1.2%
6333	Chromatin assembly or disassembly	2,34E-02	1.2%
16568	Chromatin modification	2,03E-01	0.8%
6325	Chromatin organization	9,54E-03	2.1%
6338	Chromatin remodling	4,59E-01	0.1%
6342	Chromatin silencing	2,04E-01	0.3%
31048	Chromatin silencing by small RNA	2,24E-01	0.1%
30261	Chromosome condensation	2,04E-01	0.1%
51276	Chromosome organization	4,98E-03	2.6%

7059	Chromosome segregation	1,07E-01	0.5%
7623	Circadian rhythm	2,67E-01	0.5%
6732	Coenzyme metabolic process	9,78E-01	0.1%
51187	Cofactor catabolic process	6,78E-01	0.1%
51186	Cofactor metabolic process	9,41E-01	0.5%
9631	Cold acclimation	2,24E-01	0.3%
48465	Corolla development	4,76E-01	0.1%
30865	Cortical cytoskeleton organization	4,59E-01	0.1%
43622	Cortical microtubule organization	4,43E-01	0.1%
48825	Cotyledon development	3,11E-01	0.3%
10588	Cotyledon vascular tissue pattern formation	3,89E-01	0.1%
16569	Covalent chromatin modification	2,05E-01	0.7%
42335	Cuticle development	2,03E-01	0.3%
80051	Cutin transport	1,07E-01	0.1%
910	Cytokinesis	3,05E-02	0.8%
911	Cytokinesis by cell plate formation	5,20E-01	0.1%
9736	Cytokinin mediated signaling pathway	4,03E-01	0.3%
9690	Cytokinin metabolic process	5,72E-01	0.1%
31122	Cytoplasmic microtubule organization	4,59E-01	0.1%
7010	Cytoskeleton organization	3,75E-01	0.7%
16265	Death	2,22E-01	1.5%
6952	Defense response	3,37E-01	3.5%
9870	Defense response signaling pathway, resistance gene-dependent	3,89E-01	0.1%
10204	Defense response signaling pathway, resistance gene-independent	3,69E-01	0.1%
42742	Defense response to bacterium	2,57E-01	1.4%
9816	Defense response to bacterium, incompatible interaction	6,16E-01	0.1%
50832	Defense response to fungus	4,85E-01	0.7%
51607	Defense response to virus	2,17E-01	0.3%
9814	Defense response, incompatible interaction	2,68E-01	0.8%
70988	Demethylation	2,57E-01	0.1%
9262	Deoxyribonucleotide metabolic process	2,81E-01	0.1%
16311	Dephosphorylation	8,55E-01	0.1%
9582	Detection of abiotic stimulus	5,05E-01	0.1%
16045	Detection of bacterium	2,24E-01	0.1%
9595	Detection of biotic stimulus	2,81E-01	0.1%
9729	Detection of brassinosteroid stimulus	1,66E-01	0.1%
5513	Detection of calcium ion	3,09E-01	0.1%
9593	Detection of chemical stimulus	1,71E-01	0.3%
9726	Detection of endogenous stimulus	2,81E-01	0.1%
9581	Detection of external stimulus	2,53E-01	0.3%
9720	Detection of hormone stimulus	2,81E-01	0.1%
9583	Detection of light stimulus	4,43E-01	0.1%
51606	Detection of stimulus	8,97E-02	0.7%
48262	Determination of dorsal/ventral asymmetry	1,07E-01	0.1%
9855	Determination of bilateral symetry	3,05E-02	0.3%
48263	Determination of dorsal identity	1,07E-01	0.1%
48588	Developmental cell growth	7,55E-01	0.3%
48589	Developmental growth	8,74E-02	1.9%
60560	Developmental growth involved in morphogenesis	9,08E-02	1.7%

32502	Developmental process	2,75E-05	14.3%
55066	Di-, tri-valent inorganic cation homeostasis	5,49E-01	0.3%
46451	Diaminopimelate metabolic process	3,27E-01	0.1%
43648	Dicarboxylic acid metabolic process	5,75E-01	0.3%
42938	Dipeptide transport	2,24E-01	0.1%
46351	Disaccharide biosynthetic process	6,58E-01	0.1%
5984	Disaccharide metabolic process	7,66E-01	0.1%
6305	DNA alkylation	3,57E-01	0.3%
71103	DNA conformation change	1,73E-02	1.4%
77	DNA damage checkpoint	1,07E-01	0.1%
42770	DNA damage response, signal transduction	1,07E-01	0.1%
42023	DNA endoreduplication	5,05E-01	0.1%
31570	DNA integrity checkpoint	1,66E-01	0.1%
9294	DNA mediated transformation	6,01E-01	0.1%
6259	DNA metabolic process	1,45E-06	4.9%
6306	DNA methylation	3,57E-01	0.3%
32776	DNA methylation on cytosine	2,81E-01	0.1%
10425	DNA methylation on cytosine within a CNG sequence	1,07E-01	0.1%
6304	DNA modification	3,69E-01	0.3%
6323	DNA packaging	2,53E-03	1.4%
6310	DNA recombination	5,16E-01	0.3%
6281	DNA repair	2,86E-04	2.6%
6260	DNA replication	1,75E-04	2.1%
6269	DNA replication, synthesis of RNA primer	1,66E-01	0.1%
22616	DNA strand elongation	1,07E-01	0.1%
6271	DNA strand elongation involved in DNA replication	1,07E-01	0.1%
6261	DNA-dependent DNA replication	3,72E-02	0.8%
6270	DNA-dependent DNA replication initiation	3,89E-01	0.1%
22611	Dormancy process	4,59E-01	0.1%
9953	Dorsal/ventral pattern formation	1,07E-01	0.1%
6302	Double-strand break repair	5,85E-01	0.1%
724	Double-strand break repair via homologous recombination	4,03E-01	0.1%
31050	dsRNA fragmentation	2,70E-01	0.3%
19305	dTDP-rhamnose biosynthetic process	1,66E-01	0.1%
46383	dTDP-rhamnose metabolic process	1,66E-01	0.1%
42732	D-xylose metabolic process	2,57E-01	0.1%
7398	Ectoderm development	7,17E-01	0.5%
22900	Electron transport chain	8,19E-02	0.8%
9553	Embryo sac development	7,36E-01	0.3%
9790	Embryonic development	8,25E-02	3.3%
9793	Embryonic development ending in seed dormancy	2,24E-01	2.4%
48508	Embryonic meristem development	2,24E-01	0.3%
9960	Endosperm development	4,03E-01	0.1%
15980	Energy derivation by oxidation of organic compounds	8,46E-01	0.1%
7167	Enzyme linked receptor protein signaling pathway	2,78E-04	2.1%
9913	Epidermal cell differentiation	8,80E-01	0.3%
8544	Epidermis development	7,17E-01	0.5%
51234	Establishment of localization	5,49E-01	7.0%
51649	Establishment of localization in cell	9,43E-01	1.0%

45184	Establishment of protein localization	6,88E-01	1.4%
9693	Ethylene biosynthetic process	2,38E-01	0.3%
9873	Ethylene mediated signaling pathway	7,72E-01	0.1%
9692	Ethylene metabolic process	2,38E-01	0.3%
45229	External encapsulating structure organization	6,25E-01	0.1%
6633	Fatty acid biosynthetic process	8,18E-01	0.3%
6631	Fatty acid metabolic process	5,64E-01	0.8%
15908	Fatty acid transport	1,66E-01	0.1%
10582	Floral meristem determinancy	7,64E-02	0.3%
10227	Floral organ abscission	4,90E-01	0.1%
48437	Floral organ development	1,59E-03	2.3%
48449	Floral organ formation	2,24E-01	0.3%
48444	Floral organ morphogenesis	3,11E-01	0.3%
48438	Floral whorl development	1,45E-03	2.1%
9908	Flower development	2,32E-04	3.3%
48439	Flower morphogenesis	2,57E-01	0.1%
10154	Fruit development	1,71E-02	4.0%
51318	G1 phase	3,05E-02	0.3%
80	G1 phase of mitotic cell cycle	3,05E-02	0.3%
7276	Gamete generation	5,59E-01	0.1%
48229	Gametophyte development	3,18E-01	1.4%
9450	Gamma-aminobutyric acid catabolic process	1,66E-01	0.1%
9448	Gamma-aminobutyric acid metabolic process	1,66E-01	0.1%
10467	Gene expression	1,00E+00	3.1%
16458	Gene silencing	2,24E-01	1.0%
35195	Gene silencing by miRNA	6,25E-01	0.3%
31047	Gene silencing by RNA	3,99E-01	0.7%
6091	Generation of precursor metabolites and energy	5,27E-01	1.0%
55047	Generative cell mitosis	1,66E-01	0.1%
71514	Genetic imprinting	4,03E-01	0.1%
9292	Genetic transfer	6,01E-01	0.1%
9740	Gibberellic acid mediated signaling pathway	3,04E-01	0.3%
10476	Gibberellin mediated signaling pathway	3,27E-01	0.3%
6538	Gluamate catabolic process	1,66E-01	0.1%
44042	Glucan metabolic process	9,57E-01	0.1%
6007	Glucose catabolic process	5,92E-01	0.3%
6006	Glucose metabolic process	6,08E-01	0.3%
6540	Glutamate decarboxylation to succinate	1,66E-01	0.1%
6536	Glutamate metabolic process	4,43E-01	0.1%
9065	Glutamine family amino acid catabolic process	3,09E-01	0.1%
9064	Glutamine family amino acid metabolic process	6,97E-01	0.1%
6071	Glycerol metabolic process	5,59E-01	0.1%
6072	Glycerol-3-phosphate metabolic process	2,24E-01	0.1%
6096	Glycolysis	7,38E-01	0.1%
16138	Glycoside biosynthetic process	8,51E-01	0.1%
16137	Glycoside metabolic process	9,25E-01	0.1%
48193	Golgi vesicle transport	7,22E-01	0.1%
7186	G-protein coupled receptor protein signaling pathway	5,05E-01	0.1%
9630	Gravitropism	2,36E-02	0.8%

40007	Growth	2,26E-01	1.9%
48467	Gynoecium development	1,07E-01	0.8%
35315	Hair cell differentiation	8,58E-01	0.1%
31507	Heterochromatin formation	2,24E-01	0.1%
70828	Heterochromatin organization	2,24E-01	0.1%
46700	Heterocycle catabolic process	6,25E-01	0.1%
46483	Heterocycle metabolic process	9,07E-01	1.0%
19319	Hexose biosynthetic process	1,71E-01	0.3%
19320	Hexose catabolic process	5,99E-01	0.3%
19318	Hexose metabolic process	3,50E-01	0.7%
16577	Histone demethylation	2,04E-01	0.1%
51568	Histone H3-K4 methylation	3,09E-01	0.1%
33169	Histone H3-K9 demethylation	2,04E-01	0.1%
51567	Histone H3-K9 methylation	5,91E-02	0.3%
70076	Histone lysine demethylation	2,04E-01	0.1%
34968	Histone lysine methylation	3,65E-02	0.5%
16571	Histone methylation	1,22E-01	0.5%
16570	Histone modification	1,99E-01	0.7%
42592	Homeostatic process	1,26E-01	1.7%
45143	Homologous chromosome segregation	1,66E-01	0.1%
42446	Hormone byosynthetic process	9,95E-02	0.7%
42445	Hormone metabolic process	2,34E-02	1.2%
9914	Hormone transport	5,06E-01	0.3%
9755	Hormone-mediated signaling pathway	1,16E-01	1.9%
34050	Host programmed cell death induced by symbiont	1,66E-01	0.5%
6972	Hyperosmotic response	2,81E-01	0.5%
42538	Hyperosmotic salinity response	2,24E-01	0.5%
2252	Immune efector process	1,05E-01	0.5%
6955	Immune response	1,66E-01	2.1%
2376	Immune system process	8,19E-02	2.4%
9684	Indolacetic acid biosynthetic process	3,69E-01	0.1%
42430	Indole and derivative metabolic process	4,18E-01	0.3%
42435	Indole derivative biosynthetic process	3,88E-01	0.3%
42434	Indole derivative metabolic process	4,18E-01	0.3%
42431	Indole metabolic process	3,50E-01	0.1%
9700	Indole phyloalexin biosynthetic process	3,50E-01	0.1%
46217	Indole phytoalexin metabolic process	3,50E-01	0.1%
9683	Indoleacetic acid metabolic process	3,89E-01	0.1%
45087	Innate immune response	3,50E-01	1.5%
15698	Inorganic anion transport	4,90E-01	0.3%
80060	Integument development	4,25E-02	0.3%
51701	Interaction with host	4,43E-01	0.1%
51325	Interphase	1,60E-01	0.3%
51329	Interphase of mitotic cell cycle	1,60E-01	0.3%
44419	Interspecies interaction between organisms	4,76E-01	0.1%
6886	Intracellular protein transport	7,74E-01	0.8%
35556	Intracellular signal transduction	9,27E-01	0.1%
23034	Intracellular signaling pathway	7,64E-01	0.7%
46907	Intracellular transport	9,41E-01	0.8%

6891	Intra-Golgi vesicle-mediated transport	3,89E-01	0.1%
50801	Ion homeostasis	2,05E-01	0.8%
6811	Ion transport	6,25E-01	1.5%
55072	Iron ion homeostasis	4,43E-01	0.1%
6720	Isoprenoid metabolic process	8,89E-01	0.3%
9861	Jasmonic acid and ethylene-dependent systemic resistance	3,89E-01	0.1%
9867	Jasmonic acid mediated signaling pathway	6,14E-03	1.0%
48527	Lateral root development	5,49E-01	0.3%
10102	Lateral root morphogenesis	6,01E-01	0.1%
48366	Leaf development	7,88E-03	2.4%
9965	Leaf morphogenesis	1,21E-01	1.2%
10150	Leaf senescence	6,25E-01	0.1%
10358	Leaf shaping	5,91E-02	0.3%
10305	Leaf vascular tissue pattern	2,04E-01	0.3%
9809	Lignin biosynthetic process	6,25E-01	0.1%
9808	Lignin metabolic process	7,64E-01	0.1%
8610	Lipid biosynthetic process	9,39E-01	0.8%
10876	Lipid localization	8,18E-01	0.5%
6629	Lipid metabolic process	9,10E-01	1.9%
6869	Lipid transport	7,64E-01	0.5%
51179	Localization	5,06E-01	7.4%
40011	Locomotion	3,69E-01	0.1%
7626	Locomotory behavior	3,27E-01	0.1%
15909	Long-chain fatty acid transport	1,07E-01	0.1%
9085	Lysine biosynthetic process	3,27E-01	0.1%
9089	Lysine biosynthetic process via diaminopimelate	3,27E-01	0.1%
6553	Lysine metabolic process	3,50E-01	0.1%
279	M phase	5,92E-01	0.3%
51327	M phase of meiotic cell cycle	7,58E-01	0.1%
87	M phase of mitotic cell cycle	4,90E-01	0.1%
65003	Macromolecular complex assembly	2,04E-01	1.5%
43933	Macromolecular complex subunit organization	2,26E-01	1.5%
9059	Macromolecule biosynthetic process	1,00E+00	3.8%
9057	Macromolecule catabolic process	2,58E-01	2.1%
33036	Macromolecule localization	6,70E-01	2.3%
43170	Macromolecule metabolic process	3,05E-02	22.8%
43414	Macromolecule methylation	4,23E-01	0.5%
43412	Macromolecule modification	2,42E-02	9.2%
10076	Maintenance of floral meristem identity	2,81E-01	0.1%
51235	Maintenance of location	1,07E-01	0.3%
51651	Maintenance of location in cell	9,08E-02	0.3%
10074	Maintenance of meristem identity	7,32E-02	0.5%
51457	Maintenance of protein lication in nucleus	2,04E-01	0.1%
45185	Maintenance of protein localization	9,08E-02	0.3%
32507	Maintenance of protein localization in cell	7,64E-02	0.3%
10078	Maintenance of root meristem identity	7,64E-02	0.3%
48232	Male gamete generation	4,90E-01	0.1%
7126	Meiosis	7,58E-01	0.1%
7127	Meiosis I	6,47E-01	0.1%

51321	Meiotic cell cycle	8,55E-01	0.1%
10032	Meiotic chromosome condensation	1,07E-01	0.1%
45132	Meiotic chromosome segregation	3,89E-01	0.1%
51177	Meiotic sister chromatid cohesion	2,57E-01	0.1%
51754	Meiotic sister chromatid cohesion, centromeric	1,66E-01	0.1%
61025	Membrane fusion	6,58E-01	0.1%
61024	Membrane organization	9,07E-01	0.1%
10022	Meristem determinancy	1,07E-01	0.3%
48507	Meristem development	7,34E-04	1.9%
10014	Meristem initiation	8,97E-02	0.5%
10073	Meristem maintenance	1,68E-03	1.4%
9933	Meristem structural organization	8,27E-02	0.7%
8152	Metabolic process	2,24E-01	33.2%
55065	Metal ion homeostasis	1,07E-01	0.7%
30001	Metal ion transport	5,32E-01	0.8%
9086	Methionine biosynthetic process	4,90E-01	0.1%
6555	Methionine metabolic process	2,24E-01	0.5%
32259	Methylation	4,53E-01	0.5%
10480	Microsporocyte differentiation	1,66E-01	0.1%
34453	Microtubule anchoring	1,66E-01	0.1%
226	Microtubule cytoskeleton organization	4,07E-01	0.3%
7018	Microtubule-based movement	7,66E-01	0.1%
7017	Microtubule-based process	5,98E-01	0.5%
10586	miRNA metabolic process	2,81E-01	0.1%
6298	Mismatc repair	4,59E-01	0.1%
7067	Mitosis	4,90E-01	0.1%
278	Mitotic cell cycle	1,66E-01	0.5%
44003	Modification by symbiont of host morphology or physiology	3,27E-01	0.1%
51817	Modification of morphology or physiology of other organism involved in symbiotic interaction	3,27E-01	0.1%
43632	Modification-dependent macromolecule catabolic process	1,71E-01	1.9%
19941	Modification-dependent protein catabolic process	1,71E-01	1.9%
52018	Modulation by symbiont of RNA levels in host	3,09E-01	0.1%
52249	Modulation of RNA levels in other organism involved in symbiotic interaction	3,09E-01	0.1%
32787	Monocarboxylic acid metabolic process	7,06E-01	1.2%
15718	Monocarboxylic acid transport	2,04E-01	0.1%
46364	Monosaccharide biosynthetic process	1,86E-01	0.3%
46365	Monosaccharide catabolic process	5,99E-01	0.3%
5996	Monosaccharide metabolic process	4,78E-01	0.7%
55067	Monovalent inorganic cation homeostasis	3,69E-01	0.1%
15672	Monovalent inorganic cation transport	9,51E-01	0.1%
16071	mRNA metabolic process	9,42E-01	0.1%
6397	mRNA processing	9,15E-01	0.1%
10192	Mucilage biosynthetic process	2,04E-01	0.1%
10191	Mucilage metabolic process	2,24E-01	0.1%
32504	Multicellular organism reproduction	5,75E-01	0.3%
7275	Multicellular organismal development	1,10E-04	12.9%
32501	Multicellular organismal process	8,38E-05	13.4%
9825	Multidimensional cell growth	4,67E-01	0.3%

51704	Multi-organism process	2,56E-01	4.0%
6739	NADP metabolic process	6,01E-01	0.1%
6740	NADPH regeneration	5,72E-01	0.1%
43628	ncRNA 3'-end processing	2,24E-01	0.1%
34660	ncRNA metabolic process	8,14E-01	0.7%
34470	ncRNA processing	5,90E-01	0.7%
10254	Nectary development	2,04E-01	0.1%
30835	Negative regulation of actin filament depolymerization	1,07E-01	0.1%
51100	Negative regulation of binding	1,66E-01	0.1%
48519	Negative regulation of biological process	5,74E-04	4.2%
9890	Negative regulation of biosynthetic process	7,71E-02	1.0%
43086	Negative regulation of catalytic activity	3,27E-01	0.5%
10648	Negative regulation of cell communication	2,04E-01	0.5%
45786	Negative regulation of cell cycle	4,43E-01	0.1%
10948	Negative regulation of cell cycle progress	3,89E-01	0.1%
45596	Negative regulation of cell differentiation	3,84E-02	0.7%
8285	Negative regulation of cell proliferation	2,04E-01	0.1%
31327	Negative regulation of cellular biosynthetic process	7,71E-02	1.0%
51129	Negative regulation of cellular component organization	1,07E-01	0.3%
31324	Negative regulation of cellular metabolic process	2,23E-02	1.4%
48523	Negative regulation of cellular process	2,72E-04	3.0%
32269	Negative regulation of cellular protein metabolic process	5,91E-02	0.3%
45736	Negative regulation of cyclin-dependent protein kinase activity	3,27E-01	0.1%
51494	Negative regulation of cytoskeleton organization	1,66E-01	0.1%
31348	Negative regulation of defense response	5,05E-01	0.1%
48640	Negative regulation of developmental growth	1,07E-01	0.1%
51093	Negative regulation of developmental process	4,02E-02	1.2%
43392	Negative regulation of DNA binding	1,07E-01	0.1%
32876	Negative regulation of DNA endoreduplication	2,57E-01	0.1%
51053	Negative regulation of DNA metabolic process	3,89E-01	0.1%
8156	Negative regulation of DNA replication	3,27E-01	0.1%
10105	Negative regulation of ethylene mediated signaling pathway	3,89E-01	0.1%
9910	Negative regulation of flower development	7,06E-01	0.1%
10629	Negative regulation of gene expression	1,08E-01	1.5%
45814	Negative regulation of gene expression, epigenetic	2,53E-01	0.3%
9938	Negative regulation of gibberellic acid mediated signaling pathway	9,08E-02	0.3%
45926	Negative regulation of growth	1,07E-01	0.3%
35067	Negative regulation of histone acetylation	1,07E-01	0.1%
31057	Negative regulation of histone modification	1,07E-01	0.1%
33673	Negative regulation of kinase activity	3,69E-01	0.1%
10558	Negative regulation of macromolecule biosynthetic process	6,34E-02	1.0%
10605	Negative regulation of macromolecule metabolic process	2,36E-02	2.1%
9892	Negative regulation of metabolic process	3,65E-02	2.1%
44092	Negative regulation of molecular function	2,04E-01	0.7%
51241	Negative regulation of multicellular organismal process	1,07E-01	0.3%
51172	Negative regulation of nitrogen compound metabolic process	6,34E-02	1.0%
45934	Negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	6,34E-02	1.0%
46621	Negative regulation of organ growth	2,04E-01	0.1%

10639	Negative regulation of organelle organization	1,07E-01	0.3%
50732	Negative regulation of peptidyl-tyrosine phosphorylation	1,07E-01	0.1%
45936	Negative regulation of phosphate metabolic process	1,07E-01	0.1%
10563	Negative regulation of phosphorus metabolic process	1,07E-01	0.1%
42326	Negative regulation of phosphorylation	1,07E-01	0.1%
48581	Negative regulation of post-embryonic development	3,14E-01	0.5%
1933	Negative regulation of protein amino acid phosphorylation	1,07E-01	0.1%
43242	Negative regulation of protein complex disassembly	1,07E-01	0.1%
6469	Negative regulation of protein kinase activity	3,69E-01	0.1%
51248	Negative regulation of protein metabolic process	5,91E-02	0.3%
31400	Negative regulation of protein modification process	1,89E-02	0.3%
48585	Negative regulation of response to stimulus	2,23E-01	0.7%
51253	Negative regulation of RNA metabolic process	2,70E-01	0.3%
10187	Negative regulation of seed germination	1,07E-01	0.3%
9968	Negative regulation of signal transduction	4,23E-01	0.1%
35467	Negative regulation of signaling pathway	2,01E-01	0.5%
23057	Negative regulation of signaling process	4,23E-01	0.1%
16481	Negative regulation of transcription	1,03E-01	0.8%
43433	Negative regulation of transcription factor activity	1,07E-01	0.1%
90048	Negative regulation of transcription regulator activity	1,07E-01	0.1%
45892	Negative regulation of transcription, DNA-dependent	2,70E-01	0.3%
51348	Negative regulation of transferase activity	3,69E-01	0.1%
70298	Negative regulation of two-component signal transduction system (phosphorelay)	3,89E-01	0.1%
46496	Nicotinamide nucleotide metabolic process	6,69E-01	0.1%
42128	Nitrate assimilation	3,50E-01	0.1%
42126	Nitrate metabolic process	3,50E-01	0.1%
15706	Nitrate transport	3,69E-01	0.1%
6809	Nitric oxide biosynthetic process	2,24E-01	0.1%
46209	Nitric oxide metabolic process	2,24E-01	0.1%
6807	Nitrogen compound metabolic process	1,89E-02	10.2%
280	Nuclear division	5,49E-01	0.1%
51170	Nuclear import	4,03E-01	0.3%
398	Nuclear mRNA spicing, via spliceosome	7,64E-01	0.1%
51169	Nuclear transport	5,59E-01	0.3%
90304	Nucleic acid metabolic process	3,45E-03	7.0%
46112	Nucleobase biosynthetic process	4,23E-01	0.1%
9112	Nucleobase metabolic process	5,05E-01	0.1%
15851	Nucleobase transport	5,59E-01	0.1%
34404	Nucleobase, nucleoside and nucleotide biosynthetic process	9,57E-01	0.1%
55086	Nucleobase, nucleoside and nucleotide metabolic process	8,96E-01	0.5%
34654	Nucleobase, nucleoside, nucleotide and nucleic acid biosynthetic process	9,57E-01	0.1%
6139	Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	5,70E-03	8.3%
15931	Nucleobase, nucleoside, nucleotide and nucleic acid transport	7,32E-01	0.1%
6913	Nucleocytoplasmatic transport	5,59E-01	0.3%
6753	Nucleoside phosphate metabolic process	9,20E-01	0.3%
6334	Nucleosome assembly	2,36E-02	1.0%
34728	Nucleosome organization	2,36E-02	1.0%
9117	Nucleotide metabolic process	9,20E-01	0.3%
6289	Nucleotide-excision repair	4,03E-01	0.1%

9226	Nucleotide-sugar biosynthetic process	1,07E-01	0.3%
9225	Nucleotide-sugar metabolic process	2,91E-02	0.7%
6857	Oligopeptide transport	6,28E-01	0.3%
9312	Oligosaccharide biosynthetic process	6,97E-01	0.1%
9311	Oligosaccharide metabolic process	8,26E-01	0.1%
6730	One-carbon metabolic process	3,75E-01	0.7%
48513	Organ development	6,65E-05	7.2%
48645	Organ formation	3,94E-01	0.3%
9887	Organ morphogenesis	4,25E-02	1.9%
10260	Organ senescence	6,36E-01	0.1%
48285	Organelle fission	2,58E-01	0.5%
6996	Organelle organization	3,34E-02	4.2%
16053	Organic acid biosynthetic process	9,24E-01	0.8%
16054	Organic acid catabolic process	8,89E-01	0.1%
6082	Organic acid metabolic process	9,08E-01	2.1%
15849	Organic acid transport	7,63E-03	1.4%
15711	Organic acid transport	2,24E-01	0.1%
19637	Organophosphate metabolic process	7,75E-01	0.3%
48481	Ovule development	2,14E-01	0.5%
55114	Oxidation reduction	1,07E-01	1.4%
6733	Oxidoreduction coenzyme metabolic process	7,38E-01	0.1%
43436	Oxoacid metabolic process	9,07E-01	2.1%
7389	Pattern specification process	2,83E-03	1.9%
19321	Pentose metabolic process	2,70E-01	0.3%
6098	Pentose-phosphate shunt	5,59E-01	0.1%
9051	Pentose-phosphate shunt, oxidative branch	2,81E-01	0.1%
15833	Peptide transport	6,28E-01	0.3%
48441	Petal development	4,76E-01	0.1%
9699	Phenylpropanoid biosynthetic process	8,15E-01	0.3%
9698	Phenylpropanoid metabolic process	9,04E-01	0.3%
10087	Phloem or xylem histogenesis	3,57E-01	0.3%
10233	Phloem transport	1,66E-01	0.1%
6796	Phosphate metabolic process	3,22E-03	7.7%
8654	Phospholipid biosynthetic process	8,32E-01	0.1%
6644	Phospholipid metabolic process	9,29E-01	0.1%
6793	Phosphorous metabolic process	3,22E-03	7.7%
16310	Phosphorylation	4,61E-03	7.2%
7602	Phosphotransduction	4,43E-01	0.1%
9640	Photomorphogenesis	4,85E-01	0.3%
15979	Photosynthesis	3,07E-01	0.8%
19684	Photosynthesis, light reaction	3,59E-01	0.5%
9767	Photosynthetic electron transport chain	1,07E-01	0.5%
9773	Photosynthetic electron transport chain in photosystem I	4,59E-01	0.1%
48827	Phyllome development	3,22E-03	2.8%
52315	Phytoalexin biosynthetic process	3,50E-01	0.1%
52314	Phytoalexin metabolic process	3,50E-01	0.1%
16129	Phytosteroid biosynthetic process	1,07E-01	0.3%
16128	Phytosteroid metabolic process	1,71E-01	0.3%
43476	Pigment accumulation	2,24E-01	0.1%

43478	Pigment accumulation in response to UV light	2,24E-01	0.1%
43480	Pigment accumulation in tissues	2,24E-01	0.1%
43479	Pigment accumulation in tissues in response to UV light	2,24E-01	0.1%
46148	Pigment biosynthetic process	9,06E-01	0.1%
46149	Pigment catabolic process	3,50E-01	0.1%
42440	Pigment metabolic process	7,64E-01	0.3%
43473	Pigmentation	2,24E-01	0.1%
9828	Plant-type cell wall loosening	1,81E-01	0.5%
9827	Plant-type cell wall modification	2,47E-01	0.5%
9831	Plant-type cell wall modification involved in multidimensional cell growth	2,81E-01	0.3%
9664	Plant-type cell wall organization	4,59E-01	0.5%
71669	Plant-type cell wall organization or biogenesis	7,42E-01	0.5%
9626	Plant-type hypersensitive response	1,66E-01	0.5%
43572	Plastid fission	1,71E-01	0.3%
9657	Plastid organization	2,57E-01	0.8%
9944	Polarity specification of adaxial/abaxial axis	7,74E-03	0.7%
9555	Pollen development	3,27E-01	1.0%
10584	Pollen exine formation	4,76E-01	0.1%
9865	Pollen tube adhesion	1,66E-01	0.1%
48868	Pollen tube development	7,64E-01	0.3%
9860	Pollen tube growth	6,25E-01	0.3%
10183	Pollen tube guidance	3,27E-01	0.1%
10208	Pollen wall assembly	5,36E-01	0.1%
9875	Pollen-pistil interaction	3,41E-01	0.3%
9856	Pollination	7,75E-01	0.5%
19751	Polyol metabolic process	7,06E-01	0.1%
272	Polysaccharide catabolic process	6,01E-01	0.1%
5976	Polysaccharide metabolic process	9,37E-01	0.3%
6787	Porphyrin catabolic process	4,03E-01	0.1%
6778	Porphyrin metabolic process	5,75E-01	0.3%
50918	Positive chemotaxis	3,27E-01	0.1%
9958	Positive gravitropism	3,05E-02	0.5%
48518	Positive regulation of biological process	6,39E-02	2.1%
9891	Positive regulation of biosynthetic process	1,08E-01	0.8%
45787	Positive regulation of cell cycle	2,24E-01	0.1%
90068	Positive regulation of cell cycle progress	2,24E-01	0.1%
51781	Positive regulation of cell division	2,24E-01	0.1%
8284	Positive regulation of cell proliferation	3,50E-01	0.1%
45793	Positive regulation of cell size	2,81E-01	0.1%
31328	Positive regulation of cellular biosynthetic process	1,08E-01	0.8%
31325	Positive regulation of cellular metabolic process	1,66E-01	0.8%
48522	Positive regulation of cellular process	1,85E-01	1.2%
31349	Positive regulation of defense response	3,27E-01	0.3%
45962	Positive regulation of development, heterochronic	9,08E-02	0.3%
51094	Positive regulation of developmental process	5,01E-01	0.3%
32877	Positive regulation of DNA endoreduplication	1,66E-01	0.1%
51054	Positive regulation of DNA metabolic process	2,24E-01	0.1%
45740	Positive regulation of DNA replication	2,04E-01	0.1%
40019	Positive regulation of embryonic development	1,07E-01	0.1%

9963	Positive regulation of flavonoid byosynthetic process	1,89E-02	0.3%
9911	Positive regulation of flower development	6,88E-01	0.1%
10628	Positive regulation of gene expression	1,90E-01	0.7%
50778	Positive regulation of immune response	2,89E-01	0.3%
2684	Positive regulation of immune system process	2,89E-01	0.3%
45089	Positive regulation of innate immune response	2,89E-01	0.3%
10557	Positive regulation of macromolecule biosynthetic process	2,04E-01	0.7%
10604	Positive regulation of macromolecule metabolic process	1,22E-01	0.8%
9893	Positive regulation of metabolic process	9,35E-02	1.0%
51173	Positive regulation of nitrogen compound metabolic process	2,13E-01	0.7%
45935	Positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	2,05E-01	0.7%
48582	Positive regulation of post-embryonic development	7,58E-01	0.1%
48584	Positive regulation of response to stimulus	5,59E-01	0.3%
51254	Positive regulation of RNA metabolic process	5,49E-01	0.1%
45941	Positive regulation of transcription	3,14E-01	0.5%
45893	Positive regulation of transcription, DNA dependent	5,05E-01	0.1%
9791	Post-embryonic development	1,52E-04	8.1%
9886	Post-embryonic morphogenesis	1,02E-01	1.0%
48569	Post-embryonic organ development	8,16E-03	2.4%
48563	Post-embryonic organ morphogenesis	3,11E-01	0.3%
48528	Post-embryonic root development	5,92E-01	0.3%
10101	Post-embryonic root morphogenesis	6,01E-01	0.1%
16441	Post-transcriptional gene silencing	6,01E-01	0.5%
35194	Post-transcriptional gene silencing by RNA	5,61E-01	0.5%
10608	Post-transcriptional regulation of gene expression	7,36E-01	0.5%
43687	Post-translational protein modification	5,80E-04	9.0%
55075	Potassium ion homeostasis	2,57E-01	0.1%
6813	Potassium ion transport	7,64E-01	0.1%
44238	Primary metabolic process	2,24E-01	28.1%
31053	Primary microRNA processing	2,24E-01	0.1%
80022	Primary root development	4,76E-01	0.1%
10072	Primary shoot apical meristem specification	1,60E-01	0.3%
35196	Production of miRNA involved in gene silencing by miRNA	3,09E-01	0.1%
30422	Production of siRNA involved in RNA interference	5,59E-01	0.1%
70918	Production of small RNA involved in gene silencing by RNA	2,70E-01	0.3%
12501	Programed cell death	2,14E-01	1.4%
8213	Protein amino acid alkylation	1,71E-01	0.5%
46777	Protein amino acid autophosphorylation	2,38E-01	0.3%
8214	Protein amino acid dealkylation	2,04E-01	0.1%
6482	Protein amino acid demethylation	2,04E-01	0.1%
6470	Protein amino acid dephosphorylation	8,09E-01	0.1%
6479	Protein amino acid methylation	1,71E-01	0.5%
6468	Protein amino acid phosphorylation	1,29E-03	7.2%
51865	Protein autoubiquitination	3,05E-02	0.3%
30163	Protein catabolic process	2,24E-01	1.9%
6461	Protein complex assembly	9,06E-01	0.3%
70271	Protein complex biogenesis	9,06E-01	0.3%
6457	Protein folding	2,24E-01	1.4%

51260	Protein homooligomerization	2,04E-01	0.1%
17038	Protein import	4,53E-01	0.5%
45037	Protein import into chloroplast stroma	3,27E-01	0.1%
6606	Protein import into nucleus	3,88E-01	0.3%
59	Protein import into nucleus, docking	4,59E-01	0.1%
8104	Protein localization	4,99E-01	1.7%
34504	Protein localization in nucleus	2,14E-01	0.5%
33365	Protein localization in organelle	4,00E-01	0.5%
19538	Protein metabolic process	3,50E-01	15.3%
32446	Protein modification by small protein conjugation	1,71E-01	1.0%
70647	Protein modification by small protein conjugation or removal	2,24E-01	1.0%
6464	Protein modification process	4,37E-03	9.2%
51259	Protein oligomerization	2,04E-01	0.1%
9306	Protein secretion	4,76E-01	0.1%
16925	Protein sumoylation	3,27E-01	0.1%
6605	Protein targeting	5,43E-01	0.7%
45036	Protein targeting to chloroplast	5,98E-01	0.1%
6612	Protein targeting to membrane	5,72E-01	0.1%
15031	Protein transport	6,88E-01	1.4%
16567	Protein ubiquitination	2,24E-01	0.8%
65004	Protein-DNA complex assembly	2,92E-02	1.0%
6508	Proteolysis	2,01E-01	3.8%
51603	Proteolysis involved in cellular protein catabolic process	1,81E-01	1.9%
10498	Proteosomal protein catabolic process	4,76E-01	0.1%
43161	Proteosomal ubiquitin-dependent protein catabolic process	4,23E-01	0.1%
9954	Proximal/distal pattern formation	4,25E-02	0.3%
9113	Purine base biosynthetic process	3,50E-01	0.1%
6144	Purine base metabolic process	4,03E-01	0.1%
6863	Purine transport	5,49E-01	0.1%
19362	Pyridine nucleotide metabolic process	7,06E-01	0.1%
9956	Radial pattern formation	5,91E-02	0.3%
48544	Recognition of pollen	6,01E-01	0.1%
725	Recombinational repair	4,03E-01	0.1%
10017	Red or far-red light signaling pathway	7,43E-01	0.1%
9585	Red, far-red light phototransduction	4,43E-01	0.1%
3002	Regionalization	6,23E-04	1.9%
10602	Regulation of 1-aminocydopropane-1-carboxylate metabolic process	1,07E-01	0.1%
32956	Regulation of actin cytoskeleton organization	3,27E-01	0.1%
30834	Regulation of actin filament depolymerization	2,04E-01	0.1%
30832	Regulation of actin filament legth	2,24E-01	0.1%
32970	Regulation of actin filament-based process	3,27E-01	0.1%
8064	Regulation of actin polymerization or depolymerization	2,24E-01	0.1%
22603	Regulation of anatomical structure morphogenesis	3,57E-01	0.3%
90066	Regulation of anatomical structure size	1,22E-01	2.1%
42981	Regulation of apoptosis	4,43E-01	0.1%
51098	Regulation of binding	4,25E-02	0.3%
50789	Regulation of biological process	9,22E-09	22.4%
65008	Regulation of biological quality	2,67E-03	5.3%
9889	Regulation of biosynthetic process	1,24E-05	12.9%

50790	Regulation of catalytic activity	7,17E-01	0.5%
10646	Regulation of cell communication	2,35E-01	0.8%
51726	Regulation of cell cycle	2,39E-03	1.9%
10564	Regulation of cell cycle progress	1,13E-01	0.5%
10941	Regulation of cell death	1,71E-01	0.5%
45595	Regulation of cell differentiation	1,07E-01	0.7%
51302	Regulation of cell division	2,04E-01	0.3%
1558	Regulation of cell growth	5,20E-01	0.1%
22604	Regulation of cell morphogenesis	4,03E-01	0.1%
42127	Regulation of cell proliferation	1,13E-01	0.5%
8361	Regulation of cell size	1,85E-01	1.9%
33238	Regulation of cellular amine metabolic process	5,91E-02	0.3%
6521	Regulation of cellular amino acid metabolic process	2,57E-01	0.1%
31326	Regulation of cellular biosynthetic process	1,24E-05	12.9%
51128	Regulation of cellular component organization	2,04E-01	0.5%
32535	Regulation of cellular component size	1,22E-01	2.1%
10565	Regulation of cellular ketone metabolic process	2,60E-01	0.3%
31323	Regulation of cellular metabolic process	5,58E-06	13.9%
50794	Regulation of cellular process	2,45E-07	19.6%
32268	Regulation of cellular protein metabolic process	3,11E-01	0.3%
33044	Regulation of chromosome organization	3,09E-01	0.1%
42752	Regulation of circadian rhythm	4,90E-01	0.1%
79	Regulation of cyclin-dependent protein kinase activity	3,69E-01	0.1%
51493	Regulation of cytoskeleton organization	4,03E-01	0.1%
31347	Regulation of defense response	2,17E-01	0.7%
50688	Regulation of defense response to virus	2,04E-01	0.1%
50691	Regulation of defense response to virus by host	1,66E-01	0.1%
40034	Regulation of development, heterochronic	3,50E-01	0.3%
48638	Regulation of developmental growth	1,99E-02	0.8%
50793	Regulation of developmental process	6,23E-04	3.5%
51101	Regulation of DNA binding	1,89E-02	0.3%
32875	Regulation of DNA endoreduplication	1,22E-01	0.3%
51052	Regulation of DNA metabolic process	2,81E-01	0.3%
6275	Regulation of DNA replication	2,03E-01	0.3%
90329	regulation of DNA-dependent DNA replication	1,41E-01	0.3%
45995	Regulation of embryonic development	2,24E-01	0.1%
10104	Regulation of ethylene mediated signaling pathway	1,60E-01	0.3%
9962	Regulation of flavonoid biosynthetic process	1,71E-01	0.3%
9909	Regulation of flower development	2,63E-01	0.8%
10468	Regulation of gene expression	1,15E-05	13.6%
6349	Regulation of gene expression by genetic imprinting	4,03E-01	0.1%
40029	Regulation of gene expression, epigenetic	5,06E-01	0.8%
9937	Regulation of gibberelic acid mediated signaling pathway	3,05E-02	0.5%
40008	Regulation of growth	2,36E-02	1.0%
35065	Regulation of histone acetylation	1,07E-01	0.1%
31056	Regulation of histone modification	2,04E-01	0.1%
10817	Regulation of hormone levels	3,37E-02	1.5%
32350	Regulation of hormone metabolic process	2,57E-01	0.1%
10310	Regulation of hydrogen peroxide metabolic process	3,09E-01	0.1%

2697	Regulation of immune effector process	2,04E-01	0.1%
50776	Regulation of immune response	2,22E-01	0.5%
2682	Regulation of immune system process	2,22E-01	0.5%
45088	Regulation of innate immune response	3,94E-01	0.3%
43549	Regulation of kinase activity	6,01E-01	0.1%
32879	Regulation of localization	6,47E-01	0.1%
10556	Regulation of macromolecule biosynthetic process	1,24E-05	12.7%
60255	Regulation of macromolecule metabolic process	2,73E-06	14.3%
40020	Regulation of meiosis	2,24E-01	0.1%
51445	Regulation of meiotic cell cycle	2,81E-01	0.1%
48509	Regulation of meristem development	2,04E-01	0.7%
10075	Regulation of meristem growth	1,05E-01	0.5%
9934	Regulation of meristem structural organization	5,05E-01	0.1%
19222	Regulation of metabolic process	1,45E-06	15.3%
65009	Regulation of molecular function	4,03E-01	0.8%
51239	Regulation of multicellular organismal process	4,25E-02	2.3%
43900	Regulation of multi-organism process	4,90E-01	0.1%
51171	Regulation of nitrogen compound metabolic process	1,00E-05	13.0%
6808	Regulation of nitrogen utilization	2,57E-01	0.1%
19219	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	1,64E-05	12.7%
46620	Regulation of organ growth	2,81E-01	0.1%
33043	Regulation of organelle organization	2,70E-01	0.3%
80010	Regulation of oxygen and reactive oxigen species metabolic process	1,86E-01	0.3%
50730	Regulation of peptidyl-tyrosine phosphorylation	1,07E-01	0.1%
19220	Regulation of phosphate metabolic process	3,57E-01	0.3%
51174	Regulation of phosphorus metabolic process	3,57E-01	0.3%
42325	Regulation of phosphorylation	3,27E-01	0.3%
48580	Regulation of post-embryonic development	1,66E-01	1.4%
80090	Regulation of primary metabolic process	5,58E-06	13.6%
43067	Regulation of programmed cell death	1,42E-01	0.5%
1932	Regulation of protein amino acid phosphorylation	1,66E-01	0.1%
43244	Regulation of protein complex disassembly	2,04E-01	0.1%
45859	Regulation of protein kinase activity	6,01E-01	0.1%
32880	Regulation of protein localization	3,09E-01	0.1%
51246	Regulation of protein metabolic process	4,18E-01	0.3%
31399	Regulation of protein modification process	9,08E-02	0.3%
2831	Regulation of response to biotic stimulus	3,89E-01	0.1%
48583	Regulation of response to stimulus	1,02E-01	1.5%
51252	Regulation of RNA metabolic process	6,57E-03	6.5%
43455	Regulation of secondary metabolic process	3,24E-01	0.3%
10029	Regulation of seed germination	1,64E-01	0.5%
9966	Regulation of signal transduction	2,83E-01	0.5%
35466	Regulation of signaling pathway	2,24E-01	0.8%
23051	Regulation of signaling process	2,83E-01	0.5%
10119	Regulation of stomatal movement	3,11E-01	0.3%
45449	Regulation of transcription	1,64E-05	12.3%
51090	Regulation of transcription factor activity	7,88E-03	0.3%
43619	Regulation of transcription from RNA polymerase II promoter in response to oxidative stress	1,07E-01	0.1%

43618	Regulation of transcription from RNA polymerase II promoter in response to stress	1,07E-01	0.1%
6357	Regulation of transcription from RNA polymerase II promoter	3,27E-01	0.1%
43620	Regulation of transcription in response to stress	1,66E-01	0.1%
90046	Regulation of transcription regulator activity	7,88E-03	0.3%
6355	Regulation of transcription, DNA-dependent	6,30E-03	6.5%
51338	Regulation of transferase activity	6,36E-01	0.1%
70297	Regulation of two-component signal transduction system (phosphorelay)	1,60E-01	0.3%
51510	Regulation of unidimensional cell growth	3,69E-01	0.1%
80134	Regulation to response to stress	2,78E-01	0.7%
7346	Regulationof mitotic cell cycle	4,43E-01	0.1%
48838	Release of seed from dormancy	1,66E-01	0.1%
3	Reproduction	1,35E-03	7.7%
48610	Reproductive cellular process	1,66E-01	1.2%
3006	Reproductive developmental process	2,72E-04	7.6%
22414	Reproductive process	8,77E-04	7.7%
48609	Reproductive process in a multicellular organism	5,49E-01	0.3%
48608	Reproductive structure development	5,13E-05	7.4%
45730	Respiratory burst	2,57E-01	0.1%
2679	Respiratory burst involved in defense response	2,24E-01	0.1%
22904	Respiratory electron transport chain	6,01E-01	0.1%
9628	Response to abiotic stimulus	2,72E-04	9.7%
9737	Response to abscisic acid stimulus	6,47E-01	1.2%
9646	Response to absence of light	4,43E-01	0.1%
46685	Response to arsenic	3,50E-01	0.1%
9733	Response to auxin stimulus	1,16E-01	2.3%
9617	Response to bacterium	4,07E-01	1.4%
9607	Response to biotic stimulus	2,42E-01	3.3%
9637	Response to blue light	7,96E-01	0.1%
9741	Response to brassinosteroid stimulus	1,26E-03	1.4%
46686	Response to cadmium ion	7,87E-01	1.0%
51592	Response to calcium ion	3,69E-01	0.1%
9743	Response to carbohydrate stimulus	1,90E-02	2.1%
42221	Response to chemical stimulus	1,71E-03	12.2%
10200	Response to chitin	4,95E-03	1.7%
9409	Response to cold	5,47E-01	1.2%
9735	Response to cytokinin stimulus	4,15E-01	0.5%
9269	Response to desiccation	5,05E-01	0.1%
34285	Response to disaccharide stimulus	3,75E-01	0.3%
6974	Response to DNA damage stimulus	1,75E-04	2.8%
42493	Response to drug	6,25E-01	0.1%
43331	Response to dsRNA	2,70E-01	0.3%
9719	Response to endogenous stimulus	5,80E-04	7.4%
9723	Response to ethylene stimulus	2,24E-01	1.0%
9605	Response to external stimulus	1,28E-01	1.9%
9991	Response to extracellular stimulus	9,79E-01	0.1%
10218	Response to far red light	2,22E-01	0.5%
9750	Response to fructose stimulus	3,27E-01	0.1%
9620	Response to fungus	6,69E-01	0.7%
10332	Response to gamma radiation	3,69E-01	0.1%

9739	Response to gibberellin stimulus	1,72E-01	1.0%
9749	Response to glucose stimulus	5,59E-01	0.1%
9629	Response to gravity	3,72E-02	0.8%
9408	Response to heat	7,42E-01	0.5%
9746	Response to hexose stimulus	5,72E-01	0.1%
9725	Response to hormone stimulus	1,58E-03	6.7%
1666	Response to hypoxia	2,17E-01	0.3%
10035	Response to inorganic substance	6,52E-01	1.9%
9625	Response to insect	4,90E-01	0.1%
10212	Response to ionizing radiation	4,76E-01	0.1%
9753	Response to jasmonic acid stimulus	8,07E-02	1.5%
9642	Response to light intensity	9,02E-01	0.1%
9416	Response to light stimulus	7,88E-03	4.2%
10555	Response to mannitol stimulus	2,81E-01	0.1%
9612	Response to mechanical stimulus	4,25E-02	0.5%
10038	Response to metal ion	7,56E-01	1.4%
2240	Response to molecule of oomycetes origin	1,66E-01	0.1%
34284	Response to monosaccharide stimulus	5,72E-01	0.1%
9624	Response to nematode	5,61E-01	0.3%
10167	Response to nitrate	5,36E-01	0.1%
31667	Response to nutrient levels	9,72E-01	0.1%
2239	Response to oomycetes	4,03E-01	0.1%
10033	Response to organic substance	5,67E-05	9.3%
6970	Response to osmotic stress	5,31E-01	1.9%
51707	Response to other organism	2,15E-01	3.3%
6979	Response to oxidative stress	7,03E-01	1.0%
70482	Response to oxigen levels	2,24E-01	0.3%
305	Response to oxygen radical	3,50E-01	0.1%
9314	Response to radiation	3,27E-03	4.6%
302	Response to reactive oxygen species	8,67E-01	0.1%
10114	Response to red light	3,04E-01	0.5%
9639	Response to red or far red light	5,13E-02	1.7%
9751	Response to salicylic acid stimulus	4,03E-01	0.8%
9651	Response to salt stress	6,71E-01	1.5%
42594	Response to starvation	9,52E-01	0.1%
48545	Response to steroid hormone stimulus	1,48E-02	0.8%
50896	Response to stimulus	8,32E-07	23.5%
6950	Response to stress	2,71E-02	11.6%
9744	Response to sucrose stimulus	3,69E-01	0.3%
303	Response to superoxide	3,50E-01	0.1%
9266	Response to temperature stimulus	4,44E-01	1.9%
9636	Response to toxin	4,90E-01	0.1%
9411	Response to UV	4,25E-02	1.0%
10224	Response to UV-B	4,07E-01	0.3%
9615	Response to virus	4,43E-01	0.3%
9415	Response to water	6,66E-01	0.8%
9414	Response to water deprivation	6,30E-01	0.8%
9611	Response to wounding	1,71E-01	1.2%
9410	Response to xenobiotic stimulus	9,08E-02	0.3%

10043	Response to zinc ion	6,47E-01	0.1%
19300	Rhamnose biosynthetic process	4,25E-02	0.3%
19299	Rhamnose metabolic process	4,25E-02	0.3%
48511	Rhythmic process	2,67E-01	0.5%
22618	Ribonucleoprotein complex assembly	3,50E-01	0.1%
22613	Ribonucleoprotein complex biogenesis	8,96E-01	0.3%
42254	Ribosome biogenesis	9,70E-01	0.1%
9835	Ripening	1,66E-01	0.1%
31123	RNA 3'-end processing	4,59E-01	0.1%
32774	RNA biosynthetic process	6,01E-01	0.3%
16246	RNA interference	3,24E-01	0.3%
16070	RNA metabolic process	8,18E-01	1.9%
6396	RNA processing	7,22E-01	1.4%
8380	RNA splicing	6,89E-01	0.3%
394	RNA splicing, via endonucleolytic cleavage and ligation	3,50E-01	0.1%
375	RNA splicing, via transesterification reactions	7,96E-01	0.1%
377	RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	7,96E-01	0.1%
48364	Root development	3,74E-01	1.4%
10015	Root morphogenesis	5,85E-01	0.5%
22622	Root system development	3,74E-01	1.4%
16072	rRNA metabolic process	9,38E-01	0.1%
6364	rRNA processing	9,38E-01	0.1%
9863	Salicylic acid mediated signaling pathway	2,89E-01	0.3%
31146	SCF-dependent proteosomal ubiquitin-dependent protein catabolic process	2,24E-01	0.1%
19748	Secondary metabolic process	8,96E-01	1.0%
46903	Secretion	7,66E-01	0.1%
32940	Secretion by cell	7,66E-01	0.1%
10214	Seed coat development	1,78E-02	0.5%
48316	Seed development	9,35E-02	3.3%
10162	Seed dormancy	4,59E-01	0.1%
9845	Seed germination	7,58E-01	0.1%
10431	Seed maturation	5,20E-01	0.1%
90351	Seedling development	7,72E-01	0.1%
10149	Senescence	7,43E-01	0.1%
51761	Sesquiterpene metabolic process	5,59E-01	0.1%
6714	Sesquiterpenoid metabolic process	5,59E-01	0.1%
19953	Sexual reproduction	5,99E-01	0.3%
48367	Shoot development	1,79E-03	3.5%
10016	Shoot morphogenesis	2,19E-02	1.9%
22621	Shoot system development	8,77E-04	3.7%
7165	Signal transduction	9,07E-01	1.5%
23060	Signal transmission	8,70E-01	1.9%
23052	Signaling	6,57E-03	7.7%
23033	Signaling pathway	8,26E-05	6.5%
23046	Signaling process	8,70E-01	1.9%
7062	Sister chromatid cohesion	3,05E-02	0.5%
9647	Skotomorphogenesis	2,24E-01	0.1%
7264	Small GTPase mediated signal transduction	8,46E-01	0.1%
44283	Small molecule biosynthetic process	9,08E-01	2.1%

44282	Small molecule catabolic process	8,50E-01	0.5%
44281	Small molecule metabolic process	9,55E-01	4.2%
65001	Specification of axis polarity	8,97E-03	0.7%
10093	Specification of floral organ identity	3,69E-01	0.1%
10092	Specification of organ identity	3,69E-01	0.1%
10159	Specification of organ position	1,07E-01	0.1%
9799	Specification of symmetry	2,84E-03	0.5%
245	Spliceosome assembly	2,81E-01	0.1%
48443	Stamen development	6,87E-02	0.8%
80086	Stamen filament development	2,57E-01	0.1%
48455	Stamen formation	2,24E-01	0.1%
48448	Stamen morphogenesis	2,81E-01	0.1%
10479	Stele development	1,07E-01	0.1%
48863	Stem cell developmenifferentiationt	3,07E-02	0.7%
48864	Stem cell development	2,91E-02	0.7%
19827	Stem cell maintenance	2,91E-02	0.7%
6694	Steroid biosynthetic process	3,94E-01	0.3%
43401	Steroid hormone mediated signaling pathway	1,48E-02	0.8%
8202	Steroid metabolic process	5,75E-01	0.3%
10374	Stomatal complex development	1,22E-01	0.5%
10103	Stomatal complex morphogenesis	1,86E-01	0.3%
10440	Stomatal lineage progression	2,81E-01	0.1%
10118	Stomatal movement	5,49E-01	0.1%
10345	Suberin biosynthetic process	2,57E-01	0.1%
6105	Succinate metabolic process	1,66E-01	0.1%
103	Sulfate assimilation	4,43E-01	0.1%
97	Sulfur amino acid biosynthetic process	6,88E-01	0.1%
96	Sulfur amino acid metabolic process	3,27E-01	0.5%
44272	Sulfur compound biosynthetic process	7,40E-01	0.3%
6790	Sulfur metabolic process	7,11E-01	0.7%
44403	Symbiosis, encompassing mutualism through parasitism	4,76E-01	0.1%
6949	Syncytium formation	1,66E-01	0.3%
48731	System development	3,15E-05	7.4%
9627	Systemic acquired resistance	3,27E-01	0.3%
9862	Systemic acquired resistance, salicylic acid mediated signaling pathway	4,03E-01	0.1%
48657	Tapetal cell diferentiation	2,04E-01	0.1%
48658	Tapetal layer development	3,69E-01	0.1%
48656	Tapetal layer formation	2,04E-01	0.1%
48655	Tapetal layer morphogenesis	2,24E-01	0.1%
42330	Taxis	3,27E-01	0.1%
723	Telomere maintenance	4,03E-01	0.1%
32200	Telomere organization	4,03E-01	0.1%
42214	Terpene metabolic process	7,15E-01	0.1%
6721	Terpenoid metabolic process	7,64E-01	0.3%
33015	Tetrapyrrole catabolic process	4,03E-01	0.1%
33013	Tetrapyrrole metabolic process	5,92E-01	0.3%
16108	Tetraterpenoid metabolic process	6,25E-01	0.1%
9652	Thigmotropism	2,57E-01	0.1%
9888	Tissue development	2,44E-02	2.6%

6350	Transcription	5,15E-01	0.7%
42991	Transcription factor import into nucleus	2,81E-01	0.1%
42793	Transcription from plastid promoter	2,04E-01	0.1%
6351	Transcription, DNA-dependent	8,58E-01	0.1%
6412	Translation	1,00E+00	1.0%
7169	Transmembrane receptor protein tyrosine kinase signaling pathway	2,78E-04	2.1%
6810	Transport	5,30E-01	7.0%
5992	Trehalose biosynthetic process	5,20E-01	0.1%
5991	Trehalose metabolic process	5,59E-01	0.1%
10026	Trichome differentiation	8,58E-01	0.1%
42939	Tripeptide transport	2,81E-01	0.1%
42780	tRNA 3'-end processing	2,24E-01	0.1%
8033	tRNA processing	4,07E-01	0.3%
6399	tRNAmetabolic process	7,88E-01	0.3%
9606	Tropism	4,96E-02	0.8%
15827	Tryptophan transport	1,07E-01	0.1%
35295	Tube development	7,64E-01	0.3%
160	Two-component signal transduction system (phosphorelay)	6,01E-01	0.3%
6511	Ubiquitin-dependent protein catabolic process	1,71E-01	1.9%
10253	UDP-rhamnose byosynthetic process	2,24E-01	0.1%
33478	UDP-rhamnose metabolic process	2,24E-01	0.1%
9826	Unidimensional cell growth	9,08E-02	1.7%
10136	Ureide catabolic process	1,66E-01	0.1%
10135	Ureide metabolic process	1,66E-01	0.1%
9650	UV protection	2,04E-01	0.1%
10232	Vacuolar transport	1,66E-01	0.1%
10050	Vegetative phase change	3,09E-01	0.1%
10228	Vegetative to reproductive phase transition of meristem	3,22E-01	0.7%
38	Very long-chain fatty acid metabolic process	5,59E-01	0.1%
16192	Vesicle-mediated transport	9,07E-01	0.5%
9616	Virus induced gene silencing	3,09E-01	0.1%

A.1.2 Transcripts simultaneously up-regulated in 2LD:7LD:11LD samples

Table A.2: Frequency analysis of the microarray results according to biological process, of the simultaneously up-regulated transcripts in 2LD, 7 LD and 11 LD samples, using gene ontology information; performed using the Cytoscape v2.8.3 software platform (Cline *et al*, 2007) and BiNGO v2.44 plugin (Maere *et al*, 2005)

UP-REGULATED GENES (2LD:7LD:11LD)

GO-ID	BIOLOGICAL PROCESS	p-val	Freq
9738	Abscisic acid mediated signaling pathway	2,10E-01	0.5%
9687	Abscisic acid metabolic process	5,37E-01	0.1%
9838	Abscission	5,53E-01	0.1%
6083	Acetate metabolic process	1,73E-01	0.1%
46356	Acetyl-CoA catabolic process	8,08E-02	0.4%
6084	Acetyl-CoA metabolic process	1,71E-01	0.4%
30036	Actin cytoskeleton organization	4,80E-01	0.2%
7015	Actin filament organization	5,99E-01	0.1%
30048	Actin filament-based movement	5,29E-01	0.1%
30029	Actin filament-based process	2,47E-01	0.5%
46463	Acylglycerol biosynthetic process	4,37E-02	0.2%
46464	Acylglycerol catabolic process	2,11E-01	0.1%
6639	Acylglycerol metabolic process	2,29E-02	0.4%
46086	Adenosine biosynthetic process	1,73E-01	0.1%
46085	Adenosine metabolic process	1,73E-01	0.1%
6169	Adenosine salvage	1,73E-01	0.1%
9060	Aerobic respiration	1,17E-01	0.4%
7568	Aging	7,67E-02	1.0%
46165	Alcohol biosynthetic process	1,88E-01	0.4%
46164	Alcohol catabolic process	1,82E-02	1.1%
6066	Alcohol metabolic process	1,60E-03	2.6%
19405	Alditol catabolic process	5,90E-02	0.2%
19400	Alditol metabolic process	1,21E-01	0.4%
43450	Alkene biosynthetic process	1,21E-01	0.4%
256	Allantoin catabolic process	1,72E-02	0.2%
255	Allantoin metabolic process	2,87E-02	0.2%
42886	Amide transport	2,80E-01	0.1%
9309	Amine biosynthetic process	3,44E-02	1.7%
9310	Amine catabolic process	1,08E-01	0.5%
9308	Amine metabolic process	1,72E-02	3.3%
15837	Amine transport	6,87E-01	0.2%
43102	Amino acid salvage	1,12E-01	0.2%
6865	Amino acid transport	6,81E-01	0.2%
10021	Amylopectin biosynthetic process	6,45E-03	0.4%
48532	Anatomical structure arrangement	4,98E-01	0.2%
48856	Anatomical structure development	1,49E-01	7.9%
48646	Anatomical structure formation involved in morphogenesis	9,72E-01	0.1%
9653	Anatomical structure morphogenesis	6,11E-01	2.3%
48466	Androecium development	8,52E-01	0.1%
6820	Anion transport	9,07E-01	0.1%

9901	Anther dehiscence	4,26E-01	0.1%
48653	Anther development	7,24E-01	0.1%
43288	Apocarotenoid metabolic process	5,37E-01	0.1%
6915	Apoptosis	9,87E-01	0.1%
48700	Aquisition of desiccation tolerance	1,73E-01	0.1%
19566	Arabinose metabolic process	2,11E-01	0.1%
6527	Arginine catabolic process	1,73E-01	0.1%
19544	Arginine catabolic process to glutamate	1,18E-01	0.1%
6525	Arginine metabolic process	3,59E-01	0.1%
9073	Aromatic amino acid family biosynthetic process	7,70E-01	0.1%
9095	Aromatic amino acid family biosynthetic process, prephenate pathway	4,09E-01	0.1%
9072	Aromatic amino acid family metabolic process	8,20E-01	0.1%
19438	Aromatic compound biosynthetic process	4,34E-01	1.0%
9067	Aspartate family amino acid biosynthetic process	2,11E-01	0.4%
9066	Aspartate family amino acid metabolic process	7,87E-02	0.8%
6754	ATP biosynthetic process	2,63E-02	1.0%
46034	ATP metabolic process	2,63E-02	1.0%
42773	ATP synthesis coupled electron transport	5,85E-01	0.1%
15986	ATP synthesis coupled proton transport	9,50E-02	0.5%
43044	ATP-dependent chromatin remodling	2,11E-01	0.1%
6914	Autophagy	5,85E-01	0.1%
9851	Auxin biosynthetic process	5,85E-01	0.1%
10252	Auxin homeostasis	2,36E-01	0.2%
9734	Auxin mediated signaling pathway	3,84E-01	0.2%
9850	Auxin metabolic process	7,53E-01	0.1%
9926	Auxin polar transport	3,23E-01	0.4%
60918	Auxin transport	3,34E-01	0.4%
6284	Base-excision repair	2,47E-01	0.2%
10540	Basipetal auxin transport	3,33E-01	0.1%
65007	Biological regulation	9,50E-02	17.4%
9058	Biosynthetic process	1,41E-01	13.9%
9083	Branched chain family amino acid catabolic process	5,90E-02	0.2%
9081	Branched chain family amino acid metabolic process	1,42E-01	0.4%
9742	Brassinosteroid mediated signaling pathway	2,10E-01	0.4%
15740	C4-dicarboxylate transport	2,80E-01	0.1%
55074	Calcium ion homeostasis	6,42E-01	0.1%
6816	Calcium ion transport	4,81E-01	0.1%
19722	Calcium-mediated signaling	4,98E-01	0.1%
52543	Callose deposition in cell wall	5,37E-01	0.1%
52545	Callose localization	5,71E-01	0.1%
16051	Carbohydrate biosynthetic process	6,57E-02	2.0%
16052	Carbohydrate catabolic process	1,32E-04	2.2%
9756	Carbohydrate mediated signaling	2,51E-02	0.7%
5975	Carbohydrate metabolic process	1,50E-04	7.1%
46835	Carbohydrate phosphorilation	2,11E-01	0.1%
8643	Carbohydrate transport	4,18E-01	0.2%
46394	Carboxylic acid biosynthetic process	1,18E-01	2.3%
46395	Carboxylic acid catabolic process	4,38E-02	1.0%
19752	Carboxylic acid metabolic process	3,96E-04	5.8%
46942	Carboxylic acid transport	3,33E-01	0.5%
16120	Carotene biosynthetic process	3,33E-01	0.1%

16121	Carotene catabolic process	2,11E-01	0.1%
16119	Carotene metabolic process	1,66E-01	0.2%
16117	Carotenoid biosynthetic process	2,73E-01	0.2%
16118	Carotenoid catabolic process	2,11E-01	0.1%
16116	Carotenoid metabolic process	1,71E-01	0.4%
48440	Carpel development	2,23E-01	0.5%
9056	Catabolic process	5,24E-05	6.6%
55080	Cation homeostasis	5,29E-01	0.4%
6812	Cation transport	1,04E-01	2.2%
7154	Cell communication	5,52E-01	1.0%
7049	Cell cycle	6,01E-01	0.7%
22403	Cell cycle phase	3,34E-01	0.5%
22402	Cell cycle process	6,14E-01	0.5%
8219	Cell death	9,12E-01	0.5%
48468	Cell development	7,31E-01	0.7%
30154	Cell differentiation	9,18E-01	0.8%
51301	Cell division	9,27E-01	0.1%
16049	Cell growth	1,86E-01	1.7%
48469	Cell maturation	7,81E-01	0.1%
902	Cell morphogenesis	2,47E-01	1.6%
904	Cell morphogenesis involved in differentiation	5,93E-01	0.5%
8283	Cell proliferation	4,18E-01	0.2%
8037	Cell recognition	6,42E-01	0.1%
45454	Cell redox homeostasis	1,79E-01	0.7%
7166	Cell surface receptor linked signaling pathway	9,15E-01	0.2%
9932	Cell tip growth	7,96E-01	0.2%
42546	Cell wall biogenesis	5,46E-01	0.4%
42545	Cell wall modification	9,89E-01	0.1%
71555	Cell wall organization	1,00E+00	0.1%
71554	Cell wall organization or biogenesis	9,72E-01	0.5%
52386	Cell wall thickening	5,53E-01	0.1%
6081	Cellular aldehyde metabolic process	5,71E-01	0.1%
43449	Cellular alkene metabolic process	1,21E-01	0.4%
43605	Cellular amide catabolic process	1,72E-02	0.2%
43603	Cellular amide metabolic process	7,67E-02	0.2%
44106	Cellular amine metabolic process	2,87E-02	2.9%
6519	Cellular amino acid and derivative metabolic process	1,72E-02	4.1%
8652	Cellular amino acid biosynthetic process	1,18E-01	1.3%
9063	Cellular amino acid catabolic process	9,50E-02	0.5%
42398	Cellular amino acid derivative biosynthetic process	1,36E-01	1.4%
42219	Cellular amino acid derivative catabolic process	5,99E-01	0.1%
6575	Cellular amino acid derivative metabolic process	4,32E-02	2.2%
6520	Cellular amino acid metabolic process	1,12E-01	2.3%
6725	Cellular aromatic compound metabolic process	1,41E-01	2.2%
42401	Cellular biogenic amine biosynthetic process	2,36E-01	0.4%
6576	Cellular biogenic amine metabolic process	1,86E-01	0.5%
44249	Cellular biosynthetic process	2,12E-01	12.6%
6874	Cellular calcium ion homeostasis	6,42E-01	0.1%
34637	Cellular carbohydrate biosynthetic process	5,25E-02	1.7%
44275	Cellular carbohydrate catabolic process	1,90E-04	2.0%
44262	Cellular carbohydrate metabolic process	3,56E-05	5.1%

44248	Cellular catabolic process	2,63E-02	4.1%
30003	Cellular cation homeostasis	4,63E-01	0.4%
70882	Cellular cell wall organization or biogenesis	6,02E-01	0.4%
55082	Cellular chemical homeostasis	5,37E-01	0.4%
22607	Cellular component assembly	3,08E-01	1.6%
44085	Cellular component biogenesis	6,22E-01	2.0%
22411	Cellular component disassembly	2,18E-01	0.2%
32989	Cellular component morphogenesis	3,13E-01	1.6%
16043	Cellular component organization	2,51E-02	6.6%
48869	Cellular developmental process	6,57E-01	1.9%
30005	Cellular di-,tri-valent inorganic cation homeostasis	5,37E-01	0.2%
6073	Cellular glucan metabolic process	3,56E-05	2.3%
19725	Cellular homeostasis	2,17E-01	1.1%
34754	Cellular hormone metabolic process	7,24E-01	0.1%
6873	Cellular ion homeostasis	5,36E-01	0.4%
42180	Cellular ketone metabolic process	1,68E-04	6.1%
44242	Cellular lipid catabolic process	1,14E-01	0.7%
44255	Cellular lipid metabolic process	1,44E-02	3.6%
51641	Cellular localization	2,63E-02	3.5%
34622	Cellular macromolecular complex assembly	5,85E-01	0.7%
34623	Cellular macromolecular complex disassembly	1,66E-01	0.2%
34621	Cellular macromolecular complex subunit organization	3,74E-01	1.0%
34645	Cellular macromolecule biosynthetic process	1	3.5%
44265	Cellular macromolecule catabolic process	8,15E-01	1.0%
70727	Cellular macromolecule localization	4,76E-02	2.3%
44260	Cellular macromolecule metabolic process	9,58E-01	14.3%
30026	Cellular manganese ion homeostasis	2,47E-01	0.1%
16044	Cellular membrane organization	5,36E-01	0.4%
43094	Cellular metabolic compound salvage	8,08E-02	0.7%
44237	Cellular metabolic process	5,48E-04	31.2%
6875	Cellular metal ion homeostasis	5,05E-01	0.2%
44271	Cellular nitrogen compound biosynthetic process	6,45E-03	3.6%
44270	Cellular nitrogen compound catabolic process	1,21E-01	0.4%
34641	Cellular nitrogen compound metabolic process	3,25E-03	10.4%
33692	Cellular polysaccharide biosynthesic process	2,63E-02	1.3%
44247	Cellular polysaccharide catabolic process	3,32E-03	0.7%
44264	Cellular polysaccharide metabolic process	2,94E-04	2.3%
9987	Cellular process	8,77E-05	41.8%
44257	Cellular protein catabolic process	9,27E-01	0.7%
43623	Cellular protein complex assemby	7,81E-01	0.2%
43624	Cellular protein complex disassembly	1,66E-01	0.2%
34613	Cellular protein localization	5,31E-02	2.2%
44267	Cellular protein metabolic process	1	8.5%
45333	Cellular respiration	1,26E-01	0.7%
71214	Cellular response to abiotic stimulus	2,41E-01	0.5%
71215	Cellular response to abscisic acid stimulus	2,10E-01	0.5%
71365	Cellular response to auxin stimulus	4,09E-01	0.2%
71367	Cellular response to brassinosteroid stimulus	2,10E-01	0.4%
71322	Cellular response to carbohydrate stimulus	2,51E-02	0.7%
70887	Cellular response to chemical stimulus	3,59E-02	3.0%
71495	Cellular response to endogenous stimulus	7,28E-02	2.2%

71369	Cellular response to ethylene stimulus	3,11E-01	0.4%
71496	Cellular response to external stimulus	3,35E-01	0.8%
31668	Cellular response to extracellular stimulus	3,35E-01	0.8%
71370	Cellular response to gibberellin stimulus	6,62E-01	0.1%
71333	Cellular response to glucose stimulus	2,47E-01	0.1%
34605	Cellular response to heat	2,80E-01	0.1%
71331	Cellular response to hexose stimulus	7,67E-02	0.2%
32870	Cellular response to hormone stimulus	1,12E-01	1.9%
70301	Cellular response to hydrogen peroxide	3,33E-01	0.1%
71395	Cellular response to jasmonic acid stimulus	4,66E-01	0.2%
71482	Cellular response to light stimulus	3,11E-01	0.4%
71326	Cellular response to monosaccaride stimulus	7,67E-02	0.2%
43562	Cellular response to nitrogen levels	2,18E-01	0.2%
31669	Cellular response to nutrient levels	4,11E-01	0.7%
71310	Cellular response to organic substance	4,35E-02	2.7%
71470	Cellular response to osmotic stress	4,09E-01	0.1%
34599	Cellular response to oxidative stress	5,29E-01	0.1%
16036	Cellular response to phosphate starvation	7,74E-01	0.2%
71478	Cellular response to radiation	3,11E-01	0.4%
34614	Cellular response to reactive oxygen species	4,98E-01	0.1%
71491	Cellular response to red ligh	1,12E-01	0.2%
71489	Cellular response to red or far-red ligh	2,66E-01	0.4%
71446	Cellular response to salicylic acid stimulus	3,36E-01	0.2%
71472	Cellular response to salt stress	3,84E-01	0.1%
9267	Cellular response to starvation	8,56E-01	0.2%
71383	Cellular response to steroid hormone stimulus	2,10E-01	0.4%
51716	Cellular response to stimulus	2,51E-02	5.4%
33554	Cellular response to stress	3,66E-01	2.0%
42631	Cellular response to water deprivation	4,26E-01	0.1%
30244	Cellulose biosynthetic process	6,83E-01	0.1%
30243	Cellulose metabolic process	7,15E-01	0.1%
48878	Chemical homeostasis	4,18E-01	0.7%
51026	Chiasma assembly	4,92E-02	0.4%
15994	Chlorophyll metabolic process	7,70E-01	0.1%
9658	Chloroplast organization	9,00E-01	0.1%
31425	Chloroplast RNA processing	3,59E-01	0.1%
46417	Chorismate metabolic process	7,70E-01	0.1%
31497	Chromatin assembly	6,22E-01	0.2%
6333	Chromatin assembly or disassembly	7,29E-01	0.2%
16568	Chromatin modification	4,16E-02	1.1%
6325	Chromatin organization	6,57E-02	1.6%
6338	Chromatin remodling	2,05E-01	0.2%
6342	Chromatin silencing	5,37E-01	0.1%
51276	Chromosome organization	2,63E-02	2.2%
70192	Chromosome organization involved in meiosis	2,63E-02	0.5%
7059	Chromosome segregation	3,08E-01	0.2%
10617	Circadian regulation of calcium oscillation	2,11E-01	0.1%
7623	Circadian rhythm	3,44E-02	0.8%
6101	Citrate metabolic process	2,11E-01	0.1%
15937	Coenzyme A biosynthetic process	3,33E-01	0.1%
15936	Coenzyme A metabolic process	3,33E-01	0.1%

9108	Coenzyme biosynthetic process	1,69E-03	1.4%
9109	Coenzyme catabolic process	3,31E-02	0.5%
6732	Coenzyme metabolic process	1,23E-05	2.7%
51188	Cofactor biosynthetic process	6,35E-03	1.9%
51187	Cofactor catabolic process	9,50E-02	0.5%
51186	Cofactor metabolic process	3,56E-05	3.3%
48825	Cotyledon development	6,73E-01	0.1%
16569	Covalent chromatin modification	1,50E-01	0.7%
80051	Cutin transport	1,18E-01	0.1%
19344	Cysteine biosynthetic process	4,63E-01	0.1%
6534	Cysteine metabolic process	4,63E-01	0.1%
9690	Cytokinin metabolic process	6,11E-01	0.1%
7010	Cytoskeleton organization	6,57E-01	0.4%
30705	Cytoskeleton-dependent intracellular transport	5,29E-01	0.1%
51480	Cytosolic calcium ion homeostasis	2,47E-01	0.1%
16265	Death	9,12E-01	0.5%
6952	Defense response	8,05E-01	2.4%
52542	Defense response by callose deposition	5,37E-01	0.1%
52544	Defense response by callose deposition in cell wall	5,14E-01	0.1%
52482	Defense response by cell wall thickening	5,14E-01	0.1%
42742	Defense response to bacterium	2,80E-01	1.3%
9816	Defense response to bacterium, incompatible interaction	6,55E-01	0.1%
50832	Defense response to fungus	5,86E-01	0.5%
9817	Defense response to fungus, incompatible interaction	4,38E-01	0.2%
2213	Defense response to insect	3,84E-01	0.1%
2229	Defense response to oomycetes	3,84E-01	0.1%
51607	Defense response to virus	5,53E-01	0.1%
9814	Defense response, incompatible interaction	3,59E-01	0.7%
9900	Dehiscence	2,05E-01	0.2%
16311	Dephosphorylation	1,12E-02	1.1%
9582	Detection of abiotic stimulus	2,36E-01	0.2%
5513	Detection of calcium ion	3,33E-01	0.1%
9593	Detection of chemical stimulus	6,37E-02	0.4%
9726	Detection of endogenous stimulus	3,08E-01	0.1%
9727	Detection of ethylene stimulus	2,11E-01	0.1%
9581	Detection of external stimulus	2,98E-01	0.2%
9590	Detection of gravity	1,73E-01	0.1%
9720	Detection of hormone stimulus	3,08E-01	0.1%
9583	Detection of light stimulus	4,81E-01	0.1%
9594	Detection of nutrient	1,18E-01	0.1%
51606	Detection of stimulus	4,54E-02	0.7%
48588	Developmental cell growth	8,15E-01	0.2%
48589	Developmental growth	3,08E-01	1.3%
60560	Developmental growth involved in morphogenesis	2,28E-01	1.3%
21700	Developmental maturation	8,49E-01	0.1%
32502	Developmental process	1,80E-01	9.8%
55066	Di-, tri-valent inorganic cation homeostasis	6,14E-01	0.2%
15674	Di-, tri-valent inorganic cation transporte	7,44E-01	0.1%
43648	Dicarboxylic acid metabolic process	4,18E-01	0.4%
6835	Dicarboxylic acid transport	2,80E-01	0.1%
46351	Disaccharide biosynthetic process	3,84E-01	0.2%

46352	Disaccharide catabolic process	5,90E-02	0.2%
5984	Disaccharide metabolic process	2,63E-02	0.8%
16102	Diterpenoid biosynthetic process	5,37E-01	0.1%
16101	Diterpenoid metabolic process	6,23E-01	0.1%
70838	Divalent metal ion transport	5,14E-01	0.1%
6305	DNA alkylation	4,18E-01	0.2%
6308	DNA catabolic process	3,33E-01	0.1%
738	DNA catabolic process, exonucleolytic	1,73E-01	0.1%
71103	DNA conformation change	1,66E-01	0.8%
729	DNA double-strand break processing	1,73E-01	0.1%
9294	DNA mediated transformation	1,51E-01	0.4%
6259	DNA metabolic process	3,84E-01	1.7%
6306	DNA methylation	4,18E-01	0.2%
6304	DNA modification	4,26E-01	0.2%
6323	DNA packaging	6,42E-01	0.2%
6310	DNA recombination	3,54E-01	0.4%
6281	DNA repair	4,63E-01	0.8%
6260	DNA replication	9,64E-01	0.1%
6265	DNA topological change	1,97E-02	0.5%
22611	Dormancy process	4,98E-01	0.1%
6302	Double-strand break repair	6,23E-01	0.1%
6855	Drug transmembrane transport	6,42E-01	0.1%
15893	Drug transport	6,55E-01	0.1%
7398	Ectoderm development	7,97E-01	0.4%
22900	Electron transport chain	3,84E-01	0.4%
9553	Embryo sac development	9,49E-01	0.1%
9790	Embryonic development	5,48E-01	2.0%
9793	Embryonic development ending in seed dormancy	3,90E-01	2.0%
6897	Endocytosis	1,49E-01	0.2%
10256	Endomembrane system organization	1,18E-01	0.1%
8333	Endosome to lysosome transport	2,47E-01	0.1%
16197	Endosome transport	4,81E-01	0.1%
15985	Energy coupled proton transport, down electrochemical gradient	9,50E-02	0.5%
15980	Energy derivation by oxidation of organic compounds	1,26E-01	0.7%
9649	Entrainment of circadian clock	2,47E-01	0.1%
7167	Enzyme linked receptor protein signaling pathway	8,56E-01	0.2%
9913	Epidermal cell differentiation	7,90E-01	0.4%
8544	Epidermis development	7,97E-01	0.4%
6888	ER to Golgi vesicle-mediated transport	4,45E-01	0.1%
51234	Establishment of localization	3,56E-05	12.0%
51649	Establishment of localization in cell	2,50E-02	3.3%
45184	Establishment of protein localization	7,16E-02	2.6%
42439	Ethanolamine and derivative metabolic process	3,84E-01	0.1%
9693	Ethylene biosynthetic process	1,18E-01	0.4%
9873	Ethylene mediated signaling pathway	3,08E-01	0.4%
9692	Ethylene metabolic process	1,18E-01	0.4%
6887	Exocytosis	6,62E-01	0.1%
45337	Farnesyl diphosphate biosynthetic process	1,73E-01	0.1%
45338	Farnesyl diphosphate metabolic process	2,80E-01	0.1%
42362	Fat-soluble vitamin biosynthetic process	4,63E-01	0.1%
6775	Fat-soluble vitamin metabolic process	4,63E-01	0.1%

6635	Fatty acid beta-oxidation	1,42E-01	0.4%
6633	Fatty acid biosynthetic process	3,59E-01	0.7%
9062	Fatty acid catabolic process	1,79E-01	0.4%
6631	Fatty acid metabolic process	2,11E-01	1.3%
19395	Fatty acid oxidation	1,63E-01	0.4%
15908	Fatty acid transport	1,73E-01	0.1%
9813	Flavonoid biosynthetic process	5,30E-01	0.2%
9812	Flavonoid metabolic process	5,72E-01	0.2%
10227	Floral organ abscission	5,29E-01	0.1%
48437	Floral organ development	5,53E-01	0.7%
48438	Floral whorl development	4,52E-01	0.7%
9908	Flower development	5,18E-01	1.1%
42044	Fluid transport	1,31E-01	0.2%
9396	Folic acid and derivative biosynthetic process	4,92E-02	0.4%
6760	Folic acid and derivative metabolic process	1,17E-02	0.7%
48859	Formation of anatomical boundary	3,84E-01	0.1%
10160	Formation of organ boundary	3,84E-01	0.1%
10047	Fruit dehiscence	3,59E-01	0.1%
10154	Fruit development	3,08E-01	2.6%
42353	Fucose biosynthetic process	2,47E-01	0.1%
6004	Fucose metabolic process	2,47E-01	0.1%
19375	Galactolipid biosynthetic process	3,33E-01	0.1%
19374	Galactolipid metabolic process	3,33E-01	0.1%
6012	Galactose metabolic process	3,59E-01	0.1%
48229	Gametophyte development	9,72E-01	0.4%
10467	Gene expression	1	3.3%
16458	Gene silencing	9,87E-01	0.1%
6091	Generation of precursor metabolites and energy	9,68E-03	2.3%
71514	Genetic imprinting	4,45E-01	0.1%
9292	Genetic transfer	1,51E-01	0.4%
9740	Gibberellic acid mediated signaling pathway	6,62E-01	0.1%
9686	Gibberellin biosynthetic process	5,29E-01	0.1%
10476	Gibberellin mediated signaling pathway	6,92E-01	0.1%
9685	Gibberellin metabolic process	6,11E-01	0.1%
9250	Glucan biosynthetic process	3,66E-03	1.3%
9251	Glucan catabolic process	2,63E-03	0.7%
44042	Glucan metabolic process	3,56E-05	2.3%
6094	Gluconeogenesis	2,47E-01	0.1%
6007	Glucose catabolic process	1,45E-01	0.7%
10255	Glucose mediated signaling pathway	2,47E-01	0.1%
6006	Glucose metabolic process	8,19E-02	0.8%
6536	Glutamate metabolic process	1,87E-01	0.2%
9084	Glutamine family amino acid biosynthetic process	1,01E-01	0.4%
9065	Glutamine family amino acid catabolic process	9,50E-02	0.2%
9064	Glutamine family amino acid metabolic process	1,08E-01	0.5%
6751	Glutathione catabolic process	2,47E-01	0.1%
6749	Glutathione metabolic process	1,49E-01	0.2%
34635	Glutathione transport	2,11E-01	0.1%
19682	Glyceraldehyde-3-phosphate metabolic process	3,59E-01	0.1%
19563	Glycerol catabolic process	5,90E-02	0.2%
46504	Glycerol ether biosynthetic process	4,37E-02	0.2%

44269	Glycerol ether catabolic process	2,11E-01	0.1%
6662	Glycerol ether metabolic process	2,99E-02	0.4%
6071	Glycerol metabolic process	1,18E-01	0.4%
45017	Glycerolipid biosynthetic process	1,71E-01	0.4%
46503	Glycerolipid catabolic process	2,11E-01	0.1%
46486	Glycerolipid metabolic process	1,06E-01	0.7%
6127	Glycerolphosphate shuttle	1,18E-01	0.1%
46474	Glycerophospholipid biosynthetic process	6,11E-01	0.1%
6650	Glycerophospholipid metabolic process	5,05E-01	0.2%
9247	Glycolipid biosynthetic process	4,98E-01	0.1%
6664	Glycolipid metabolic process	5,37E-01	0.1%
6096	Glycolysis	2,57E-01	0.4%
9101	Glycoprotein biosynthetic process	8,62E-01	0.1%
6516	Glycoprotein catabolic process	2,47E-01	0.1%
9100	Glycoprotein metabolic process	6,42E-01	0.2%
16138	Glycoside biosynthetic process	6,42E-01	0.2%
16139	Glycoside catabolic process	5,29E-01	0.1%
16137	Glycoside metabolic process	2,63E-01	0.7%
70085	Glycosylation	8,62E-01	0.1%
10111	Glyoxysome organization	1,18E-01	0.1%
51645	Golgi localization	2,11E-01	0.1%
7030	Golgi organization	3,08E-01	0.1%
6896	Golgi to vacuole transport	5,90E-02	0.2%
48193	Golgi vesicle transport	4,54E-02	0.7%
9630	Gravitropism	1,72E-02	0.8%
6752	Group transfer coenzyme metabolic process	1,12E-02	0.8%
40007	Growth	1,17E-01	2.2%
8617	Guanosine metabolic process	2,11E-01	0.1%
15969	Guanosine tetraphosphate metabolic process	1,73E-01	0.1%
48467	Gynoecium development	2,62E-01	0.5%
35315	Hair cell differentiation	4,29E-01	0.4%
10286	Heat acclimation	2,11E-01	0.2%
6783	Heme biosynthetic process	3,59E-01	0.1%
42168	Heme metabolic process	4,26E-01	0.1%
18130	Heterocycle biosynthetic process	1,19E-01	1.1%
46700	Heterocycle catabolic process	6,37E-02	0.5%
46483	Heterocycle metabolic process	1,68E-04	4.1%
9747	Hexokinase-dependent signaling	1,73E-01	0.1%
19319	Hexose biosynthetic process	2,05E-01	0.2%
19320	Hexose catabolic process	7,60E-02	0.8%
9757	Hexose mediated signaling	7,67E-02	0.2%
19318	Hexose metabolic process	2,51E-02	1.3%
15712	Hexose phosphate transport	2,11E-01	0.1%
8645	Hexose transport	2,47E-01	0.1%
16575	Histone deacetylation	4,63E-01	0.1%
51567	Histone H3-K9 methylation	3,08E-01	0.1%
34968	Histone lysine methylation	4,81E-01	0.1%
16571	Histone methylation	3,36E-01	0.2%
16570	Histone modification	1,32E-01	0.7%
42592	Homeostatic process	2,11E-01	1.4%
42446	Hormone byosynthetic process	7,77E-01	0.1%

42445	Hormone metabolic process	7,29E-01	0.2%
9914	Hormone transport	3,43E-01	0.4%
9755	Hormone-mediated signaling pathway	1,06E-01	1.9%
34050	Host programmed cell death induced by symbiont	4,09E-01	0.2%
50665	Hydrogen peroxide biosynthetic process	2,11E-01	0.1%
42744	Hydrogen peroxide catabolic process	3,08E-01	0.1%
42743	Hydrogen peroxide metabolic process	1,18E-01	0.2%
6818	Hydrogen transport	5,90E-02	0.7%
6972	Hyperosmotic response	1,01E-01	0.7%
42538	Hyperosmotic salinity response	6,37E-02	0.7%
2252	Immune efector process	6,11E-01	0.1%
6955	Immune response	7,90E-01	1.0%
2376	Immune system process	8,18E-01	1.0%
6917	Induction of apoptosis	2,47E-01	0.1%
12502	Induction of programmed cell death	2,80E-01	0.1%
45087	Innate immune response	8,49E-01	0.8%
15698	Inorganic anion transport	8,32E-01	0.1%
7243	Intracellular protein kinase cascade	4,26E-01	0.1%
6886	Intracellular protein transport	4,18E-02	2.2%
35556	Intracellular signal transduction	6,10E-01	0.4%
23034	Intracellular signaling pathway	7,28E-01	0.7%
46907	Intracellular transport	1,06E-02	3.2%
6891	Intra-Golgi vesicle-mediated transport	4,26E-01	0.1%
50801	Ion homeostasis	6,14E-01	0.4%
34220	Ion transmembrane transport	1,25E-01	0.5%
6811	Ion transport	1,86E-01	2.3%
18283	Iron incorporation into metallo-sulfur cluster	1,18E-01	0.1%
16226	Iron-sulfur cluster assembly	2,57E-01	0.2%
6102	Isocitrate metabolic process	1,49E-01	0.2%
9240	Isopentenyl diphosphate biosynthetic process	4,09E-01	0.1%
19288	Isopentenyl diphosphate biosynthetic process, mevalonate-independent pathway	3,33E-01	0.1%
46490	Isopentenyl diphosphate metabolic process	4,09E-01	0.1%
8299	Isoprenoid biosynthetic process	3,54E-01	0.7%
8300	Isoprenoid catabolic process	4,26E-01	0.1%
6720	Isoprenoid metabolic process	2,16E-01	1.0%
9861	Jasmonic acid and ethylene-dependent systemic resistance	4,26E-01	0.1%
9871	Jasmonic acid and ethylene-dependent systemic resistance, ethylene mediated signaling pathway	2,11E-01	0.1%
9695	Jasmonic acid biosynthetic process	2,57E-01	0.2%
9867	Jasmonic acid mediated signaling pathway	4,66E-01	0.2%
9694	Jasmonic acid metabolic process	2,98E-01	0.2%
48527	Lateral root development	8,62E-01	0.1%
48366	Leaf development	5,35E-01	1.0%
9965	Leaf morphogenesis	5,86E-01	0.5%
10150	Leaf senescence	6,37E-02	0.5%
10305	Leaf vascular tissue pattern	5,37E-01	0.1%
6552	Leucine catabolic process	4,37E-02	0.2%
6551	Leucine metabolic process	1,73E-01	0.2%
9809	Lignin biosynthetic process	3,52E-01	0.2%
9808	Lignin metabolic process	5,14E-01	0.2%
8610	Lipid biosynthetic process	1,06E-01	2.4%

16042	Lipid catabolic process	2,63E-02	1.0%
30259	Lipid glycosylation	1,73E-01	0.1%
10876	Lipid localization	4,62E-01	0.8%
6629	Lipid metabolic process	1,72E-02	4.6%
30258	Lipid modification	2,63E-02	0.7%
34440	Lipid oxidation	1,63E-01	0.4%
19915	Lipid storage	5,14E-01	0.1%
6869	Lipid transport	5,29E-01	0.7%
9107	Lipoate biosynthetic process	2,47E-01	0.1%
9106	Lipoate metabolic process	2,47E-01	0.1%
9105	Lipoic acid biosynthetic process	2,80E-01	0.1%
273	Lipoic acid metabolic process	3,08E-01	0.1%
71265	L-methionine biosynthetic process	1,18E-01	0.2%
71267	L-methionine salvage	1,12E-01	0.2%
19509	L-methionine salvage from methylthioadenosine	1,12E-01	0.2%
51179	Localization	3,56E-05	12.3%
15909	Long-chain fatty acid transport	1,18E-01	0.1%
48571	Long-day photoperiodism	2,80E-01	0.1%
48574	Long-day photoperiodism, flowering	2,80E-01	0.1%
7041	Lysosomal transport	2,47E-01	0.1%
279	M phase	2,55E-01	0.5%
51327	M phase of meiotic cell cycle	1,48E-01	0.5%
65003	Macromolecular complex assembly	4,16E-01	1.1%
43933	Macromolecular complex subunit organization	2,62E-01	1.4%
9059	Macromolecule biosynthetic process	1	3.5%
9057	Macromolecule catabolic process	2,55E-01	2.0%
43413	Macromolecule glycosylation	8,62E-01	0.1%
33036	Macromolecule localization	9,71E-02	3.6%
43170	Macromolecule metabolic process	9,48E-01	16.2%
43414	Macromolecule methylation	5,13E-01	0.4%
43412	Macromolecule modification	8,57E-01	5.4%
32984	Macromoleular complex disassembly	1,73E-01	0.2%
10216	Maintenance of DNA methylation	3,33E-01	0.1%
10076	Maintenance of floral meristem identity	3,08E-01	0.1%
34090	Maintenance of meiotic sister chromatid cohesion	1,18E-01	0.1%
10074	Maintenance of meristem identity	5,53E-01	0.1%
34086	Maintenance of sister chromatid cohesion	1,18E-01	0.1%
6108	Malate metabolic process	1,31E-01	0.2%
15743	Malate transport	2,80E-01	0.1%
25	Maltose catabolic process	1,18E-01	0.1%
23	Maltose metabolic process	2,11E-01	0.1%
55071	Manganese ion homeostasis	3,08E-01	0.1%
7126	Meiosis	1,48E-01	0.5%
7127	Meiosis I	7,67E-02	0.5%
51321	Meiotic cell cycle	2,47E-01	0.5%
45132	Meiotic chromosome segregation	4,26E-01	0.1%
42138	Meiotic DNA double-strand break formation	5,90E-02	0.2%
706	Meiotic DNA double-strand break processing	1,73E-01	0.1%
51177	Meiotic sister chromatid cohesion	2,80E-01	0.1%
22406	Membrane docking	6,62E-01	0.1%
10324	Membrane invagination	1,49E-01	0.2%

46467	Membrane lipid biosynthetic process	6,34E-01	0.1%
6643	Membrane lipid metabolic process	7,33E-01	0.1%
61024	Membrane organization	5,36E-01	0.4%
48507	Meristem development	6,46E-01	0.4%
10073	Meristem maintenance	8,62E-01	0.1%
9933	Meristem structural organization	4,47E-01	0.2%
8152	Metabolic process	2,63E-04	38.6%
18282	Metal incorporation into metallo-sulfur cluster	1,18E-01	0.1%
55065	Metal ion homeostasis	5,05E-01	0.2%
30001	Metal ion transport	3,74E-01	1.0%
31163	Metallo-sulfur cluster assembly	2,57E-01	0.2%
9086	Methionine biosynthetic process	8,08E-02	0.4%
6555	Methionine metabolic process	2,51E-02	0.8%
32259	Methylation	5,36E-01	0.4%
7018	Microtubule-based movement	8,08E-01	0.1%
7017	Microtubule-based process	9,72E-01	0.1%
6298	Mismatc repair	4,98E-01	0.1%
51646	Mitochondria localization	2,11E-01	0.1%
42775	Mitochondrial ATP synthesis coupled electron transport	5,85E-01	0.1%
6121	Mitochondrial electron ttransport, succinate to ubiquinone	3,33E-01	0.1%
6839	Mitochondrial transport	3,02E-01	0.4%
31930	Mitochondria-nucleus signaling pathway	2,11E-01	0.1%
7005	Mitochondrion organization	6,92E-01	0.1%
43632	Modification-dependent macromolecule catabolic process	9,10E-01	0.7%
19941	Modification-dependent protein catabolic process	9,10E-01	0.7%
32324	Molybdopterin cofactor biosynthetic process	3,08E-01	0.1%
43545	Molybdopterin cofactor metabolic process	3,08E-01	0.1%
6777	Mo-molybopterin cofactor biosynthetic process	3,08E-01	0.1%
19720	Mo-molybopterin cofactor metabolic process	3,08E-01	0.1%
32787	Monocarboxylic acid metabolic process	1,32E-02	2.9%
15718	Monocarboxylic acid transport	2,11E-01	0.1%
46364	Monosaccharide biosynthetic process	2,11E-01	0.2%
46365	Monosaccharide catabolic process	7,60E-02	0.8%
5996	Monosaccharide metabolic process	1,69E-02	1.6%
15749	Monosaccharide transport	3,33E-01	0.1%
15672	Monovalent inorganic cation transport	8,63E-02	1.1%
6402	mRNA catabolic process	4,09E-01	0.1%
16071	mRNA metabolic process	3,32E-01	0.7%
6397	mRNA processing	3,78E-01	0.5%
32504	Multicellular organism reproduction	4,18E-01	0.4%
7275	Multicellular organismal development	3,33E-01	8.3%
32501	Multicellular organismal process	2,11E-01	9.2%
9825	Multidimensional cell growth	3,08E-01	0.4%
51704	Multi-organism process	4,17E-02	4.9%
15798	Myo-inositol transport	1,73E-01	0.1%
6739	NADP metabolic process	3,33E-01	0.2%
6740	NADPH regeneration	2,98E-01	0.2%
43628	ncRNA 3'-end processing	2,47E-01	0.1%
34660	ncRNA metabolic process	9,91E-01	0.2%
34470	ncRNA processing	9,48E-01	0.2%
48519	Negative regulation of biological process	4,50E-01	1.9%

9890	Negative regulation of biosynthetic process	1,18E-01	0.8%
43086	Negative regulation of catalytic activity	8,72E-01	0.1%
10648	Negative regulation of cell communication	7,62E-01	0.1%
45596	Negative regulation of cell differentiation	6,62E-01	0.1%
8285	Negative regulation of cell proliferation	2,11E-01	0.1%
31327	Negative regulation of cellular biosynthetic process	1,18E-01	0.8%
31324	Negative regulation of cellular metabolic process	1,79E-01	0.8%
48523	Negative regulation of cellular process	3,73E-01	1.1%
32269	Negative regulation of cellular protein metabolic process	3,08E-01	0.1%
51093	Negative regulation of developmental process	1,51E-01	0.8%
10105	Negative regulation of ethylene mediated signaling pathway	4,26E-01	0.1%
9910	Negative regulation of flower development	1,12E-01	0.5%
10629	Negative regulation of gene expression	5,19E-01	0.8%
45814	Negative regulation of gene expression, epigenetic	2,98E-01	0.2%
32582	Negative regulation of gene-specific transcription	2,47E-01	0.1%
33673	Negative regulation of kinase activity	4,09E-01	0.1%
10558	Negative regulation of macromolecule biosynthetic process	1,12E-01	0.8%
10605	Negative regulation of macromolecule metabolic process	4,63E-01	1.0%
43407	Negative regulation of MAP kinase activity	1,73E-01	0.1%
9892	Negative regulation of metabolic process	5,24E-01	1.0%
44092	Negative regulation of molecular function	8,89E-01	0.1%
51172	Negative regulation of nitrogen compound metabolic process	1,91E-01	0.7%
45934	Negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	1,91E-01	0.7%
10100	Negative regulation of photomorphogenesis	3,59E-01	0.1%
48581	Negative regulation of post-embryonic development	1,18E-01	0.7%
6469	Negative regulation of protein kinase activity	4,09E-01	0.1%
51248	Negative regulation of protein metabolic process	3,08E-01	0.1%
48585	Negative regulation of response to stimulus	6,81E-01	0.2%
51253	Negative regulation of RNA metabolic process	4,93E-02	0.5%
9968	Negative regulation of signal transduction	4,63E-01	0.1%
35467	Negative regulation of signaling pathway	7,53E-01	0.1%
23057	Negative regulation of signaling process	4,63E-01	0.1%
16481	Negative regulation of transcription	1,39E-01	0.7%
16480	Negative regulation of transcription from RNA polymerase III promoter	1,18E-01	0.1%
45892	Negative regulation of transcription, DNA-dependent	4,93E-02	0.5%
51348	Negative regulation of transferase activity	4,09E-01	0.1%
17148	Negative regulation of translation	2,11E-01	0.1%
70298	Negative regulation of two-component signal transduction system (phosphorelay)	4,26E-01	0.1%
46460	Neutral lipid biosynthetic process	4,37E-02	0.2%
46461	Neutral lipid catabolic process	2,11E-01	0.1%
6638	Neutral lipid metabolic process	2,29E-02	0.4%
46496	Nicotinamide nucleotide metabolic process	3,99E-01	0.2%
19357	Nicotinate nucleotide biosynthetic process	1,73E-01	0.1%
46497	Nicotinate nucleotide metabolic process	1,73E-01	0.1%
19358	Nicotinate nucleotide salvage	1,73E-01	0.1%
6807	Nitrogen compound metabolic process	1,68E-03	11.0%
280	Nuclear division	5,85E-01	0.1%
51170	Nuclear import	7,70E-01	0.1%
398	Nuclear mRNA spicing, via spliceosome	8,01E-01	0.1%
51169	Nuclear transport	6,22E-01	0.2%

956	Nuclear-transcribed mRNA catabolic process	3,84E-01	0.1%
184	Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	2,11E-01	0.1%
90304	Nucleic acid metabolic process	4,63E-01	4.2%
90305	Nucleic acid phosphodiester bond hydrolysis	2,11E-01	0.2%
46113	Nucleobase catabolic process	2,47E-01	0.1%
9112	Nucleobase metabolic process	2,36E-01	0.2%
34404	Nucleobase, nucleoside and nucleotide biosynthetic process	2,63E-02	1.4%
34656	Nucleobase, nucleoside and nucleotide catabolic process	3,33E-01	0.1%
55086	Nucleobase, nucleoside and nucleotide metabolic process	5,21E-04	2.6%
34654	Nucleobase, nucleoside, nucleotide and nucleic acid biosynthetic process	2,63E-02	1.4%
34655	Nucleobase, nucleoside, nucleotide and nucleic acid catabolic process	3,33E-01	0.1%
6139	Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	8,08E-02	6.9%
6913	Nucleocytoplasmatic transport	6,22E-01	0.2%
9163	Nucleoside biosynthetic process	2,11E-01	0.1%
33865	Nucleoside biphosphate meabolic process	1,18E-01	0.2%
9116	Nucleoside metabolic process	1,25E-01	0.5%
9123	Nucleoside monophosphate metabolic process	4,81E-01	0.1%
6753	Nucleoside phosphate metabolic process	3,84E-03	2.0%
43174	Nucleoside salvage	1,73E-01	0.1%
9142	Nucleoside triphosphate biosynthetic process	2,74E-02	1.0%
9141	Nucleoside triphosphate metabolic process	2,74E-02	1.0%
6334	Nucleosome assembly	6,08E-01	0.2%
34728	Nucleosome organization	6,08E-01	0.2%
9165	Nucleotide biosynthetic process	3,22E-02	1.3%
9117	Nucleotide metabolic process	3,84E-03	2.0%
43173	Nucleotide salvage	2,11E-01	0.1%
6289	Nucleotide-excision repair	4,45E-01	0.1%
15780	Nucleotide-sugar transport	4,09E-01	0.1%
6857	Oligopeptide transport	6,93E-01	0.2%
9312	Oligosaccharide biosynthetic process	4,26E-01	0.2%
9313	Oligosaccharide catabolic process	5,90E-02	0.2%
9311	Oligosaccharide metabolic process	4,92E-02	0.8%
6730	One-carbon metabolic process	6,57E-01	0.4%
10199	Organ boundary specification between lateral organs and the meristem	3,33E-01	0.1%
48513	Organ development	3,56E-01	3.8%
48645	Organ formation	7,62E-01	0.1%
35265	Organ growth	3,84E-01	0.1%
9887	Organ morphogenesis	5,71E-01	0.8%
10260	Organ senescence	7,04E-02	0.5%
48285	Organelle fission	8,26E-01	0.1%
51640	Organelle localization	5,37E-01	0.1%
6996	Organelle organization	4,83E-03	4.6%
16053	Organic acid biosynthetic process	1,18E-01	2.3%
16054	Organic acid catabolic process	4,38E-02	1.0%
6082	Organic acid metabolic process	3,96E-04	5.8%
15849	Organic acid transport	3,33E-01	0.5%
15850	Organic alcohol transport	2,11E-01	0.1%
18904	Organic ether metabolic process	2,99E-02	0.4%
19637	Organophosphate metabolic process	1,18E-01	1.0%
6593	Ornithine catabolic process	1,18E-01	0.1%
6591	Ornithine metabolic process	2,11E-01	0.1%

48481	Ovule development	1,25E-01	0.5%
55114	Oxidation reduction	3,81E-01	0.8%
6119	Oxidative phosphorylation	1,14E-01	0.7%
6733	Oxidoreduction coenzyme metabolic process	5,30E-02	0.7%
43436	Oxoacid metabolic process	3,96E-04	5.8%
6800	Oxygen and reactive oxygen species metabolic process	1,73E-01	0.4%
31408	Oxylipin biosynthetic process	3,08E-01	0.2%
31407	Oxylipin metabolic process	1,73E-01	0.4%
7389	Pattern specification process	4,11E-01	0.7%
19321	Pentose metabolic process	3,17E-01	0.2%
6098	Pentose-phosphate shunt	2,81E-01	0.2%
9051	Pentose-phosphate shunt, oxidative branch	3,08E-01	0.1%
43171	Peptide catabolic process	2,47E-01	0.1%
6518	Peptide metabolic process	2,98E-01	0.2%
15833	Peptide transport	6,93E-01	0.2%
18193	Peptidyl-amino acid modification	2,57E-01	0.2%
18202	Peptidyl-histidine modification	2,11E-01	0.1%
18106	Peptidyl-histidine phosphorylation	2,11E-01	0.1%
18022	Peptidyl-lysine methylation	1,18E-01	0.1%
18205	Peptidyl-lysine modification	1,73E-01	0.1%
43574	Peroxisomal transport	3,59E-02	0.4%
60151	Peroxisome localization	2,11E-01	0.1%
7031	Peroxisome organization	5,90E-02	0.5%
9699	Phenylpropanoid biosynthetic process	5,15E-01	0.5%
9698	Phenylpropanoid metabolic process	6,63E-01	0.5%
6796	Phosphate metabolic process	4,98E-01	4.6%
6817	Phosphate transport	4,81E-01	0.1%
6656	Phosphatidylcholine biosynthetic process	2,47E-01	0.1%
46470	Phosphatidylcholine metabolic process	3,59E-01	0.1%
46488	Phosphatidylinositol metabolic process	4,63E-01	0.1%
30384	Phosphoinositide metabolic process	6,62E-01	0.1%
8654	Phospholipid biosynthetic process	1,18E-01	0.7%
6644	Phospholipid metabolic process	1,01E-01	1.0%
15914	Phospholipid transport	1,73E-01	0.2%
6793	Phosphorous metabolic process	4,99E-01	4.6%
16310	Phosphorylation	8,47E-01	3.5%
7602	Phosphotransduction	4,81E-01	0.1%
9640	Photomorphogenesis	3,23E-01	0.4%
9648	Photoperiodism	4,92E-02	0.7%
48573	Photoperiodism, flowering	1,12E-01	0.5%
9853	Photorespiration	6,62E-01	0.1%
15979	Photosynthesis	1,12E-01	1.1%
9765	Photosynthesis, light harvesting	2,73E-01	0.2%
9769	Photosynthesis, light harvesting in photosystem II	1,73E-01	0.1%
19684	Photosynthesis, light reaction	2,62E-01	0.5%
9767	Photosynthetic electron transport chain	6,34E-01	0.1%
80005	Photosystem stoichiometry adjustment	1,73E-01	0.1%
9638	Phototropism	1,66E-01	0.2%
48827	Phyllome development	4,98E-01	1.1%
42372	Phylloquinone biosynthetic process	2,80E-01	0.1%
42374	Phyloquinone metabolic process	2,80E-01	0.1%

46148	Pigment biosynthetic process	5,29E-01	0.4%
42440	Pigment metabolic process	6,33E-01	0.4%
9832	Plant-type cell wall biogenesis	4,18E-01	0.4%
71669	Plant-type cell wall organization or biogenesis	8,20E-01	0.4%
9626	Plant-type hypersensitive response	3,99E-01	0.2%
48227	Plasma membrane to endosome transport	1,18E-01	0.1%
9657	Plastid organization	5,05E-01	0.5%
9555	Pollen development	8,74E-01	0.4%
9846	Pollen germination	7,03E-01	0.1%
48868	Pollen tube development	9,61E-01	0.1%
9860	Pollen tube growth	9,07E-01	0.1%
9875	Pollen-pistil interaction	7,03E-01	0.1%
9856	Pollination	8,53E-01	0.4%
6596	Polyamine biosynthetic process	3,59E-02	0.4%
6595	Polyamine metabolic process	6,37E-02	0.4%
46174	Polyol catabolic process	5,90E-02	0.2%
19751	Polyol metabolic process	2,23E-01	0.4%
15791	Polyol transport	2,11E-01	0.1%
16094	Polyprenol biosynthetic process	3,08E-01	0.1%
16093	Polyprenol metabolic process	3,84E-01	0.1%
271	Polysaccharide biosynthetic process	3,13E-02	1.3%
272	Polysaccharide catabolic process	3,15E-03	0.8%
33037	Polysaccharide localization	5,85E-01	0.1%
5976	Polysaccharide metabolic process	3,22E-04	2.4%
6779	Porphyrin biosynthetic process	8,01E-01	0.1%
6778	Porphyrin metabolic process	6,42E-01	0.2%
9958	Positive gravitropism	1,73E-01	0.2%
35468	Positive regulaf signaling pathwaytion	2,57E-01	0.2%
48518	Positive regulation of biological process	3,05E-02	2.2%
9891	Positive regulation of biosynthetic process	2,80E-01	0.5%
10647	Positive regulation of cell communication	2,98E-01	0.2%
90068	Positive regulation of cell cycle progress	2,47E-01	0.1%
10942	Positive regulation of cell death	4,09E-01	0.1%
8284	Positive regulation of cell proliferation	3,84E-01	0.1%
31328	Positive regulation of cellular biosynthetic process	2,80E-01	0.5%
31325	Positive regulation of cellular metabolic process	3,58E-01	0.5%
48522	Positive regulation of cellular process	1,17E-01	1.3%
51094	Positive regulation of developmental process	3,67E-02	0.8%
9963	Positive regulation of flavonoid byosynthetic process	2,11E-01	0.1%
9911	Positive regulation of flower development	1,01E-01	0.5%
10628	Positive regulation of gene expression	4,11E-01	0.4%
10557	Positive regulation of macromolecule biosynthetic process	4,26E-01	0.4%
10604	Positive regulation of macromolecule metabolic process	4,81E-01	0.4%
45836	Positive regulation of meiosis	1,18E-01	0.1%
9893	Positive regulation of metabolic process	3,78E-01	0.5%
51240	Positive regulation of multicellular organismal process	9,50E-02	0.2%
51173	Positive regulation of nitrogen compound metabolic process	4,45E-01	0.4%
45935	Positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	4,39E-01	0.4%
48582	Positive regulation of post-embryonic development	2,51E-02	0.8%
43068	Positive regulation of programmed cell death	3,59E-01	0.1%
48584	Positive regulation of response to stimulus	6,22E-01	0.2%

51254	Positive regulation of RNA metabolic process	5,85E-01	0.1%
10030	Positive regulation of seed germination	9,50E-02	0.2%
45941	Positive regulation of transcription	3,84E-01	0.4%
45893	Positive regulation of transcription, DNA dependent	5,37E-01	0.1%
9789	Positive regulator of abscisic acid mediated signaling pathway	1,73E-01	0.2%
43065	Positive regulator of apoptosis	2,47E-01	0.1%
9791	Post-embryonic development	1,25E-01	5.4%
48569	Post-embryonic organ development	6,64E-01	0.8%
48528	Post-embryonic root development	8,89E-01	0.1%
6892	Post-Golgi vesicle-mediated transport	5,90E-02	0.2%
10608	Post-transcriptional regulation of gene expression	9,89E-01	0.1%
43687	Post-translational protein modification	6,42E-01	4.8%
6813	Potassium ion transport	2,92E-01	0.4%
16091	Prenol biosynthetic process	3,08E-01	0.1%
16090	Prenol metabolic process	3,84E-01	0.1%
9833	Primary cell wall biogenesis	2,47E-01	0.1%
44238	Primary metabolic process	2,79E-02	30.3%
80022	Primary root development	5,14E-01	0.1%
12501	Programed cell death	8,38E-01	0.5%
6561	Proline biosynthetic process	1,13E-02	0.4%
6562	Proline catabolic process	2,11E-01	0.1%
10133	Proline catabolic process to glutamate	1,18E-01	0.1%
6560	Proline metabolic process	2,86E-03	0.5%
51189	Prosthetic group metabolic process	3,08E-01	0.1%
8213	Protein amino acid alkylation	4,18E-01	0.2%
6476	Protein amino acid deacetylation	4,98E-01	0.1%
6470	Protein amino acid dephosphorylation	1,72E-02	1.0%
6486	Protein amino acid glycosylation	8,62E-01	0.1%
6479	Protein amino acid methylation	4,18E-01	0.2%
6493	Protein amino acid O-linked glycosylation	1,18E-01	0.1%
6468	Protein amino acid phosphorylation	9,72E-01	2.6%
30163	Protein catabolic process	9,44E-01	0.7%
6461	Protein complex assembly	5,14E-01	0.7%
70271	Protein complex biogenesis	5,14E-01	0.7%
43241	Protein complex disassembly	1,73E-01	0.2%
16579	Protein deubiquitination	2,80E-01	0.1%
6457	Protein folding	2,47E-01	1.3%
17038	Protein import	5,36E-01	0.4%
16558	Protein import into peroxisome matrix	1,12E-01	0.2%
16560	Protein import into peroxisome matrix, docking	1,18E-01	0.1%
71108	Protein K48-linked deubiquitination	1,18E-01	0.1%
70536	Protein K63-limked deubiquitination	1,18E-01	0.1%
8104	Protein localization	9,50E-02	2.6%
70585	Protein localization in mitochondrion	5,37E-01	0.1%
33365	Protein localization in organelle	9,12E-01	0.1%
19538	Protein metabolic process	1	10.2%
32446	Protein modification by small protein conjugation	9,74E-01	0.1%
70647	Protein modification by small protein conjugation or removal	9,18E-01	0.2%
70646	Protein modification by small protein removal	5,29E-01	0.1%
6464	Protein modification process	7,77E-01	5.1%
9306	Protein secretion	5,14E-01	0.1%

6605	Protein targeting	1,49E-01	1.1%
6626	Protein targeting to mitochondrion	5,37E-01	0.1%
6625	Protein targeting to peroxisome	3,59E-02	0.4%
6623	Protein targeting to vacuole	2,36E-02	0.5%
15031	Protein transport	7,16E-02	2.6%
16567	Protein ubiquitination	9,67E-01	0.1%
65004	Protein-DNA complex assembly	6,30E-01	0.2%
6508	Proteolysis	8,27E-01	2.3%
51603	Proteolysis involved in cellular protein catabolic process	9,15E-01	0.7%
15992	Proton transport	5,90E-02	0.7%
42559	Pteridine and derivative biosynthetic process	1,66E-01	0.2%
42558	Pteridine and derivative metabolic process	2,51E-02	0.5%
6144	Purine base metabolic process	4,45E-01	0.1%
42451	Purine nucleoside biosynthetic process	1,73E-01	0.1%
42278	Purine nucleoside metabolic process	2,51E-02	0.5%
9145	Purine nucleoside triphosphate biosynthetic process	2,63E-02	1.0%
9144	Purine nucleoside triphosphate metabolic process	2,63E-02	1.0%
6164	Purine nucleotide biosynthetic process	5,42E-02	1.0%
6163	Purine nucleotide metabolic process	6,64E-02	1.0%
46129	Purine ribonucleoside biosynthetic process	1,73E-01	0.1%
46128	Purine ribonucleoside metabolic process	2,51E-02	0.5%
6166	Purine ribonucleoside salvage	1,73E-01	0.1%
9206	Purine ribonucleoside triphosphate biosynthetic process	2,63E-02	1.0%
9205	Purine ribonucleoside triphosphate metabolic process	2,63E-02	1.0%
9152	Purine ribonucleotide byosynthetic process	4,14E-02	1.0%
9150	Purine ribonucleotide metabolic process	4,92E-02	1.0%
43101	Purine salvage	3,33E-01	0.1%
9446	Putrescine biosynthetic process	1,73E-01	0.1%
9445	Putrescine metabolic process	1,73E-01	0.1%
19363	Pyridine nucleotide biosynthetic process	1,49E-01	0.2%
19362	Pyridine nucleotide metabolic process	1,12E-01	0.5%
19365	Pyridine nucleotide salvage	2,11E-01	0.1%
8615	Pyridoxine biosynthetic process	2,80E-01	0.1%
8614	Pyridoxine metabolic process	2,80E-01	0.1%
6208	Pyrimidine base catabolic process	2,11E-01	0.1%
6206	Pyrimidine base metabolic process	3,08E-01	0.1%
9129	Pyrimidine nucleoside monophosphate metabolic process	1,73E-01	0.1%
6220	Pyrimidine nucleotide metabolic process	4,09E-01	0.1%
9173	Pyrimidine ribonucleoside monophosphate metabolic process	1,18E-01	0.1%
9218	Pyrimidine ribonucleotide metabolic process	2,80E-01	0.1%
6090	Pyruvate metabolic process	6,37E-02	0.4%
45426	Quinone cofactor biosynthetic process	1,73E-01	0.2%
42375	Quinone cofactor metabolic process	1,73E-01	0.2%
9956	Radial pattern formation	3,08E-01	0.1%
7131	Reciprocal meiotic recombination	2,11E-01	0.2%
48544	Recognition of pollen	6,42E-01	0.1%
10161	Red light signaling pathway	1,12E-01	0.2%
10017	Red or far-red light signaling pathway	2,66E-01	0.4%
9585	Red, far-red light phototransduction	4,81E-01	0.1%
3002	Regionalization	4,52E-01	0.5%
9787	Regulation of abscisic acid mediated signaling pathway	4,09E-01	0.2%

80143	Regulation of amino acid export	3,33E-01	0.1%
51952	Regulation of amino acid transport	3,33E-01	0.1%
51955	Regulation of amino acid transport	3,33E-01	0.1%
90066	Regulation of anatomical structure size	2,17E-01	1.7%
42981	Regulation of apoptosis	1,87E-01	0.2%
50789	Regulation of biological process	1,18E-01	14.9%
65008	Regulation of biological quality	1,18E-01	3.8%
9889	Regulation of biosynthetic process	4,27E-01	7.4%
9894	Regulation of catabolic process	2,78E-01	0.4%
50790	Regulation of catalytic activity	4,66E-01	0.7%
10646	Regulation of cell communication	1,18E-01	1.0%
51726	Regulation of cell cycle	3,94E-01	0.7%
10564	Regulation of cell cycle progress	6,42E-01	0.1%
10941	Regulation of cell death	4,18E-01	0.2%
45595	Regulation of cell differentiation	7,96E-01	0.1%
42127	Regulation of cell proliferation	3,33E-01	0.2%
8361	Regulation of cell size	2,14E-01	1.7%
31326	Regulation of cellular biosynthetic process	4,27E-01	7.4%
31329	Regulation of cellular catabolic process	2,47E-01	0.4%
32535	Regulation of cellular component size	2,17E-01	1.7%
31323	Regulation of cellular metabolic process	2,65E-01	8.6%
50794	Regulation of cellular process	1,56E-01	13.0%
32268	Regulation of cellular protein metabolic process	6,73E-01	0.1%
42752	Regulation of circadian rhythm	2,18E-01	0.2%
31347	Regulation of defense response	6,73E-01	0.2%
50688	Regulation of defense response to virus	2,11E-01	0.1%
50793	Regulation of developmental process	2,09E-01	1.9%
45995	Regulation of embryonic development	2,47E-01	0.1%
10104	Regulation of ethylene mediated signaling pathway	4,63E-01	0.1%
9962	Regulation of flavonoid biosynthetic process	4,98E-01	0.1%
9909	Regulation of flower development	8,22E-02	1.1%
10468	Regulation of gene expression	4,40E-01	7.9%
6349	Regulation of gene expression by genetic imprinting	4,45E-01	0.1%
40029	Regulation of gene expression, epigenetic	6,29E-01	0.7%
43467	Regulation of generation of precursor metabolites an energy	4,98E-01	0.1%
32583	Regulation of gene-specific transcription	5,14E-01	0.1%
33124	Regulation of GTP catabolic process	1,88E-01	0.4%
43087	Regulation of GTPase activity	1,88E-01	0.4%
10817	Regulation of hormone levels	4,63E-01	0.7%
51336	Regulation of hydrolase activity	2,10E-01	0.4%
2697	Regulation of immune effector process	2,11E-01	0.1%
50776	Regulation of immune response	7,81E-01	0.1%
2682	Regulation of immune system process	7,81E-01	0.1%
43549	Regulation of kinase activity	6,42E-01	0.1%
32879	Regulation of localization	6,83E-01	0.1%
10556	Regulation of macromolecule biosynthetic process	4,26E-01	7.3%
60255	Regulation of macromolecule metabolic process	4,57E-01	8.0%
43405	Regulation of MAP kinase activity	2,11E-01	0.1%
40020	Regulation of meiosis	2,47E-01	0.1%
51445	Regulation of meiotic cell cycle	3,08E-01	0.1%
19222	Regulation of metabolic process	2,77E-01	9.3%

65009	Regulation of molecular function	5,24E-01	0.7%
51239	Regulation of multicellular organismal process	1,66E-01	1.7%
43900	Regulation of multi-organism process	5,29E-01	0.1%
51171	Regulation of nitrogen compound metabolic process	3,44E-01	7.7%
6808	Regulation of nitrogen utilization	2,80E-01	0.1%
19219	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	3,25E-01	7.7%
30811	Regulation of nucleotide catabolic process	1,88E-01	0.4%
6140	Regulation of nucleotide metabolic process	2,01E-01	0.4%
32890	Regulation of organic acid transport	3,33E-01	0.1%
80010	Regulation of oxygen and reactive oxigen species metabolic process	5,14E-01	0.1%
19220	Regulation of phosphate metabolic process	7,24E-01	0.1%
51174	Regulation of phosphorus metabolic process	7,24E-01	0.1%
42325	Regulation of phosphorylation	6,92E-01	0.1%
10099	Regulation of photomorphogenesis	4,81E-01	0.1%
10109	Regulation of photosynthesis	1,01E-01	0.4%
42548	Regulation of photosynthesis, light reaction	4,81E-01	0.1%
48580	Regulation of post-embryonic development	3,22E-02	1.7%
80090	Regulation of primary metabolic process	3,38E-01	8.0%
43067	Regulation of programmed cell death	3,61E-01	0.2%
45859	Regulation of protein kinase activity	6,42E-01	0.1%
51246	Regulation of protein metabolic process	4,86E-01	0.2%
33121	Regulation of purine nucleotide catabolic process	1,88E-01	0.4%
32313	Regulation of Rab GTPase activity	9,23E-02	0.4%
32483	Regulation of Rab protein signal transduction	9,23E-02	0.4%
32318	Regulation of Ras GTPase activity	1,71E-01	0.4%
46578	Regulation of Ras protein signal transduction	1,71E-01	0.4%
47484	Regulation of response to osmotic stress	1,18E-01	0.1%
48583	Regulation of response to stimulus	4,80E-01	0.8%
32784	Regulation of RNA elongation	2,47E-01	0.1%
51252	Regulation of RNA metabolic process	4,82E-01	3.9%
43455	Regulation of secondary metabolic process	6,83E-01	0.1%
10029	Regulation of seed germination	1,88E-01	0.4%
9966	Regulation of signal transduction	1,06E-01	0.7%
35466	Regulation of signaling pathway	1,13E-01	1.0%
23051	Regulation of signaling process	1,06E-01	0.7%
51056	Regulation of small GTPase mediated signal transduction	1,71E-01	0.4%
10119	Regulation of stomatal movement	7,04E-02	0.5%
45449	Regulation of transcription	3,71E-01	7.3%
6359	Regulation of transcription from RNA polymerase III promoter	1,18E-01	0.1%
6355	Regulation of transcription, DNA-dependent	4,80E-01	3.9%
51338	Regulation of transferase activity	6,73E-01	0.1%
6417	Regulation of translation	5,71E-01	0.1%
51049	Regulation of transport	6,34E-01	0.1%
70297	Regulation of two-component signal transduction system (phosphorelay)	4,63E-01	0.1%
50792	Regulation of viral reproduction	1,73E-01	0.1%
80134	Regulation to response to stress	5,37E-01	0.4%
2831	Reguo biotic stimuluslation of response t	4,26E-01	0.1%
3	Reproduction	2,11E-01	5.2%
48610	Reproductive cellular process	9,29E-01	0.2%
3006	Reproductive developmental process	2,03E-01	4.8%
22414	Reproductive process	1,92E-01	5.2%

48609	Reproductive process in a multicellular organism	3,84E-01	0.4%
48608	Reproductive structure development	1,52E-01	4.5%
22904	Respiratory electron transport chain	3,33E-01	0.2%
9628	Response to abiotic stimulus	2,71E-04	9.3%
9737	Response to abscisic acid stimulus	1,29E-03	3.2%
46685	Response to arsenic	3,84E-01	0.1%
9733	Response to auxin stimulus	3,85E-01	1.6%
9617	Response to bacterium	1,28E-01	1.9%
9607	Response to biotic stimulus	3,22E-02	4.2%
9637	Response to blue light	1,86E-01	0.5%
9741	Response to brassinosteroid stimulus	2,10E-01	0.5%
46686	Response to cadmium ion	1,69E-03	3.2%
51592	Response to calcium ion	4,09E-01	0.1%
9743	Response to carbohydrate stimulus	1,56E-01	1.4%
43157	Response to cation stress	2,11E-01	0.1%
42221	Response to chemical stimulus	3,09E-04	12.4%
10200	Response to chitin	9,73E-01	0.1%
9409	Response to cold	3,43E-01	1.4%
10201	Response to continuous far red light stimulus by the high-irradiance response system	1,73E-01	0.1%
46688	Response to copper ion	5,71E-01	0.1%
9269	Response to desiccation	9,23E-02	0.4%
34285	Response to disaccharide stimulus	2,23E-01	0.4%
6974	Response to DNA damage stimulus	5,05E-01	0.8%
42493	Response to drug	6,62E-01	0.1%
9719	Response to endogenous stimulus	6,37E-02	5.5%
9723	Response to ethylene stimulus	4,66E-01	0.7%
9605	Response to external stimulus	4,16E-02	2.2%
9991	Response to extracellular stimulus	2,80E-01	1.0%
10218	Response to far red light	1,31E-01	0.5%
9620	Response to fungus	6,20E-01	0.7%
9739	Response to gibberellin stimulus	3,72E-01	0.7%
9749	Response to glucose stimulus	3,67E-02	0.5%
9629	Response to gravity	8,01E-03	1.0%
9408	Response to heat	3,54E-01	0.8%
80027	Response to herbivore	2,11E-01	0.1%
9746	Response to hexose stimulus	4,16E-02	0.5%
9644	Response to high light intensity	7,96E-01	0.1%
9725	Response to hormone stimulus	5,42E-02	5.2%
42542	Response to hydrogen peroxide	4,86E-01	0.2%
1666	Response to hypoxia	5,53E-01	0.1%
10035	Response to inorganic substance	5,14E-04	4.5%
9625	Response to insect	8,08E-02	0.4%
9753	Response to jasmonic acid stimulus	4,35E-01	0.8%
10288	Response to lead ion	2,11E-01	0.1%
9642	Response to light intensity	2,06E-01	0.7%
9416	Response to light stimulus	3,73E-01	2.4%
10202	Response to low fluence red light stimulus	1,18E-01	0.1%
9645	Response to low light intensity stimulus	3,33E-01	0.1%
10042	Response to manganese ion	1,73E-01	0.1%
10555	Response to mannitol stimulus	3,08E-01	0.1%
10038	Response to metal ion	4,94E-04	3.9%

10188	Response to microbial phytotoxin	3,08E-01	0.1%
2237	Response to molecule of bacterial origin	4,09E-01	0.1%
34284	Response to monosaccharide stimulus	4,16E-02	0.5%
9624	Response to nematode	5,90E-02	0.8%
7584	Response to nutrient	3,33E-01	0.1%
31667	Response to nutrient levels	3,33E-01	0.8%
2239	Response to oomycetes	4,45E-01	0.1%
10033	Response to organic substance	4,14E-02	6.9%
6970	Response to osmotic stress	9,88E-04	4.1%
51707	Response to other organism	2,51E-02	4.2%
6979	Response to oxidative stress	3,66E-01	1.4%
70482	Response to oxigen levels	5,85E-01	0.1%
9314	Response to radiation	4,17E-01	2.4%
302	Response to reactive oxygen species	2,70E-01	0.5%
10114	Response to red light	1,14E-01	0.7%
9639	Response to red or far red light	4,86E-01	0.8%
9751	Response to salicylic acid stimulus	2,54E-01	1.0%
9651	Response to salt stress	7,19E-04	3.9%
10269	Response to selenium ion	1,73E-01	0.1%
304	Response to singlet oxygen	9,50E-02	0.2%
42594	Response to starvation	8,75E-01	0.2%
48545	Response to steroid hormone stimulus	2,10E-01	0.4%
50896	Response to stimulus	1,19E-06	22.7%
6950	Response to stress	1,91E-03	12.6%
9744	Response to sucrose stimulus	2,14E-01	0.4%
10477	Response to sulfur dioxide	1,18E-01	0.1%
9266	Response to temperature stimulus	2,11E-01	2.3%
9636	Response to toxin	5,29E-01	0.1%
10353	Response to trehalose stimulus	1,18E-01	0.1%
9411	Response to UV	9,00E-01	0.1%
10224	Response to UV-B	7,77E-01	0.1%
10203	Response to very low fluence red light stimulus	1,18E-01	0.1%
55122	Response to very low light intensity stimulus	1,73E-01	0.1%
9615	Response to virus	1,48E-01	0.5%
9415	Response to water	1,82E-02	2.2%
9414	Response to water deprivation	1,39E-02	2.2%
9611	Response to wounding	2,47E-01	1.0%
6890	Retrograde vesicle-mediated transport, Golgi to ER	2,47E-01	0.1%
48511	Rhythmic process	3,44E-02	0.8%
22618	Ribonucleoprotein complex assembly	3,84E-01	0.1%
22613	Ribonucleoprotein complex biogenesis	9,89E-01	0.1%
42455	Ribonucleoside biosynthetic process	2,11E-01	0.1%
9119	Ribonucleoside metabolic process	6,37E-02	0.5%
9201	Ribonucleoside triphosphate biosynthetic process	2,63E-02	1.0%
9199	Ribonucleoside triphosphate metabolic process	2,63E-02	1.0%
9260	Ribonucleotide biosynthetic process	5,76E-02	1.0%
9259	Ribonucleotide metabolic process	3,22E-02	1.1%
9161	Ribosnucleoside monophosphate metabolic process	4,26E-01	0.1%
31123	RNA 3'-end processing	4,98E-01	0.1%
32774	RNA biosynthetic process	4,45E-01	0.4%
6401	RNA catabolic process	4,81E-01	0.1%

16070	RNA metabolic process	6,74E-01	2.2%
9451	RNA modification	9,79E-01	0.1%
6396	RNA processing	5,63E-01	1.6%
8380	RNA splicing	7,56E-01	0.2%
375	RNA splicing, via transesterification reactions	8,38E-01	0.1%
377	RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	8,38E-01	0.1%
48364	Root development	7,74E-01	0.8%
10053	Root epidermal cell differentiation	8,52E-01	0.1%
48765	Root hair cell differentiation	7,81E-01	0.1%
48768	Root hair cell tip growth	4,45E-01	0.1%
48767	Root hair elongation	6,34E-01	0.1%
10015	Root morphogenesis	9,71E-01	0.1%
22622	Root system development	7,74E-01	0.8%
46500	S-adeosylmethionine metabolic process	2,80E-01	0.1%
9863	Salicylic acid mediated signaling pathway	3,36E-01	0.2%
10265	SCF complex assembly	1,18E-01	0.1%
19748	Secondary metabolic process	3,54E-01	1.9%
19932	Second-messenger-mediated signaling	5,37E-01	0.1%
46903	Secretion	5,24E-01	0.2%
32940	Secretion by cell	5,24E-01	0.2%
48316	Seed development	3,33E-01	2.4%
10162	Seed dormancy	4,98E-01	0.1%
9845	Seed germination	7,96E-01	0.1%
10431	Seed maturation	5,53E-01	0.1%
90351	Seedling development	8,15E-01	0.1%
1887	Selenium metabolic process	1,18E-01	0.1%
10149	Senescence	2,29E-02	0.8%
9070	Serine family amino acid biosynthetic process	5,37E-01	0.1%
9069	Serine family amino acid metabolic process	7,24E-01	0.1%
51761	Sesquiterpene metabolic process	5,99E-01	0.1%
6714	Sesquiterpenoid metabolic process	5,99E-01	0.1%
48367	Shoot development	6,55E-01	1.3%
10016	Shoot morphogenesis	6,23E-01	0.7%
22621	Shoot system development	6,64E-01	1.3%
48572	Short-day photoperiodism	2,87E-02	0.2%
48575	Short-day photoperiodism, flowering	2,87E-02	0.2%
7165	Signal transduction	6,84E-01	2.0%
23060	Signal transmission	5,92E-01	2.4%
23014	Signal transmission via phosphorylation event	4,26E-01	0.1%
23052	Signaling	3,08E-01	5.4%
23033	Signaling pathway	1,51E-01	3.9%
23046	Signaling process	5,92E-01	2.4%
7062	Sister chromatid cohesion	4,63E-01	0.1%
7264	Small GTPase mediated signal transduction	8,75E-01	0.1%
44283	Small molecule biosynthetic process	2,48E-04	6.0%
44282	Small molecule catabolic process	4,94E-04	2.4%
44281	Small molecule metabolic process	2,10E-10	12.9%
33528	S-methylmethionine cycle	1,18E-01	0.1%
33477	S-methylmetionine metabolic process	1,18E-01	0.1%
245	Spliceosome assembly	3,08E-01	0.1%
48443	Stamen development	8,52E-01	0.1%

19252	Starch biosynthetic process	1,72E-02	0.5%
5983	Starch catabolic process	1,91E-03	0.7%
5982	Starch metabolic process	3,51E-06	1.6%
48864	Stem cell development	6,34E-01	0.1%
48863	Stem cell differentiation	6,42E-01	0.1%
19827	Stem cell maintenance	6,34E-01	0.1%
10222	Stem vascular tissue pattern formation	1,73E-01	0.1%
6694	Steroid biosynthetic process	4,60E-01	0.2%
6706	Steroid catabolic process	1,18E-01	0.1%
34433	Steroid esterification	1,18E-01	0.1%
43401	Steroid hormone mediated signaling pathway	2,10E-01	0.4%
8202	Steroid metabolic process	4,18E-01	0.4%
16126	Sterol biosynthetic process	6,11E-01	0.1%
16127	Sterol catabolic process	1,18E-01	0.1%
34434	Sterol esterification	1,18E-01	0.1%
16125	Sterol metabolic process	3,36E-01	0.2%
10374	Stomatal complex development	6,55E-01	0.1%
10118	Stomatal movement	5,85E-01	0.1%
5986	Sucrose biosynthetic process	4,26E-01	0.1%
5985	Sucrose metabolic process	1,11E-01	0.4%
10182	Sugar mediated signaling pathway	2,51E-02	0.7%
97	Sulfur amino acid biosynthetic process	1,01E-01	0.5%
96	Sulfur amino acid metabolic process	2,51E-02	1.0%
44272	Sulfur compound biosynthetic process	2,57E-01	0.7%
44273	Sulfur compound catabolic process	5,37E-01	0.1%
6790	Sulfur metabolic process	2,51E-02	1.9%
7129	Synapsis	2,63E-02	0.5%
48731	System development	3,58E-01	3.8%
9627	Systemic acquired resistance	6,92E-01	0.1%
9862	Systemic acquired resistance, salicylic acid mediated signaling pathway	4,45E-01	0.1%
46246	Terpene biosynthetic process	6,34E-01	0.1%
46247	Terpene catabolic process	2,80E-01	0.1%
42214	Terpene metabolic process	2,36E-01	0.4%
16114	Terpenoid biosynthetic process	4,90E-01	0.4%
16115	Terpenoid catabolic process	4,26E-01	0.1%
6721	Terpenoid metabolic process	2,83E-01	0.7%
46654	Tetrahydrofolate bosynthetic process	2,80E-01	0.1%
46653	Tetrahydrofolate metabolic process	7,67E-02	0.2%
46900	Tetrahydrofolylpolyglutamate metabolic process	1,73E-01	0.1%
33014	Tetrapyrrole biosynthetic process	8,26E-01	0.1%
33013	Tetrapyrrole metabolic process	6,55E-01	0.2%
16109	Tetraterpenoid biosynthetic process	2,73E-01	0.2%
16110	Tetraterpenoid catabolic process	2,11E-01	0.1%
16108	Tetraterpenoid metabolic process	1,71E-01	0.4%
9888	Tissue development	8,53E-01	0.8%
9407	Toxin catabolic process	1,69E-01	0.5%
9404	Toxin metabolic process	1,69E-01	0.5%
6350	Transcription	4,59E-01	0.7%
6366	Transcription from RNA polymerase II promoter	5,37E-01	0.1%
6352	Transcription initiation	2,80E-01	0.4%
6367	Transcription initiation from RNA polymerase II promoter	4,81E-01	0.1%

6351	Transcription, DNA-dependent	4,29E-01	0.4%
6412	Translation	1	1.1%
6413	Translational initiation	8,93E-01	0.1%
6415	Translational termination	1,31E-01	0.2%
7169	Transmembrane receptor protein tyrosine kinase signaling pathway	8,56E-01	0.2%
55085	Transmembrane transport	2,19E-01	0.7%
10148	Transpiration	1,18E-01	0.1%
6810	Transport	3,56E-05	12.0%
5992	Trehalose biosynthetic process	5,53E-01	0.1%
5993	Trehalose catabolic process	1,18E-01	0.1%
5991	Trehalose metabolic process	2,81E-01	0.2%
6099	Tricarboxylic acid cycle	8,08E-02	0.4%
10054	Trichoblast differentiation	8,01E-01	0.1%
48764	Trichoblast maturation	7,81E-01	0.1%
10091	Trichome branching	5,37E-01	0.1%
10026	Trichome differentiation	4,29E-01	0.4%
10090	Trichome morphogenesis	3,11E-01	0.4%
19432	Triglyceride biosynthetic process	4,37E-02	0.2%
19433	Triglyceride catabolic process	2,11E-01	0.1%
6641	Triglyceride metabolic process	2,29E-02	0.4%
42939	Tripeptide transport	3,08E-01	0.1%
42780	tRNA 3'-end processing	2,47E-01	0.1%
6400	tRNA modification	3,33E-01	0.1%
8033	tRNA processing	4,80E-01	0.2%
2100	tRNA wobble adenosine to inosine editing	1,18E-01	0.1%
2097	tRNA wobble base modification	1,73E-01	0.1%
6399	tRNAmetabolic process	8,52E-01	0.2%
9606	Tropism	3,22E-02	0.8%
35295	Tube development	9,61E-01	0.1%
160	Two-component signal transduction system (phosphorelay)	4,45E-01	0.4%
6571	Tyrosine biosynthetic process	2,80E-01	0.1%
6570	Tyrosine metabolic process	3,08E-01	0.1%
6744	Ubiquinone biosynthetic process	2,47E-01	0.1%
6743	Ubiquinone metabolic process	2,47E-01	0.1%
6511	Ubiquitin-dependent protein catabolic process	9,10E-01	0.7%
9826	Unidimensional cell growth	2,28E-01	1.3%
6636	Unsaturated fatty acid biosynthetic process	1,12E-01	0.2%
33559	Unsaturated fatty acid metabolic process	1,31E-01	0.2%
6212	Uracil catabolic process	2,11E-01	0.1%
19860	Uracil metabolic process	2,11E-01	0.1%
15840	Urea transport	2,47E-01	0.1%
7034	Vacuolar transport	3,67E-02	0.5%
7033	Vacuole organization	4,92E-02	0.4%
6573	Valine metabolic process	1,73E-01	0.1%
10050	Vegetative phase change	3,33E-01	0.1%
10228	Vegetative to reproductive phase transition of meristem	1,63E-01	0.8%
10048	Vernalization response	4,45E-01	0.1%
48278	Vesicle docking	6,62E-01	0.1%
6904	Vesicle docking involved in exocytosis	6,55E-01	0.1%
16192	Vesicle-mediated transport	5,96E-03	2.3%
19079	Viral genome replication	2,47E-01	0.1%

19058	Viral infectious cycle	2,47E-01	0.1%
46786	Viral replication complex formation and maintenance	2,47E-01	0.1%
16032	Viral reproduction	3,59E-01	0.1%
22415	Viral reproductive process	3,59E-01	0.1%
42819	Vitamin B6 biosynthetic process	3,08E-01	0.1%
42816	Vitamin B6 metabolic process	3,08E-01	0.1%
9110	Vitamin biosynthetic process	2,32E-01	0.5%
42371	Vitamin K biosynthetic process	3,08E-01	0.1%
42373	Vitamin K metbolic process	3,08E-01	0.1%
6766	Vitamin metabolic process	2,62E-01	0.5%
6833	Water transport	1,31E-01	0.2%
42364	Water-soluble vitamin biosynthetic process	3,02E-01	0.4%
6767	Water-soluble vitamin metabolic process	3,34E-01	0.4%
16123	Xanthophyll biosynthetic process	3,08E-01	0.1%
16124	Xanthophyll catabolic process	2,11E-01	0.1%
16122	Xanthophyll metabolic process	1,66E-01	0.2%
10051	Xylem and phloem pattern formation	2,80E-01	0.4%
9969	Xyloglucan biosynthetic process	5,90E-02	0.2%

A.1.3 Transcripts simultaneously down-regulated in 7LD:11LD samples

Table A.3: Frequency analysis of the microarray results according to biological process, of the simultaneously down-regulated transcripts in 7 LD and 11 LD samples, using gene ontology information; performed using the Cytoscape v2.8.3 software platform (Cline *et al*, 2007) and BiNGO v2.44 plugin (Maere *et al*, 2005)

DOWN-REGULATED GENES (7LD:11LD)

GO-ID	BIOLOGICAL PROCESS	p-val	Freq
9738	Abscisic acid mediated signaling pathway	5,23E-01	0.4%
9838	Abscission	1,47E-01	0.9%
30036	Actin cytoskeleton organization	4,62E-01	0.4%
30029	Actin filament-based process	5,40E-01	0.4%
32147	Activation of protein kinase activitiy	2,83E-01	0.4%
7205	Activation of protein kinase C activity by G-protein coupled receptor protein signaling pathway	2,83E-01	0.4%
9943	Adaxial/abaxial axis specification	3,41E-01	0.4%
9955	Adaxial/abaxial pattern formation	1,68E-01	0.9%
7568	Aging	2,03E-01	1.3%
9309	Amine biosynthetic process	3,71E-01	1.3%
9310	Amine catabolic process	4,38E-01	0.4%
9308	Amine metabolic process	4,42E-01	2.2%
15837	Amine transport	1,73E-01	1.3%
6865	Amino acid transport	1,73E-01	1.3%
48532	Anatomical structure arrangement	4,69E-01	0.4%
48856	Anatomical structure development	9,52E-02	10.8%
48646	Anatomical structure formation involved in morphogenesis	7,00E-01	0.4%
9653	Anatomical structure morphogenesis	2,47E-01	4.0%
48466	Androecium development	5,23E-01	0.4%
6820	Anion transport	1,73E-01	1.3%
48653	Anther development	4,30E-01	0.4%
6915	Apoptosis	2,97E-01	1.3%
9073	Aromatic amino acid family biosynthetic process	4,58E-01	0.4%
9072	Aromatic amino acid family metabolic process	4,91E-01	0.4%
19438	Aromatic compound biosynthetic process	4,06E-01	1.3%
19439	Aromatic compound catabolic process	3,41E-01	0.4%
9067	Aspartate family amino acid biosynthetic process	4,30E-01	0.4%
9066	Aspartate family amino acid metabolic process	5,51E-01	0.4%
6754	ATP biosynthetic process	5,40E-01	0.4%
46034	ATP metabolic process	5,40E-01	0.4%
51313	Attachment of spindle microtubules to chromosome	1,59E-01	0.4%
8608	Attachment of spindle microtubules to kinetochore	1,59E-01	0.4%
51455	Attachment of spindle microtubules to kinetochore involved in homologous chromosome segregation	1,59E-01	0.4%

51316	Attachment of spindle microtubules to kinetochore involved in meioticchromosome segregation	1,59E-01	0.4%
9734	Auxin mediated signaling pathway	1,90E-01	0.9%
9798	Axis specification	3,92E-01	0.4%
6284	Base-excision repair	3,41E-01	0.4%
15802	Basic amino acid transport	2,03E-01	0.4%
22610	Biological adhesion	3,12E-01	0.4%
65007	Biological regulation	9,19E-02	21.1%
9058	Biosynthetic process	4,86E-01	12.6%
31324	Bnegative regulation of cellular metabolic process	1,41E-01	1.8%
55074	Calcium ion homeostasis	3,85E-01	0.4%
16051	Carbohydrate biosynthetic process	2,47E-01	2.2%
9756	Carbohydrate mediated signaling	4,00E-01	0.4%
5975	Carbohydrate metabolic process	9,02E-01	2.2%
46394	Carboxylic acid biosynthetic process	9,05E-02	4.0%
46395	Carboxylic acid catabolic process	3,27E-01	0.9%
19752	Carboxylic acid metabolic process	1,68E-01	5.4%
46942	Carboxylic acid transport	1,90E-01	1.3%
16117	Carotenoid biosynthetic process	3,56E-01	0.4%
16116	Carotenoid metabolic process	3,99E-01	0.4%
48440	Carpel development	2,87E-01	0.9%
9056	Catabolic process	4,62E-01	3.6%
55080	Cation homeostasis	1,91E-01	1.3%
6812	Cation transport	3,10E-01	2.2%
7155	Cell adhesion	3,12E-01	0.4%
7049	Cell cycle	5,40E-01	0.9%
22403	Cell cycle phase	3,41E-01	0.9%
22402	Cell cycle process	4,69E-01	0.9%
8219	Cell death	8,90E-02	3.6%
48468	Cell development	2,87E-01	1.8%
30154	Cell differentiation	1,18E-01	3.6%
23033	Cell division	3,41E-01	4.0%
51301	Cell division	3,41E-01	0.9%
16049	Cell growth	7,36E-01	0.9%
48469	Cell maturation	4,62E-01	0.4%
902	Cell morphogenesis	7,35E-01	0.9%
904	Cell morphogenesis involved in differentiation	7,37E-01	0.4%
7166	Cell surface receptor linked signaling pathway	1,82E-01	1.8%
42546	Cell wall biogenesis	3,53E-01	0.9%
44038	Cell wall macromolecule biosynthetic process	2,87E-01	0.4%
44036	Cell wall macromolecule metabolic process	4,62E-01	0.4%
42545	Cell wall modification	7,57E-01	0.4%
71555	Cell wall organization	8,26E-01	0.4%
71554	Cell wall organization or biogenesis	5,57E-01	1.3%

70592	Cell wall polysaccharide biosynthetic process	2,87E-01	0.4%
10383	Cell wall polysaccharide metabolic process	3,41E-01	0.4%
44106	Cellular amine metabolic process	5,23E-01	1.8%
6519	Cellular amino acid and derivative metabolic process	3,68E-01	3.1%
8652	Cellular amino acid biosynthetic process	3,41E-01	1.3%
9063	Cellular amino acid catabolic process	4,30E-01	0.4%
42398	Cellular amino acid derivative biosynthetic process	3,99E-01	1.3%
42219	Cellular amino acid derivative catabolic process	3,63E-01	0.4%
6575	Cellular amino acid derivative metabolic process	3,64E-01	1.8%
6520	Cellular amino acid metabolic process	4,72E-01	1.8%
6725	Cellular aromatic compound metabolic process	3,41E-01	2.2%
42401	Cellular biogenic amine biosynthetic process	4,47E-01	0.4%
6576	Cellular biogenic amine metabolic process	5,10E-01	0.4%
44249	Cellular biosynthetic process	4,48E-01	12.1%
6874	Cellular calcium ion homeostasis	3,85E-01	0.4%
34637	Cellular carbohydrate biosynthetic process	2,68E-01	1.8%
44262	Cellular carbohydrate metabolic process	6,82E-01	1.8%
44248	Cellular catabolic process	4,00E-01	3.1%
30003	Cellular cation homeostasis	3,12E-01	0.9%
10382	Cellular cell wall macromolecule metabolic process	3,03E-01	0.4%
70882	Cellular cell wall organization or biogenesis	3,72E-01	0.9%
55082	Cellular chemical homeostasis	3,48E-01	0.9%
22607	Cellular component assembly	9,34E-01	0.4%
44085	Cellular component biogenesis	4,41E-01	2.7%
22411	Cellular component disassembly	3,27E-01	0.4%
70589	Cellular component macromolecule biosynthetic process	2,87E-01	0.4%
32989	Cellular component morphogenesis	7,65E-01	0.9%
16043	Cellular component organization	3,14E-01	5.8%
48869	Cellular developmental process	1,75E-01	4.0%
30005	Cellular di-,tri-valent inorganic cation homeostasis	2,51E-01	0.9%
6073	Cellular glucan metabolic process	7,14E-01	0.4%
19725	Cellular homeostasis	5,40E-01	0.9%
34754	Cellular hormone metabolic process	4,30E-01	0.4%
6873	Cellular ion homeostasis	3,47E-01	0.9%
42180	Cellular ketone metabolic process	1,73E-01	5.4%
44242	Cellular lipid catabolic process	5,27E-01	0.4%
44255	Cellular lipid metabolic process	1,60E-01	4.0%
51641	Cellular localization	3,89E-01	2.7%
34622	Cellular macromolecular complex assembly	8,00E-01	0.4%
34623	Cellular macromolecular complex disassembly	2,87E-01	0.4%
34621	Cellular macromolecular complex subunit organization	5,57E-01	0.9%
34645	Cellular macromolecule biosynthetic process	9,38E-01	4.5%
44265	Cellular macromolecule catabolic process	6,04E-01	1.3%
70727	Cellular macromolecule localization	7,62E-01	0.9%

44260	Cellular macromolecule metabolic process	3,86E-01	18.4%
43094	Cellular metabolic compound salvage	4,91E-01	0.4%
44237	Cellular metabolic process	2,63E-01	28.3%
6875	Cellular metal ion homeostasis	4,72E-01	0.4%
44271	Cellular nitrogen compound biosynthetic process	4,59E-01	2.2%
34641	Cellular nitrogen compound metabolic process	1,91E-01	9.9%
33692	Cellular polysaccharide biosynthetic process	3,89E-01	0.9%
44264	Cellular polysaccharide metabolic process	5,15E-01	0.9%
9987	Cellular process	4,88E-02	44.1%
44257	Cellular protein catabolic process	5,57E-01	1.3%
43624	Cellular protein complex disassembly	2,87E-01	0.4%
34613	Cellular protein localization	7,36E-01	0.9%
44267	Cellular protein metabolic process	8,14E-01	10.8%
71214	Cellular response to abiotic stimulus	5,40E-01	0.4%
71215	Cellular response to abscisic acid stimulus	5,23E-01	0.4%
71365	Cellular response to auxin stimulus	1,98E-01	0.9%
71216	Cellular response to biotic stimulus	2,59E-01	0.4%
71322	Cellular response to carbohydrate stimulus	4,00E-01	0.4%
70887	Cellular response to chemical stimulus	2,47E-01	3.1%
70417	Cellular response to cold	2,47E-01	0.4%
71368	Cellular response to cytokinin stimulus	2,26E-01	0.9%
71359	Cellular response to dsRNA	1,73E-01	0.9%
71495	Cellular response to endogenous stimulus	5,47E-01	1.3%
34605	Cellular response to heat	2,03E-01	0.4%
71331	Cellular response to hexose stimulus	2,26E-01	0.4%
32870	Cellular response to hormone stimulus	5,10E-01	1.3%
71482	Cellular response to light stimulus	4,91E-01	0.4%
71326	Cellular response to monosaccaride stimulus	2,26E-01	0.4%
71310	Cellular response to organic substance	2,01E-01	3.1%
71445	Cellular response to protein stimulus	2,47E-01	0.4%
71478	Cellular response to radiation	4,91E-01	0.4%
71491	Cellular response to red ligh	2,47E-01	0.4%
71489	Cellular response to red or far-red ligh	4,62E-01	0.4%
51716	Cellular response to stimulus	2,87E-01	4.9%
33554	Cellular response to stress	4,39E-01	2.2%
34620	Cellular response to unfolded protein	2,47E-01	0.4%
6882	Cellular zinc ion homeostasis	1,73E-01	0.4%
30244	Cellulose biosynthetic process	4,06E-01	0.4%
30243	Cellulose metabolic process	4,30E-01	0.4%
48878	Chemical homeostasis	2,87E-01	1.3%
6821	Chloride transport	2,44E-01	0.4%
10239	Chloroplast mRNA processing	1,18E-01	0.4%
9658	Chloroplast organization	5,56E-01	0.4%
31425	Chloroplast RNA processing	2,47E-01	0.4%

46417	Chorismate metabolic process	4,58E-01	0.4%
16568	Chromatin modification	6,43E-01	0.4%
6325	Chromatin organization	5,52E-01	0.9%
6342	Chromatin silencing	3,35E-01	0.4%
31048	Chromatin silencing by small RNA	1,90E-01	0.4%
30261	Chromosome condensation	1,73E-01	0.4%
51276	Chromosome organization	6,82E-01	0.9%
7059	Chromosome segregation	1,73E-01	0.9%
51304	Chromosome separation	1,90E-01	0.4%
7623	Circadian rhythm	2,59E-01	0.9%
9805	Coumarin biosynthetic process	1,73E-01	0.4%
9804	Coumarin metabolic process	1,73E-01	0.4%
16569	Covalent chromatin modification	5,48E-01	0.4%
42335	Cuticle development	3,27E-01	0.4%
19344	Cysteine biosynthetic process	2,94E-01	0.4%
6534	Cysteine metabolic process	2,94E-01	0.4%
9823	Cytokinin catabolic process	2,44E-01	0.4%
9736	Cytokinin mediated signaling pathway	2,26E-01	0.9%
9690	Cytokinin metabolic process	3,67E-01	0.4%
7010	Cytoskeleton organization	2,47E-01	1.3%
16265	Death	8,90E-02	3.6%
6952	Defense response	4,42E-01	3.6%
42742	Defense response to bacterium	3,02E-01	1.8%
9816	Defense response to bacterium, incompatible interaction	3,92E-01	0.4%
9814	Defense response, incompatible interaction	7,05E-01	0.4%
9900	Dehiscence	3,12E-01	0.4%
48588	Developmental cell growth	6,55E-01	0.4%
48589	Developmental growth	6,65E-01	0.9%
60560	Developmental growth involved in morphogenesis	8,49E-01	0.4%
21700	Developmental maturation	5,21E-01	0.4%
32502	Developmental process	9,05E-02	13.5%
55066	Di-, tri-valent inorganic cation homeostasis	2,87E-01	0.9%
43648	Dicarboxylic acid metabolic process	5,40E-01	0.4%
42938	Dipeptide transport	1,90E-01	0.4%
46351	Disaccharide biosynthetic process	1,90E-01	0.9%
5984	Disaccharide metaboli process	2,47E-01	0.9%
16103	Diterpenoid catabolic process	2,26E-01	0.4%
16101	Diterpenoid metabolic process	3,72E-01	0.4%
6305	DNA alkylation	2,01E-01	0.9%
71103	DNA conformation change	6,47E-01	0.4%
9294	DNA mediated transformation	3,85E-01	0.4%
6259	DNA metabolic process	2,68E-01	2.7%
6306	DNA methylation	2,01E-01	0.9%
6304	DNA modification	2,03E-01	0.9%

6323	DNA packaging	5,40E-01	0.4%
6310	DNA recombination	2,68E-01	0.9%
6281	DNA repair	5,42E-01	0.9%
6260	DNA replication	6,73E-01	0.4%
6302	Double-strand break repair	3,72E-01	0.4%
724	Double-strand break repair via homologous recombination	2,87E-01	0.4%
6855	Drug transmembrane transport	1,73E-01	0.9%
15893	Drug transport	1,73E-01	0.9%
31050	dsRNA fragmentation	1,73E-01	0.9%
7398	Ectoderm development	4,74E-01	0.9%
9559	Embryo sac central cell differentiation	3,47E-01	0.4%
9553	Embryo sac development	3,68E-01	0.9%
9790	Embryonic development	8,22E-01	1.3%
9793	Embryonic development ending in seed dormancy	9,78E-01	0.4%
7167	Enzyme linked receptor protein signaling pathway	2,48E-01	1.3%
9913	Epidermal cell differentiation	4,72E-01	0.9%
8544	Epidermis development	4,74E-01	0.9%
51234	Establishment of localization	9,19E-02	11.7%
51649	Establishment of localization in cell	4,62E-01	2.2%
45184	Establishment of protein localization	6,88E-01	1.3%
6633	Fatty acid biosynthetic process	6,91E-02	2.7%
6631	Fatty acid metabolic process	8,90E-02	3.1%
9813	Flavonoid biosynthetic process	4,87E-01	0.4%
9812	Flavonoid metabolic process	5,15E-01	0.4%
10582	Floral meristem determinancy	8,90E-02	0.9%
10227	Floral organ abscission	1,30E-01	0.9%
48437	Floral organ development	1,45E-01	2.2%
48449	Floral organ formation	3,56E-01	0.4%
48444	Floral organ morphogenesis	4,00E-01	0.4%
48438	Floral whorl development	1,85E-01	1.8%
9908	Flower development	8,90E-02	3.6%
10047	Fruit dehiscence	2,47E-01	0.4%
10154	Fruit development	9,49E-01	0.9%
7276	Gamete generation	3,63E-01	0.4%
48229	Gametophyte development	4,74E-01	1.3%
10467	Gene expression	5,78E-01	7.2%
16458	Gene silencing	2,94E-01	1.3%
31047	Gene silencing by RNA	2,51E-01	1.3%
9292	Genetic transfer	3,85E-01	0.4%
45487	Gibberellin catabolic process	2,26E-01	0.4%
9685	Gibberellin metabolic process	3,67E-01	0.4%
9250	Glucan biosynthetic process	5,51E-01	0.4%
44042	Glucan metabolic process	7,19E-01	0.4%
10417	Glucuronoxylan biosynthetic process	2,68E-01	0.4%

10413	Glucuronoxylan metabolic process	2,68E-01	0.4%
6546	Glycine catabolic process	2,47E-01	0.4%
6544	Glycine metabolic process	3,12E-01	0.4%
16138	Glycoside biosynthetic process	2,94E-01	0.9%
16137	Glycoside metabolic process	3,72E-01	0.9%
48193	Golgi vesicle transport	4,52E-01	0.4%
7186	G-protein coupled receptor protein signaling pathway	3,35E-01	0.4%
40007	Growth	7,94E-01	0.9%
48467	Gynoecium development	3,03E-01	0.9%
10410	Hemicellulose metabolic process	2,94E-01	0.4%
18130	Heterocycle biosynthetic process	7,41E-01	0.4%
46483	Heterocycle metabolic process	5,40E-01	1.8%
9747	Hexokinase-dependent signaling	1,59E-01	0.4%
9757	Hexose mediated signaling	2,26E-01	0.4%
51567	Histone H3-K9 methylation	2,26E-01	0.4%
34968	Histone lysine methylation	3,03E-01	0.4%
16571	Histone methylation	3,92E-01	0.4%
16570	Histone modification	5,40E-01	0.4%
42592	Homeostatic process	4,57E-01	1.3%
45143	Homologous chromosome segregation	1,59E-01	0.4%
42447	Hormone catabolic process	2,44E-01	0.4%
42445	Hormone metabolic process	5,89E-01	0.4%
9755	Hormone-mediated signaling pathway	5,02E-01	1.3%
34050	Host programmed cell death induced by symbiont	1,98E-01	0.9%
6972	Hyperosmotic response	5,15E-01	0.4%
42538	Hyperosmotic salinity response	4,72E-01	0.4%
6955	Immune response	4,30E-01	1.8%
2376	Immune system process	4,48E-01	1.8%
46219	Indolalhylamine biosynthetic process	3,47E-01	0.4%
6586	Indolalhylamine metabolic process	3,63E-01	0.4%
42430	Indole and derivative metabolic process	4,62E-01	0.4%
42435	Indole derivative biosynthetic process	4,47E-01	0.4%
42434	Indole derivative metabolic process	4,62E-01	0.4%
45087	Innate immune response	4,03E-01	1.8%
15698	Inorganic anion transport	2,59E-01	0.9%
48219	Inter-Golgi cisterna vesicle-mediated transport	1,18E-01	0.4%
6886	Intracellular protein transport	7,19E-01	0.9%
23034	Intracellular signaling pathway	8,53E-01	0.4%
46907	Intracellular transport	3,85E-01	2.2%
50801	Ion homeostasis	2,28E-01	1.3%
6811	Ion transport	2,26E-01	3.1%
8299	Isoprenoid biosynthetic process	7,03E-01	0.4%
8300	Isoprenoid catabolic process	2,83E-01	0.4%
6720	Isoprenoid metabolic process	4,75E-01	0.9%

46864	Isoprenoid transport	1,18E-01	0.4%
9695	Jasmonic acid biosynthetic process	3,47E-01	0.4%
9694	Jasmonic acid metabolic process	3,67E-01	0.4%
741	Karyogamy	3,47E-01	0.4%
48527	Lateral root development	5,37E-01	0.4%
15692	Lead ion transport	1,90E-01	0.4%
48366	Leaf development	6,61E-01	0.9%
9965	Leaf morphogenesis	7,36E-01	0.4%
10150	Leaf senescence	3,99E-01	0.4%
46274	Lignin catabolic process	2,87E-01	0.4%
9808	Lignin metabolic process	4,75E-01	0.4%
8610	Lipid biosynthetic process	1,98E-01	3.1%
16042	Lipid catabolic process	5,40E-01	0.4%
10876	Lipid localization	5,40E-01	0.9%
6629	Lipid metabolic process	1,75E-01	4.9%
6869	Lipid transport	5,13E-01	0.9%
51179	Localization	9,05E-02	12.1%
279	M phase	3,02E-01	0.9%
51327	M phase of meiotic cell cycle	2,44E-01	0.9%
65003	Macromolecular complex assembly	8,78E-01	0.4%
43933	Macromolecular complex subunit organization	6,93E-01	0.9%
9059	Macromolecule biosynthetic process	9,39E-01	4.5%
9057	Macromolecule catabolic process	6,87E-01	1.3%
33036	Macromolecule localization	5,27E-01	2.7%
43170	Macromolecule metabolic process	4,00E-01	20.2%
43414	Macromolecule methylation	3,35E-01	0.9%
43412	Macromolecule modification	3,18E-01	8.1%
32984	Macromoleular complex disassembly	2,94E-01	0.4%
10076	Maintenance of floral meristem identity	8,90E-02	0.9%
51235	Maintenance of location	2,68E-01	0.4%
51651	Maintenance of location in cell	2,47E-01	0.4%
10074	Maintenance of meristem identity	1,47E-01	0.9%
45185	Maintenance of protein localization	2,47E-01	0.4%
32507	Maintenance of protein localization in cell	2,44E-01	0.4%
9561	Megagametogenesis	2,47E-01	0.9%
7126	Meiosis	2,44E-01	0.9%
7127	Meiosis I	1,90E-01	0.9%
51321	Meiotic cell cycle	2,97E-01	0.9%
10032	Meiotic chromosome condensation	1,18E-01	0.4%
45132	Meiotic chromosome segregation	9,19E-02	0.9%
51307	Meiotic chromosome separation	1,90E-01	0.4%
51177	Meiotic sister chromatid cohesion	2,03E-01	0.4%
51754	Meiotic sister chromatid cohesion, centromeric	1,59E-01	0.4%
10022	Meristem determinancy	9,05E-02	0.9%

48507	Meristem development	1,74E-02	3.1%
10073	Meristem maintenance	1,46E-02	2.7%
9933	Meristem structural organization	4,47E-01	0.4%
8152	Metabolic process	2,03E-01	36.0%
55065	Metal ion homeostasis	4,72E-01	0.4%
30001	Metal ion transport	2,51E-01	1.8%
9086	Methionine biosynthetic process	3,27E-01	0.4%
6555	Methionine metabolic process	4,72E-01	0.4%
32259	Methylation	3,47E-01	0.9%
10480	Microsporocyte differentiation	1,59E-01	0.4%
34453	Microtubule anchoring	1,59E-01	0.4%
226	Microtubule cytoskeleton organization	4,62E-01	0.4%
7017	Microtubule-based process	2,51E-01	1.3%
6839	Mitochondrial transport	2,47E-01	0.9%
7005	Mitochondrion organization	4,14E-01	0.4%
43632	Modification-dependent macromolecule catabolic process	5,40E-01	1.3%
19941	Modification-dependent protein catabolic process	5,40E-01	1.3%
32787	Monocarboxylic acid metabolic process	1,18E-01	3.6%
55067	Monovalent inorganic cation homeostasis	2,68E-01	0.4%
15672	Monovalent inorganic cation transport	4,30E-01	0.9%
31124	mRNA 3'-end processing	2,03E-01	0.4%
6379	mRNA cleavage	2,59E-01	0.4%
16071	mRNA metabolic process	2,47E-01	1.3%
16556	mRNA modification	2,03E-01	0.4%
6378	mRNA polyadenylation	1,90E-01	0.4%
6397	mRNA processing	3,61E-01	0.9%
32504	Multicellular organism reproduction	2,94E-01	0.9%
7275	Multicellular organismal development	1,44E-01	11.7%
32501	Multicellular organismal process	1,73E-01	11.7%
51704	Multi-organism process	6,07E-01	3.1%
34660	ncRNA metabolic process	4,52E-01	1.3%
34470	ncRNA processing	3,31E-01	1.3%
10254	Nectary development	1,73E-01	0.4%
48519	Negative regulation of biological process	1,20E-01	4.0%
9890	Negative regulation of biosynthetic process	1,01E-01	1.8%
60548	Negative regulation of cell death	3,03E-01	0.4%
45596	Negative regulation of cell differentiation	1,76E-01	0.9%
8285	Negative regulation of cell proliferation	4,88E-02	0.9%
31327	Negative regulation of cellular biosynthetic process	1,01E-01	1.8%
48523	Negative regulation of cellular process	9,05E-02	3.1%
51093	Negative regulation of developmental process	3,67E-01	0.9%
9910	Negative regulation of flower development	4,42E-01	0.4%
10629	Negative regulation of gene expression	9,68E-02	2.7%
45814	Negative regulation of gene expression, epigenetic	3,67E-01	0.4%

32582	Negative regulation of gene-specific transcription	1,90E-01	0.4%
10558	Negative regulation of macromolecule biosynthetic process	9,45E-02	1.8%
10605	Negative regulation of macromolecule metabolic process	1,19E-01	2.7%
9892	Negative regulation of metabolic process	1,46E-01	2.7%
51172	Negative regulation of nitrogen compound metabolic process	9,45E-02	1.8%
45934	Negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	9,45E-02	1.8%
48581	Negative regulation of post-embryonic development	5,37E-01	0.4%
43069	Negative regulation of programmed cell death	2,87E-01	0.4%
51253	Negative regulation of RNA metabolic process	8,90E-02	1.3%
16481	Negative regulation of transcription	9,05E-02	1.8%
45892	Negative regulation of transcription, DNA-dependent	8,90E-02	1.3%
6807	Nitrogen compound metabolic process	1,90E-01	10.3%
10213	Non-photoreactive DNA repair	1,59E-01	0.4%
90304	Nucleic acid metabolic process	1,40E-01	7.2%
90305	Nucleic acid phosphodiester bond hydrolysis	3,18E-01	0.4%
34404	Nucleobase, nucleoside and nucleotide biosynthetic process	4,47E-01	0.9%
55086	Nucleobase, nucleoside and nucleotide metabolic process	6,08E-01	0.9%
34654	Nucleobase, nucleoside, nucleotide and nucleic acid biosynthetic process	4,47E-01	0.9%
6139	Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	1,73E-01	8.1%
9116	Nucleoside metabolic process	4,62E-01	0.4%
9124	Nucleoside monophosphate biosynthetic process	2,94E-01	0.4%
9123	Nucleoside monophosphate metabolic process	3,03E-01	0.4%
6753	Nucleoside phosphate metabolic process	5,33E-01	0.9%
9142	Nucleoside triphosphate biosynthetic process	5,42E-01	0.4%
9141	Nucleoside triphosphate metabolic process	5,42E-01	0.4%
9165	Nucleotide biosynthetic process	4,06E-01	0.9%
9117	Nucleotide metabolic process	5,33E-01	0.9%
6289	Nucleotide-excision repair	2,87E-01	0.4%
6294	Nucleotide-excision repair, preincision complex assembly	1,18E-01	0.4%
6997	Nucleus organization	3,85E-01	0.4%
6857	Oligopeptide transport	1,75E-01	1.3%
9312	Oligosaccharide biosynthetic process	2,03E-01	0.9%
9311	Oligosaccharide metabolic process	2,83E-01	0.9%
6730	One-carbon metabolic process	4,00E-01	0.9%
48513	Organ development	4,88E-02	8.1%
48645	Organ formation	4,52E-01	0.4%
9887	Organ morphogenesis	1,82E-01	2.2%
10260	Organ senescence	4,00E-01	0.4%
48285	Organelle fission	4,98E-01	0.4%
48284	Organelle fusion	3,47E-01	0.4%
6996	Organelle organization	2,52E-01	4.0%
16053	Organic acid biosynthetic process	9,05E-02	4.0%
16054	Organic acid catabolic process	3,27E-01	0.9%

6082	Organic acid metabolic process	1,68E-01	5.4%
15849	Organic acid transport	1,90E-01	1.3%
15822	Ornithine transport	1,18E-01	0.4%
48481	Ovule development	4,62E-01	0.4%
55114	Oxidation reduction	7,73E-01	0.4%
43436	Oxoacid metabolic process	1,68E-01	5.4%
31408	Oxylipin biosynthetic process	3,72E-01	0.4%
31407	Oxylipin metabolic process	4,00E-01	0.4%
7389	Pattern specification process	2,85E-01	1.3%
15833	Peptide transport	1,75E-01	1.3%
18193	Peptidyl-amino acid modification	3,47E-01	0.4%
18202	Peptidyl-histidine modification	1,73E-01	0.4%
18106	Peptidyl-histidine phosphorylation	1,73E-01	0.4%
16559	Peroxisome fission	2,68E-01	0.4%
7031	Peroxisome organization	1,73E-01	0.9%
9699	Phenylpropanoid biosynthetic process	4,26E-01	0.9%
46271	Phenylpropanoid catabolic process	2,87E-01	0.4%
9698	Phenylpropanoid metabolic process	3,18E-01	1.3%
6796	Phosphate metabolic process	4,19E-01	5.4%
6793	Phosphorous metabolic process	4,20E-01	5.4%
16310	Phosphorylation	3,63E-01	5.4%
9640	Photomorphogenesis	4,98E-01	0.4%
9648	Photoperiodism	4,58E-01	0.4%
48573	Photoperiodism, flowering	4,42E-01	0.4%
9853	Photorespiration	3,99E-01	0.4%
15979	Photosynthesis	7,25E-01	0.4%
48827	Phyllome development	7,05E-01	0.9%
46148	Pigment biosynthetic process	6,03E-01	0.4%
42440	Pigment metabolic process	6,65E-01	0.4%
9832	Plant-type cell wall biogenesis	2,94E-01	0.9%
9828	Plant-type cell wall loosening	4,38E-01	0.4%
9827	Plant-type cell wall modification	4,87E-01	0.4%
9664	plant-type cell wall organization	6,08E-01	0.4%
71669	Plant-type cell wall organization or biogenesis	3,14E-01	1.3%
9626	Plant-type hypersensitive response	1,91E-01	0.9%
9657	Plastid organization	6,97E-01	0.4%
10197	Polar nucleus fusion	3,41E-01	0.4%
9944	Polarity specification of adaxial/abaxial axis	3,18E-01	0.4%
271	Polysaccharide biosynthetic process	4,04E-01	0.9%
5976	Polysaccharide metabolic process	5,46E-01	0.9%
48518	Positive regulation of biological process	3,41E-01	1.8%
9891	Positive regulation of biosynthetic process	5,57E-01	0.4%
43085	Positive regulation of catalytic activity	4,14E-01	0.4%
31328	Positive regulation of cellular biosynthetic process	5,57E-01	0.4%

31325	Positive regulation of cellular metabolic process	6,08E-01	0.4%
48522	Positive regulation of cellular process	7,74E-01	0.4%
42753	Positive regulation of circadian rhythm	1,59E-01	0.4%
51094	Positive regulation of developmental process	2,65E-01	0.9%
9911	Positive regulation of flower development	2,01E-01	0.9%
10628	Positive regulation of gene expression	5,40E-01	0.4%
10557	Positive regulation of macromolecule biosynthetic process	5,42E-01	0.4%
10604	Positive regulation of macromolecule metabolic process	5,71E-01	0.4%
9893	Positive regulation of metabolic process	6,20E-01	0.4%
44093	Positive regulation of molecular function	4,22E-01	0.4%
51173	Positive regulation of nitrogen compound metabolic process	5,51E-01	0.4%
45935	Positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	5,48E-01	0.4%
48582	Positive regulation of post-embryonic development	2,44E-01	0.9%
45860	Positive regulation of protein kinase activity	3,03E-01	0.4%
33674	Positive regulation of protein kinase activity	3,03E-01	0.4%
45941	Positive regulation of transcription	5,37E-01	0.4%
51347	Positive regulation of transferase activity	3,27E-01	0.4%
9791	Post-embryonic development	2,28E-01	6.3%
9886	Post-embryonic morphogenesis	6,29E-01	0.4%
48569	Post-embryonic organ development	9,05E-02	3.1%
48563	Post-embryonic organ morphogenesis	4,00E-01	0.4%
48528	Post-embryonic root development	5,46E-01	0.4%
16441	Post-transcriptional gene silencing	2,53E-01	1.3%
35194	Post-transcriptional gene silencing by RNA	2,46E-01	1.3%
10608	Post-transcriptional regulation of gene expression	3,12E-01	1.3%
43687	Post-translational protein modification	4,38E-01	5.8%
44238	Primary metabolic process	2,47E-01	30.1%
30422	Production of siRNA involved in RNA interference	1,59E-01	0.9%
70918	Production of small RNA involved in gene silencing by RNA	1,73E-01	0.9%
12501	Programed cell death	1,18E-01	2.7%
8213	Protein amino acid alkylation	4,30E-01	0.4%
6479	Protein amino acid methylation	4,30E-01	0.4%
6468	Protein amino acid phosphorylation	3,12E-01	5.4%
30163	Protein catabolic process	5,81E-01	1.3%
43241	Protein complex disassembly	2,94E-01	0.4%
6457	Protein folding	6,13E-01	0.9%
17038	Protein import	6,06E-01	0.4%
8104	Protein localization	5,40E-01	1.8%
70585	Protein localization in mitochondrion	3,35E-01	0.4%
33365	Protein localization in organelle	5,71E-01	0.4%
19538	Protein metabolic process	7,94E-01	12.6%
6464	Protein modification process	5,47E-01	5.8%
6605	Protein targeting	7,57E-01	0.4%
6626	Protein targeting to mitochondrion	3,35E-01	0.4%

15031	Protein transport	6,88E-01	1.3%
65004	Protein-DNA complex assembly	5,40E-01	0.4%
6508	Proteolysis	5,23E-01	3.1%
51603	Proteolysis involved in cellular protein catabolic process	5,46E-01	1.3%
1522	Pseudouridine synthesis	1,30E-01	0.9%
9145	Purine nucleoside triphosphate biosynthetic process	5,40E-01	0.4%
9144	Purine nucleoside triphosphate metabolic process	5,40E-01	0.4%
6164	Purine nucleotide biosynthetic process	5,93E-01	0.4%
6163	Purine nucleotide metabolic process	6,12E-01	0.4%
9206	Purine ribonucleoside triphosphate biosynthetic process	5,40E-01	0.4%
9205	Purine ribonucleoside triphosphate metabolic process	5,40E-01	0.4%
9152	Purine ribonucleotide biosynthetic process	5,67E-01	0.4%
9150	Purine ribonucleotide metabolic process	5,84E-01	0.4%
7131	Reciprocal meiotic recombination	3,18E-01	0.4%
725	Recombinational repair	2,87E-01	0.4%
10161	Red light signaling pathway	2,47E-01	0.4%
10017	Red or far-red light signaling pathway	4,62E-01	0.4%
3002	Regionalization	2,44E-01	1.3%
22603	Regulation of anatomical structure morphogenesis	4,30E-01	0.4%
90066	Regulation of anatomical structure size	7,57E-01	0.9%
50789	Regulation of biological process	1,20E-01	18.0%
65008	Regulation of biological quality	4,65E-01	3.1%
9889	Regulation of biosynthetic process	9,05E-02	12.1%
50790	Regulation of catalytic activity	7,54E-01	0.4%
51726	Regulation of cell cycle	7,19E-01	0.4%
10564	Regulation of cell cycle progress	3,85E-01	0.4%
10941	Regulation of cell death	4,30E-01	0.4%
45595	Regulation of cell differentiation	2,44E-01	0.9%
42127	Regulation of cell proliferation	8,90E-02	1.3%
8361	Regulation of cell size	7,57E-01	0.9%
31326	Regulation of cellular biosynthetic process	9,05E-02	12.1%
32535	Regulation of cellular component size	7,57E-01	0.9%
10565	Regulation of cellular ketone metabolic process	3,72E-01	0.4%
31323	Regulation of cellular metabolic process	8,94E-02	13.0%
50794	Regulation of cellular process	1,73E-01	15.3%
42752	Regulation of circadian rhythm	1,30E-01	0.9%
50793	Regulation of develomental process	3,12E-01	2.2%
48638	Regulation of developmental growth	4,42E-01	0.4%
9909	Regulation of flower development	2,59E-01	1.3%
10468	Regulation of gene expression	9,05E-02	12.6%
40029	Regulation of gene expression, epigenetic	3,69E-01	1.3%
32583	Regulation of gene-specific transcription	3,18E-01	0.4%
10371	Regulation of gibberellin biosynthetic process	1,59E-01	0.4%
40008	Regulation of growth	5,33E-01	0.4%

10817	Regulation of hormone levels	7,52E-01	0.4%
10310	Regulation of hydrogen peroxide metabolic process	2,44E-01	0.4%
19747	Regulation of isoprenoid metabolic process	2,44E-01	0.4%
43549	Regulation of kinase activity	3,85E-01	0.4%
46890	Regulation of lipid biosynthetic process	2,87E-01	0.4%
19216	Regulation of lipid metabolic process	3,27E-01	0.4%
10556	Regulation of macromolecule biosynthetic process	9,05E-02	11.7%
60255	Regulation of macromolecule metabolic process	9,19E-02	12.6%
40020	Regulation of meiosis	1,90E-01	0.4%
51445	Regulation of meiotic cell cycle	2,26E-01	0.4%
48509	Regulation of meristem development	5,42E-01	0.4%
10075	Regulation of meristem growth	3,67E-01	0.4%
9934	Regulation of meristem structural organization	3,35E-01	0.4%
19222	Regulation of metabolic process	8,94E-02	13.9%
65009	Regulation of molecular function	7,73E-01	0.4%
51239	Regulation of multicellular organismal process	3,63E-01	1.8%
51171	Regulation of nitrogen compound metabolic process	9,52E-02	11.7%
19219	Regulation of nucleobase, nucleoside, nucleotide and nuclei acid metabolic process	9,19E-02	11.7%
80010	Regulation of oxygen and reactive oxigen species metabolic process	3,18E-01	0.4%
6885	Regulation of pH	2,03E-01	0.4%
19220	Regulation of phosphate metabolic process	4,30E-01	0.4%
51174	Regulation of phosphorus metabolic process	4,30E-01	0.4%
42325	Regulation of phosphorylation	4,14E-01	0.4%
48580	Regulation of post-embryonic development	3,67E-01	1.3%
80090	Regulation of primary metabolic process	9,19E-02	12.1%
43067	Regulation of programmed cell death	4,00E-01	0.4%
45859	Regulation of protein kinase activity	3,85E-01	0.4%
51252	Regulation of RNA metabolic process	2,41E-01	5.8%
43455	Regulation of secondary metabolic process	4,06E-01	0.4%
90322	Regulation of superoxide metabolic process	1,59E-01	0.4%
45449	Regulation of transcription	9,05E-02	11.7%
6355	Regulation of transcription, DNA-dependent	2,37E-01	5.8%
51338	Regulation of transferase activity	4,00E-01	0.4%
3	Reproduction	3,78E-01	5.4%
48610	Reproductive cellular process	7,57E-01	0.4%
3006	Reproductive developmental process	3,67E-01	4.9%
22414	Reproductive process	3,64E-01	5.4%
48609	Reproductive process in a multicellular organism	2,87E-01	0.9%
48608	Reproductive structure development	2,94E-01	4.9%
712	Resolution of meiotic recombination intermediates	1,73E-01	0.4%
9628	Response to abiotic stimulus	9,05E-02	9.9%
9737	Response to abscisic acid stimulus	4,30E-01	1.8%
9733	Response to auxin stimulus	4,47E-01	1.8%
9617	Response to bacterium	2,68E-01	2.2%

9607	Response to biotic stimulus	2,80E-01	4.0%
46686	Response to cadmium ion	3,18E-01	2.2%
9743	Response to carbohydrate stimulus	2,82E-01	1.8%
42221	Response to chemical stimulus	1,86E-01	11.2%
10200	Response to chitin	2,59E-01	1.3%
9409	Response to cold	1,20E-01	3.1%
9735	Response to cytokinin stimulus	3,34E-01	0.9%
9269	Response to desiccation	3,35E-01	0.4%
6974	Response to DNA damage stimulus	5,57E-01	0.9%
42493	Response to drug	1,76E-01	0.9%
43331	Response to dsRNA	1,73E-01	0.9%
9719	Response to endogenous stimulus	5,40E-01	4.0%
9723	Response to ethylene stimulus	4,74E-01	0.9%
10332	Response to gamma radiation	2,68E-01	0.4%
9739	Response to gibberellin stimulus	7,11E-01	0.4%
9408	Response to heat	3,14E-01	1.3%
9746	Response to hexose stimulus	3,67E-01	0.4%
9725	Response to hormone stimulus	4,75E-01	4.0%
10035	Response to inorganic substance	4,14E-01	2.7%
10212	Response to ionizing radiation	3,18E-01	0.4%
9753	Response to jasmonic acid stimulus	5,40E-01	0.9%
9416	Response to light stimulus	1,90E-01	4.0%
10038	Response to metal ion	3,12E-01	2.7%
34284	Response to monosaccharide stimulus	3,67E-01	0.4%
9624	Response to nematode	5,40E-01	0.4%
10033	Response to organic substance	2,26E-01	7.2%
6970	Response to osmotic stress	7,73E-01	1.3%
51707	Response to other organism	4,22E-01	3.1%
10193	Response to ozone	3,67E-01	0.4%
51789	Response to protein stimulus	2,83E-01	0.4%
9314	Response to radiation	2,03E-01	4.0%
10114	Response to red light	5,27E-01	0.4%
9639	Response to red or far red light	3,71E-01	1.3%
9751	Response to salicylic acid stimulus	3,25E-01	1.3%
9651	Response to salt stress	7,40E-01	1.3%
50896	Response to stimulus	9,52E-02	20.7%
6950	Response to stress	3,83E-01	9.9%
9266	Response to temperature stimulus	9,05E-02	4.5%
9636	Response to toxin	3,27E-01	0.4%
6986	Response to unfolded protein	2,47E-01	0.4%
9411	Response to UV	1,73E-01	1.3%
10224	Response to UV-B	2,30E-01	0.9%
9615	Response to virus	4,72E-01	0.4%
9415	Response to water	4,47E-01	1.3%

9414	Response to water deprivation	4,30E-01	1.3%
9611	Response to wounding	7,67E-01	0.4%
10043	Response to zinc ion	4,06E-01	0.4%
48511	Rhythmic process	2,59E-01	0.9%
22613	Ribonucleoprotein complex biogenesis	3,12E-01	1.3%
9201	Ribonucleoside triphosphate biosynthetic process	5,40E-01	0.4%
9199	Ribonucleoside triphosphate metabolic process	5,40E-01	0.4%
9260	Ribonucleotide biosynthetic process	3,41E-01	0.9%
9259	Ribonucleotide metabolic process	3,61E-01	0.9%
9156	Ribosnucleoside monophosphate biosynthetic process	2,68E-01	0.4%
9161	Ribosnucleoside monophosphate metabolic process	2,83E-01	0.4%
42254	Ribosome biogenesis	2,97E-01	1.3%
31123	RNA 3'-end processing	3,12E-01	0.4%
16246	RNA interference	1,90E-01	0.9%
16070	RNA metabolic process	9,19E-02	5.4%
9451	RNA modification	1,73E-01	1.8%
43631	RNA polyadenylation	2,47E-01	0.4%
6396	RNA processing	8,90E-02	4.5%
48364	Root development	7,19E-01	0.9%
10053	Root epidermal cell differentiation	5,23E-01	0.4%
48765	Root hair cell differentiation	4,62E-01	0.4%
48767	Root hair elongation	3,81E-01	0.4%
10015	Root morphogenesis	6,88E-01	0.4%
22622	Root system development	7,19E-01	0.9%
16072	rRNA metabolic process	3,96E-01	0.9%
6364	rRNA processing	3,96E-01	0.9%
9834	Secondary cell wall biogenesis	3,56E-01	0.4%
19748	Secondary metabolic process	1,59E-01	3.6%
48316	Seed development	9,39E-01	0.9%
9845	Seed germination	2,44E-01	0.9%
90351	Seedling development	2,47E-01	0.9%
10149	Senescence	4,62E-01	0.4%
9070	Serine family amino acid biosynthetic process	3,35E-01	0.4%
9071	Serine family amino acid catabolic process	2,47E-01	0.4%
9069	Serine family amino acid metabolic process	2,01E-01	0.9%
19953	Sexual reproduction	3,03E-01	0.9%
48367	Shoot development	8,22E-01	0.9%
10016	Shoot morphogenesis	8,14E-01	0.4%
22621	Shoot system development	8,26E-01	0.9%
7165	Signal transduction	8,71E-01	1.3%
23060	Signal transmission	8,13E-01	1.8%
23052	Signaling	5,40E-01	4.9%
23046	Signaling process	8,13E-01	1.8%
7062	Sister chromatid cohesion	2,94E-01	0.4%

44283	Small molecule biosynthetic process	1,18E-01	5.8%
44282	Small molecule catabolic process	3,81E-01	1.3%
44281	Small molecule metabolic process	2,01E-01	8.5%
6814	Sodium ion transport	1,59E-01	0.9%
65001	Specification of axis polarity	3,27E-01	0.4%
10093	Specification of floral organ identity	2,68E-01	0.4%
10092	Specification of organ identity	2,68E-01	0.4%
48443	Stamen development	5,23E-01	0.4%
48864	Stem cell development	1,73E-01	0.9%
19827	Stem cell maintenance	1,73E-01	0.9%
48863	Stm cell differentiation	1,73E-01	0.9%
48479	Style development	1,73E-01	0.4%
5986	Sucrose biosynthetic process	2,83E-01	0.4%
5985	Sucrose metabolic process	3,47E-01	0.4%
10182	Sugar mediated signaling pathway	4,00E-01	0.4%
8272	Sulfate transport	3,12E-01	0.4%
97	Sulfur amino acid biosynthetic process	2,01E-01	0.9%
96	Sulfur amino acid metabolic process	2,87E-01	0.9%
44272	Sulfur compound biosynthetic process	3,71E-01	0.9%
6790	Sulfur metabolic process	5,67E-01	0.9%
48731	System development	4,88E-02	8.1%
16114	Terpenoid biosynthetic process	5,75E-01	0.4%
16115	Terpenoid catabolic process	2,83E-01	0.4%
6721	Terpenoid metabolic process	3,85E-01	0.9%
46865	Terpenoid transport	1,18E-01	0.4%
16109	Tetraterpenoid biosynthetic process	3,56E-01	0.4%
16108	Tetraterpenoid metabolic process	3,99E-01	0.4%
9888	Tissue development	8,90E-02	4.0%
9407	Toxin catabolic process	4,87E-01	0.4%
9404	Toxin metabolic process	4,87E-01	0.4%
6350	Transcription	7,50E-01	0.4%
41	Transition metal ion transport	5,10E-01	0.4%
6412	Translation	9,70E-01	2.7%
6415	Translational termination	2,68E-01	0.4%
7169	Transmembrane receptor protein tyrosine kinase signaling pathway	2,48E-01	1.3%
55085	Transmembrane transport	3,53E-01	0.9%
6810	Transport	9,05E-02	11.7%
5992	Trehalose biosynthetic process	3,41E-01	0.4%
5991	Trehalose metabolic process	3,63E-01	0.4%
10054	Trichoblast differentiation	4,75E-01	0.4%
48764	Trichoblast maturation	4,62E-01	0.4%
42939	Tripeptide transport	2,26E-01	0.4%
8033	tRNA processing	4,62E-01	0.4%
6399	tRNAmetabolic process	6,87E-01	0.4%

162	Tryptophan biosynthetic process	3,47E-01	0.4%
6568	Tryptophan metabolic process	3,63E-01	0.4%
160	Two-component signal transduction system (phosphorelay)	5,51E-01	0.4%
6511	Ubiquitin-dependent protein catabolic process	5,40E-01	1.3%
9826	Unidimensional cell growth	8,49E-01	0.4%
10228	Vegetative to reproductive phase transition of meristem	2,26E-01	1.3%
10048	Vernalization response	2,87E-01	0.4%
42761	Very long-chain fatty acid biosynthetic process	1,90E-01	0.4%
38	Very long-chain fatty acid metabolic process	1,59E-01	0.9%
16192	Vesicle-mediated transport	8,63E-01	0.4%
45492	Xylan biosynthetic process	2,68E-01	0.4%
45491	Xylan metabolic process	2,94E-01	0.4%
55069	Zinc ion homeostasis	2,03E-01	0.4%
6829	Zinc ion transport	2,94E-01	0.4%

A.1.4 Transcripts simultaneously up-regulated in 7LD:11LD samples

Table A.4: Frequency analysis of the microarray results according to biological process, of the simultaneously up-regulated transcripts in 7 LD and 11 LD samples, using gene ontology information; performed using the Cytoscape v2.8.3 software platform (Cline *et al*, 2007) and BiNGO v2.44 plugin (Maere *et al*, 2005)

UP-REGULATED (7LD:11LD)

GO-ID	BIOLOGICAL PROCESS	p-val	Freq
9838	Abscission	1,61E-01	0.7%
6083	Acetate metabolic process	1,61E-01	0.3%
9943	Adaxial/abaxial axis specification	3,82E-01	0.3%
9955	Adaxial/abaxial pattern formation	4,15E-01	0.3%
7568	Aging	4,34E-01	0.7%
46164	Alcohol catabolic process	6,29E-01	0.3%
6066	Alcohol metabolic process	5,48E-01	1.1%
19400	Alditol metabolic process	4,15E-01	0.3%
9309	Amine biosynthetic process	8,73E-01	0.3%
9310	Amine catabolic process	6,19E-02	1.4%
9308	Amine metabolic process	3,48E-01	2.6%
43038	Amino acid activation	5,99E-01	0.3%
9660	Amyloplast organization	1,95E-01	0.3%
48532	Anatomical structure arrangement	5,32E-01	0.3%
48856	Anatomical structure development	1,61E-01	9.7%
48646	Anatomical structure formation involved in morphogenesis	7,57E-01	0.3%
9653	Anatomical structure morphogenesis	3,48E-01	3.3%
48466	Androecium development	5,77E-01	0.3%
6915	Apoptosis	8,07E-01	0.3%
19438	Aromatic compound biosynthetic process	5,07E-01	1.1%
6754	ATP biosynthetic process	3,48E-01	0.7%
46034	ATP metabolic process	3,48E-01	0.7%
42773	ATP synthesis coupled electron transport	4,03E-01	0.3%
15986	ATP synthesis coupled proton transport	4,86E-01	0.3%
10252	Auxin homeostasis	3,74E-01	0.3%
9798	Axis specification	4,43E-01	0.3%
7610	Behavior	1,29E-01	0.7%
19484	Beta-alanine catabolic process	1,42E-01	0.3%
19482	Beta-alanine metabolic process	1,42E-01	0.3%
22610	Biological adhesion	3,48E-01	0.3%
65007	Biological regulation	4,34E-01	16.1%
9058	Biosynthetic process	5,77E-01	11.9%
15691	Cadmium ion transport	2,71E-01	0.3%
6816	Calcium ion transport	3,48E-01	0.3%
16051	Carbohydrate biosynthetic process	6,01E-01	1.1%
16052	Carbohydrate catabolic process	3,48E-01	1.1%
5975	Carbohydrate metabolic process	2,14E-01	5.6%
8643	Carbohydrate transport	2,34E-01	0.7%
46394	Carboxylic acid biosynthetic process	7,58E-01	1.1%

46395	Carboxylic acid catabolic process	6,96E-02	1.8%
19752	Carboxylic acid metabolic process	3,26E-01	4.1%
48440	Carpel development	6,19E-02	1.8%
9056	Catabolic process	1,30E-01	5.9%
55080	Cation homeostasis	6,62E-01	0.3%
6812	Cation transport	2,05E-01	2.6%
7155	Cell adhesion	3,48E-01	0.3%
7154	Cell communication	2,88E-01	1.8%
7049	Cell cycle	6,21E-01	0.7%
22403	Cell cycle phase	4,06E-01	0.7%
22402	Cell cycle process	5,48E-01	0.7%
8219	Cell death	9,31E-01	0.3%
48468	Cell development	3,58E-01	1.4%
30154	Cell differentiation	2,36E-01	2.6%
51301	Cell division	6,59E-01	0.3%
45165	Cell fate commitment	4,73E-01	0.3%
1708	Cell fate specification	4,11E-01	0.3%
16049	Cell growth	4,84E-01	1.4%
902	Cell morphogenesis	4,79E-01	1.4%
904	Cell morphogenesis involved in differentiation	3,49E-01	1.1%
45454	Cell redox homeostasis	6,38E-01	0.3%
9932	Cell tip growth	2,56E-01	1.1%
44036	Cell wall macromolecule metabolic process	5,27E-01	0.3%
42545	Cell wall modification	8,16E-01	0.3%
42547	Cell wall modification involved in multidimensional cell growth	4,53E-01	0.3%
71555	Cell wall organization	8,80E-01	0.3%
71554	Cell wall organization or biogenesis	8,45E-01	0.7%
10383	Cell wall polysaccharide metabolic process	3,82E-01	0.3%
16337	Cell-cell adhesion	2,05E-01	0.3%
6081	Cellular aldehyde metabolic process	3,93E-01	0.3%
44106	Cellular amine metabolic process	3,78E-01	2.2%
6519	Cellular amino acid and derivative metabolic process	2,36E-01	3.7%
9063	Cellular amino acid catabolic process	1,34E-01	1.1%
42398	Cellular amino acid derivative biosynthetic process	2,26E-01	1.8%
42219	Cellular amino acid derivative catabolic process	1,86E-01	0.7%
6575	Cellular amino acid derivative metabolic process	1,61E-01	2.6%
6520	Cellular amino acid metabolic process	5,88E-01	1.4%
6725	Cellular aromatic compound metabolic process	5,78E-01	1.4%
42401	Cellular biogenic amine biosynthetic process	5,07E-01	0.3%
42402	Cellular biogenic amine catabolic process	2,05E-01	0.3%
6576	Cellular biogenic amine metabolic process	3,21E-01	0.7%
44249	Cellular biosynthetic process	5,32E-01	11.6%
34637	Cellular carbohydrate biosynthetic process	5,03E-01	1.1%
44275	Cellular carbohydrate catabolic process	5,03E-01	0.7%
44262	Cellular carbohydrate metabolic process	3,43E-01	2.9%
44248	Cellular catabolic process	2,05E-01	4.1%
30003	Cellular cation homeostasis	6,23E-01	0.3%
55082	Cellular chemical homeostasis	6,67E-01	0.3%
22607	Cellular component assembly	1,98E-01	2.6%

44085	Cellular component biogenesis	4,68E-01	2.6%
22411	Cellular component disassembly	3,67E-01	0.3%
32989	Cellular component morphogenesis	5,18E-01	1.4%
16043	Cellular component organization	1,61E-01	7.1%
48869	Cellular developmental process	3,48E-01	2.9%
6073	Cellular glucan metabolic process	7,70E-01	0.3%
19725	Cellular homeostasis	6,20E-01	0.7%
34754	Cellular hormone metabolic process	4,97E-01	0.3%
6873	Cellular ion homeostasis	6,66E-01	0.3%
42180	Cellular ketone metabolic process	3,38E-01	4.1%
44242	Cellular lipid catabolic process	3,38E-01	0.7%
44255	Cellular lipid metabolic process	2,26E-01	3.3%
51641	Cellular localization	6,28E-01	1.8%
34622	Cellular macromolecular complex assembly	2,92E-01	1.4%
34621	Cellular macromolecular complex subunit organization	3,34E-01	1.4%
34645	Cellular macromolecule biosynthetic process	9,76E-01	4.1%
44265	Cellular macromolecule catabolic process	5,52E-01	1.4%
70727	Cellular macromolecule localization	3,77E-01	1.8%
44260	Cellular macromolecule metabolic process	4,02E-01	18.3%
6944	Cellular membrane fusion	4,73E-01	0.3%
16044	Cellular membrane organization	4,14E-01	0.7%
44237	Cellular metabolic process	1,08E-01	31.4%
6875	Cellular metal ion homeostasis	5,34E-01	0.3%
44271	Cellular nitrogen compound biosynthetic process	3,50E-01	2.6%
34641	Cellular nitrogen compound metabolic process	1,42E-01	10.4%
33692	Cellular polysaccharide biosynthesic process	7,22E-01	0.3%
44264	Cellular polysaccharide metabolic process	5,90E-01	0.7%
9987	Cellular process	6,96E-02	41.9%
44257	Cellular protein catabolic process	5,21E-01	1.4%
43623	Cellular protein complex assemby	2,52E-01	1.1%
34613	Cellular protein localization	3,48E-01	1.8%
44267	Cellular protein metabolic process	5,69E-01	12.7%
45333	Cellular respiration	5,99E-01	0.3%
71214	Cellular response to abiotic stimulus	5,99E-01	0.3%
70887	Cellular response to chemical stimulus	4,39E-01	2.2%
71345	Cellular response to cytokinin stimulus	1,42E-01	0.3%
71368	Cellular response to cytokinin stimulus	5,16E-01	0.3%
71495	Cellular response to endogenous stimulus	6,53E-01	1.1%
71369	Cellular response to ethylene stimulus	5,49E-01	0.3%
71496	Cellular response to external stimulus	2,36E-01	1.4%
31668	Cellular response to extracellular stimulus	2,36E-01	1.4%
34605	Cellular response to heat	2,27E-01	0.3%
32870	Cellular response to hormone stimulus	7,93E-01	0.7%
71241	Cellular response to inorganic substance	2,71E-01	0.3%
71395	Cellular response to jasmonic acid stimulus	5,16E-01	0.3%
71248	Cellular response to metal ion	2,27E-01	0.3%
31669	Cellular response to nutrient levels	3,48E-01	1.1%
71310	Cellular response to organic substance	6,20E-01	1.4%
71470	Cellular response to osmotic stress	3,11E-01	0.3%

16036	Cellular response to phosphate starvation	4,23E-01	0.7%
71472	Cellular response to salt stress	2,88E-01	0.3%
71291	Cellular response to selenium ion	1,42E-01	0.3%
9267	Cellular response to starvation	3,21E-01	1.1%
51716	Cellular response to stimulus	3,48E-01	4.4%
33554	Cellular response to stress	3,48E-01	2.6%
43617	Cellular response to sucrose starvation	1,42E-01	0.3%
42631	Cellular response to water deprivation	3,21E-01	0.3%
48878	Chemical homeostasis	5,34E-01	0.7%
6935	Chemotaxis	1,00E-01	0.7%
51026	Chiasma assembly	3,43E-01	0.3%
15995	Chlorophyll biosynthetic process	2,03E-01	0.7%
15994	Chlorophyll metabolic process	2,54E-01	0.7%
9658	Chloroplast organization	6,20E-01	0.3%
16568	Chromatin modification	7,03E-01	0.3%
6325	Chromatin organization	8,73E-01	0.3%
51276	Chromosome organization	7,61E-01	0.7%
70192	Chromosome organization involved in meiosis	3,82E-01	0.3%
7623	Circadian rhythm	5,57E-01	0.3%
15937	Coenzyme A biosynthetic process	2,54E-01	0.3%
15936	Coenzyme A metabolic process	2,54E-01	0.3%
9108	Coenzyme biosynthetic process	2,19E-01	1.1%
6732	Coenzyme metabolic process	2,56E-01	1.4%
51188	Cofactor biosynthetic process	6,66E-02	2.6%
51186	Cofactor metabolic process	1,14E-01	2.9%
48825	Cotyledon development	2,05E-01	0.7%
10388	Cullin deneddylation	1,00E-01	0.7%
10190	Cytochrome B6F complex assembly	2,36E-01	0.3%
17004	Cytochrome complex assembly	3,48E-01	0.3%
9691	Cytokinin biosynthetic process	3,11E-01	0.3%
9736	Cytokinin mediated signaling pathway	5,16E-01	0.3%
9690	Cytokinin metabolic process	4,15E-01	0.3%
7010	Cytoskeleton organization	7,40E-01	0.3%
16265	Death	9,31E-01	0.3%
6952	Defense response	9,86E-01	1.1%
42742	Defense response to bacterium	5,37E-01	1.1%
9816	Defense response to bacterium, incompatible interaction	4,43E-01	0.3%
50832	Defense response to fungus	7,93E-01	0.3%
9814	Defense response, incompatible interaction	5,07E-01	0.7%
9900	Dehiscence	3,48E-01	0.3%
16311	Dephosphorylation	3,33E-03	2.6%
9582	Detection of abiotic stimulus	3,74E-01	0.3%
9593	Detection of chemical stimulus	3,48E-01	0.3%
9722	Detection of cytokinin stimulus	1,42E-01	0.3%
9726	Detection of endogenous stimulus	2,36E-01	0.3%
9581	Detection of external stimulus	4,15E-01	0.3%
9590	Detection of gravity	1,61E-01	0.3%
9720	Detection of hormone stimulus	2,36E-01	0.3%
51606	Detection of stimulus	2,52E-01	0.7%

48588	Developmental cell growth	2,71E-01	1.1%
48589	Developmental growth	4,06E-01	1.4%
60560	Developmental growth involved in morphogenesis	3,49E-01	1.4%
32502	Developmental process	1,30E-01	12.7%
15674	Di-, tri-valent inorganic cation transporte	5,04E-01	0.3%
43648	Dicarboxylic acid metabolic process	6,01E-01	0.3%
46351	Disaccharide biosynthetic process	4,73E-01	0.3%
46352	Disaccharide catabolic process	2,27E-01	0.3%
5984	Disaccharide metabolic process	2,88E-01	0.7%
16103	Diterpenoid catabolic process	2,36E-01	0.3%
16101	Diterpenoid metabolic process	4,27E-01	0.3%
70838	Divalent metal ion transport	3,55E-01	0.3%
71103	DNA conformation change	7,06E-01	0.3%
6259	DNA metabolic process	6,01E-01	1.4%
6281	DNA repair	6,25E-01	0.7%
6260	DNA replication	4,73E-01	0.7%
6265	DNA topological change	3,55E-01	0.3%
22611	Dormancy process	3,48E-01	0.3%
7398	Ectoderm development	5,54E-01	0.7%
22900	Electron transport chain	5,88E-01	0.3%
9790	Embryonic development	6,51E-01	1.8%
9793	Embryonic development ending in seed dormancy	5,67E-01	1.8%
15985	Energy coupled proton transport, down electrochemical gradient	4,86E-01	0.3%
15980	Energy derivation by oxidation of organic compounds	5,99E-01	0.3%
9649	Entrainment of circadian clock	2,05E-01	0.3%
43153	Entrainment of circadian clock by photoperiod	1,95E-01	0.3%
9913	Epidermal cell differentiation	5,49E-01	0.7%
8544	Epidermis development	5,54E-01	0.7%
51234	Establishment of localization	6,22E-01	6.7%
51649	Establishment of localization in cell	5,85E-01	1.8%
45184	Establishment of protein localization	5,04E-01	1.8%
9873	Ethylene mediated signaling pathway	5,46E-01	0.3%
6635	Fatty acid beta-oxidation	4,34E-01	0.3%
33542	Fatty acid beta-oxidation, unsaturated, even number	1,42E-01	0.3%
6633	Fatty acid biosynthetic process	3,33E-01	1.1%
9062	Fatty acid catabolic process	4,68E-01	0.3%
6631	Fatty acid metabolic process	2,26E-01	1.8%
19395	Fatty acid oxidation	4,43E-01	0.3%
9813	Flavonoid biosynthetic process	5,46E-01	0.3%
9812	Flavonoid metabolic process	5,66E-01	0.3%
10227	Floral organ abscission	1,61E-01	0.7%
48437	Floral organ development	6,96E-02	2.6%
48449	Floral organ formation	4,03E-01	0.3%
48444	Floral organ morphogenesis	4,63E-01	0.3%
48438	Floral whorl development	1,59E-01	1.8%
9908	Flower development	6,19E-02	3.7%
48439	Flower morphogenesis	2,27E-01	0.3%
10047	Fruit dehiscence	2,71E-01	0.3%
10154	Fruit development	3,68E-01	2.9%

51319	G2 phase	2,05E-01	0.3%
85	G2 phase of mitotic cell cycle	2,05E-01	0.3%
19375	Galactolipid biosynthetic process	2,54E-01	0.3%
19374	Galactolipid metabolic process	2,54E-01	0.3%
48229	Gametophyte development	9,32E-01	0.3%
9450	Gamma-aminobutyric acid catabolic process	1,61E-01	0.3%
9448	Gamma-aminobutyric acid metabolic process	1,61E-01	0.3%
10467	Gene expression	9,75E-01	4.4%
16458	Gene silencing	5,46E-01	0.7%
6091	Generation of precursor metabolites and energy	7,40E-01	0.7%
45487	Gibberellin catabolic process	2,36E-01	0.3%
9685	Gibberellin metabolic process	4,15E-01	0.3%
6538	Gluamate catabolic process	1,61E-01	0.3%
44042	Glucan metabolic process	7,79E-01	0.3%
6007	Glucose catabolic process	6,10E-01	0.3%
6006	Glucose metabolic process	6,20E-01	0.3%
19761	Glucosinolate biosynthetic process	4,68E-01	0.3%
19760	Glucosinolate metabolic process	5,44E-01	0.3%
6540	Glutamate decarboxylation to succinate	1,61E-01	0.3%
6536	Glutamate metabolic process	3,48E-01	0.3%
9065	Glutamine family amino acid catabolic process	2,54E-01	0.3%
9064	Glutamine family amino acid metabolic process	5,03E-01	0.3%
6071	Glycerol metabolic process	4,11E-01	0.3%
9247	Glycolipid biosynthetic process	3,48E-01	0.3%
6664	Glycolipid metabolic process	3,74E-01	0.3%
9101	Glycoprotein biosynthetic process	5,88E-01	0.3%
9100	Glycoprotein metabolic process	6,01E-01	0.3%
16138	Glycoside biosynthetic process	3,48E-01	0.7%
16139	Glycoside catabolic process	3,67E-01	0.3%
16137	Glycoside metabolic process	2,66E-01	1.1%
19758	Glycosinolate biosynthetic process	4,68E-01	0.3%
19757	Glycosinolate metabolic process	5,44E-01	0.3%
70085	Glycosylation	5,88E-01	0.3%
6097	Glyoxylate cycle	1,61E-01	0.3%
46487	Glyoxylate metabolic process	1,95E-01	0.3%
6896	Golgi to vacuole transport	2,27E-01	0.3%
48193	Golgi vesicle transport	2,52E-01	0.7%
9630	Gravitropism	5,10E-01	0.3%
6752	Group transfer coenzyme metabolic process	2,34E-01	0.7%
40007	Growth	3,21E-01	2.2%
48467	Gynoecium development	6,19E-02	1.8%
35315	Hair cell differentiation	6,10E-01	0.3%
10286	Heat acclimation	3,55E-01	0.3%
6783	Heme biosynthetic process	2,71E-01	0.3%
42168	Heme metabolic process	3,21E-01	0.3%
10410	Hemicellulose metabolic process	3,43E-01	0.3%
18130	Heterocycle biosynthetic process	3,55E-01	1.1%
46700	Heterocycle catabolic process	4,53E-01	0.3%
46483	heterocycle metabolic process	2,23E-01	2.9%

19320	Hexose catabolic process	6,13E-01	0.3%
19318	Hexose metabolic process	7,20E-01	0.3%
6548	Histidine catabolic process	1,61E-01	0.3%
9077	Histidine family amino acid catabolic process	1,61E-01	0.3%
9075	Histidine family amino acid metabolic process	3,36E-01	0.3%
6547	Histidine metabolic process	3,36E-01	0.3%
42592	Homeostatic process	5,48E-01	1.1%
10289	Homogalacturonan biosynthetic process	1,95E-01	0.3%
42446	Hormone byosynthetic process	5,21E-01	0.3%
42445	Hormone metabolic process	6,51E-01	0.3%
9755	Hormone-mediated signaling pathway	7,90E-01	0.7%
6818	Hydrogen transport	5,32E-01	0.3%
6972	Hyperosmotic response	5,66E-01	0.3%
42538	Hyperosmotic salinity response	5,34E-01	0.3%
6955	Immune response	8,62E-01	0.7%
2376	Immune system process	8,73E-01	0.7%
45087	Innate immune response	8,41E-01	0.7%
51325	Interphase	3,43E-01	0.3%
51329	Interphase of mitotic cell cycle	3,43E-01	0.3%
6886	Intracellular protein transport	3,43E-01	1.8%
35556	Intracellular signal transduction	7,10E-01	0.3%
23034	Intracellular signaling pathway	9,03E-01	0.3%
46907	Intracellular transport	5,04E-01	1.8%
6891	Intra-Golgi vesicle-mediated transport	3,21E-01	0.3%
50801	Ion homeostasis	7,13E-01	0.3%
34220	Ion transmembrane transport	5,21E-01	0.3%
6811	Ion transport	3,21E-01	2.6%
16226	Iron-sulfur cluster assembly	3,93E-01	0.3%
8299	Isoprenoid biosynthetic process	7,58E-01	0.3%
8300	Isoprenoid catabolic process	3,21E-01	0.3%
6720	Isoprenoid metabolic process	5,57E-01	0.7%
9861	Jasmonic acid and ethylene-dependent systemic resistance	3,21E-01	0.3%
9695	Jasmonic acid biosynthetic process	3,93E-01	0.3%
9867	Jasmonic acid mediated signaling pathway	5,16E-01	0.3%
9694	Jasmonic acid metabolic process	4,15E-01	0.3%
48527	Lateral root development	5,88E-01	0.3%
10102	Lateral root morphogenesis	4,39E-01	0.3%
48366	Leaf development	5,49E-01	1.1%
9965	Leaf morphogenesis	7,93E-01	0.3%
10150	Leaf senescence	4,53E-01	0.3%
9809	Lignin biosynthetic process	4,53E-01	0.3%
9808	Lignin metabolic process	5,39E-01	0.3%
8610	Lipid biosynthetic process	5,01E-01	1.8%
16042	Lipid catabolic process	1,95E-01	1.1%
6629	Lipid metabolic process	3,48E-01	3.7%
30258	Lipid modification	4,73E-01	0.3%
34440	Lipid oxidation	4,43E-01	0.3%
51179	Localization	6,66E-01	6.7%
40011	Locomotion	1,29E-01	0.7%

7626	Locomotory behavior	1,00E-01	0.7%
279	M phase	6,10E-01	0.3%
51327	M phase of meiotic cell cycle	5,34E-01	0.3%
65003	Macromolecular complex assembly	1,95E-01	2.2%
43933	Macromolecular complex subunit organization	2,05E-01	2.2%
9059	Macromolecule biosynthetic process	9,76E-01	4.1%
9057	Macromolecule catabolic process	3,74E-01	2.2%
43413	Macromolecule glycosylation	5,88E-01	0.3%
33036	Macromolecule localization	7,90E-01	1.8%
43170	Macromolecule metabolic process	3,67E-01	20.5%
43412	Macromolecule modification	2,36E-01	8.6%
10076	Maintenance of floral meristem identity	2,36E-01	0.3%
48497	Maintenance of floral organ identity	1,61E-01	0.3%
10074	Maintenance of meristem identity	3,82E-01	0.3%
48496	Maintenance of organ identity	1,61E-01	0.3%
10231	Maintenance of seed dormancy	2,05E-01	0.3%
6828	Manganese ion transport	2,54E-01	0.3%
7126	Meiosis	5,34E-01	0.3%
7127	Meiosis I	4,68E-01	0.3%
51321	Meiotic cell cycle	6,06E-01	0.3%
30397	Membrane disassembly	1,42E-01	0.3%
61025	Membrane fusion	4,73E-01	0.3%
46467	Membrane lipid biosynthetic process	4,34E-01	0.3%
6643	Membrane lipid metabolic process	2,36E-01	0.7%
61024	Membrane organization	4,14E-01	0.7%
48507	Meristem development	7,33E-01	0.3%
10073	Meristem maintenance	5,88E-01	0.3%
9933	Meristem structural organization	5,07E-01	0.3%
8152	Metabolic process	1,00E-01	38.5%
55065	Metal ion homeostasis	5,34E-01	0.3%
30001	Metal ion transport	4,73E-01	1.1%
31163	Metallo-sulfur cluster assembly	3,93E-01	0.3%
42775	Mitochondrial ATP synthesis coupled electron transport	4,03E-01	0.3%
6122	Mitochondrial electron transport, ubiquinol to cytochrome C	2,27E-01	0.3%
278	Mitotic cell cycle	4,79E-01	0.3%
43632	Modification-dependent macromolecule catabolic process	5,04E-01	1.4%
19941	Modification-dependent protein catabolic process	5,04E-01	1.4%
32787	Monocarboxylic acid metabolic process	2,31E-01	2.6%
46365	Monosaccharide catabolic process	6,13E-01	0.3%
5996	Monosaccharide metabolic process	7,91E-01	0.3%
15749	Monosaccharide transport	2,54E-01	0.3%
15672	Monovalent inorganic cation transport	5,07E-01	0.7%
16071	mRNA metabolic process	1,32E-01	1.8%
6397	mRNA processing	1,00E-01	1.8%
32504	Multicellular organism reproduction	1,95E-01	1.1%
7275	Multicellular organismal development	1,42E-01	11.6%
32501	Multicellular organismal process	1,42E-01	11.9%
9825	Multidimensional cell growth	5,46E-01	0.3%
51704	Multi-organism process	4,79E-01	3.7%

6741	NADP biosynthetic process	1,61E-01	0.3%
6739	NADP metabolic process	2,03E-01	0.7%
6740	NADPH regeneration	4,15E-01	0.3%
34660	ncRNA metabolic process	9,21E-01	0.3%
10254	Nectary development	1,95E-01	0.3%
9959	Negative gravitropism	2,54E-01	0.3%
48519	Negative regulation of biological process	3,43E-01	2.6%
9890	Negative regulation of biosynthetic process	6,62E-01	0.3%
10648	Negative regulation of cell communication	5,10E-01	0.3%
45596	Negative regulation of cell differentiation	2,05E-01	0.7%
8285	Negative regulation of cell proliferation	1,95E-01	0.3%
31327	Negative regulation of cellular biosynthetic process	6,62E-01	0.3%
31324	Negative regulation of cellular metabolic process	7,22E-01	0.3%
48523	Negative regulation of cellular process	3,75E-01	1.4%
31348	Negative regulation of defense response	3,74E-01	0.3%
51093	Negative regulation of developmental process	4,39E-01	0.7%
10629	Negative regulation of gene expression	4,79E-01	1.1%
9938	Negative regulation of gibberellic acid mediated signaling pathway	2,71E-01	0.3%
10558	Negative regulation of macromolecule biosynthetic process	6,47E-01	0.3%
10605	Negative regulation of macromolecule metabolic process	5,16E-01	1.1%
9892	Negative regulation of metabolic process	5,46E-01	1.1%
51172	Negative regulation of nitrogen compound metabolic process	6,47E-01	0.3%
45934	Negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	6,47E-01	0.3%
48585	Negative regulation of response to stimulus	3,68E-01	0.7%
51253	Negative regulation of RNA metabolic process	4,34E-01	0.3%
35467	Negative regulation of signaling pathway	5,07E-01	0.3%
16481	Negative regulation of transcription	6,06E-01	0.3%
45892	Negative regulation of transcription, DNA-dependent	4,34E-01	0.3%
19359	Nicotinamide nucleotide biosynthetic process	2,54E-01	0.3%
46496	Nicotinamide nucleotide metabolic process	2,27E-01	0.7%
6807	Nitrogen compound metabolic process	1,42E-01	10.8%
398	Nuclear mRNA spicing, via spliceosome	1,59E-01	1.1%
90304	Nucleic acid metabolic process	3,48E-01	5.2%
34404	Nucleobase, nucleoside and nucleotide biosynthetic process	3,48E-01	1.1%
55086	Nucleobase, nucleoside and nucleotide metabolic process	1,61E-01	2.2%
34654	Nucleobase, nucleoside, nucleotide and nucleic acid biosynthetic process	3,48E-01	1.1%
6139	Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	2,05E-01	7.4%
33865	Nucleoside biphosphate meabolic process	2,88E-01	0.3%
9116	Nucleoside metabolic process	5,21E-01	0.3%
6753	Nucleoside phosphate metabolic process	1,42E-01	2.2%
9142	Nucleoside triphosphate biosynthetic process	3,48E-01	0.7%
9141	Nucleoside triphosphate metabolic process	3,48E-01	0.7%
9165	Nucleotide biosynthetic process	3,16E-01	1.1%
9117	Nucleotide metabolic process	1,42E-01	2.2%
6857	Oligopeptide transport	6,29E-01	0.3%
9312	Oligosaccharide biosynthetic process	5,03E-01	0.3%
9313	Oligosaccharide catabolic process	2,27E-01	0.3%
9311	Oligosaccharide metabolic process	3,43E-01	0.7%

48513	Organ development	1,81E-01	5.6%
48645	Organ formation	5,10E-01	0.3%
9887	Organ morphogenesis	6,89E-01	0.7%
10260	Organ senescence	4,63E-01	0.3%
48285	Organelle fission	5,53E-01	0.3%
6996	Organelle organization	5,54E-01	2.6%
16053	Organic acid biosynthetic process	7,58E-01	1.1%
16054	Organic acid catabolic process	6,96E-02	1.8%
6082	Organic acid metabolic process	3,27E-01	4.1%
19637	Organophosphate metabolic process	7,33E-01	0.3%
48481	Ovule development	1,42E-01	1.1%
55114	Oxidation reduction	4,03E-01	1.1%
6119	Oxidative phosphorylation	3,38E-01	0.7%
6733	Oxidoreduction coenzyme metabolic process	2,56E-01	0.7%
43436	Oxoacid metabolic process	3,26E-01	4.1%
31408	Oxylipin biosynthetic process	1,95E-01	0.7%
31407	Oxylipin metabolic process	2,05E-01	0.7%
15940	Pantothenate biosynthetic process	2,05E-01	0.3%
15939	Pantothenate metabolic process	2,05E-01	0.3%
7389	Pattern specification process	2,08E-01	1.4%
45489	Pectin biosynthetic process	3,21E-01	0.3%
45488	Pectin metabolic process	3,48E-01	0.3%
19321	Pentose metabolic process	4,34E-01	0.3%
6098	Pentose-phosphate shunt	4,11E-01	0.3%
9052	Pentose-phosphate shunt, non-oxidative branch	1,42E-01	0.3%
15833	Peptide transport	6,29E-01	0.3%
16559	Peroxisome fission	3,11E-01	0.3%
7031	Peroxisome organization	4,43E-01	0.3%
6432	Phenylalanyl-tRNA aminoacylation	2,36E-01	0.3%
9699	Phenylpropanoid biosynthetic process	3,27E-01	1.1%
9698	Phenylpropanoid metabolic process	3,93E-01	1.1%
6796	Phosphate metabolic process	1,52E-01	7.4%
6644	Phospholipid metabolic process	7,13E-01	0.3%
6793	Phosphorous metabolic process	1,52E-01	7.4%
16310	Phosphorylation	4,64E-01	4.8%
9640	Photomorphogenesis	1,61E-01	1.1%
9648	Photoperiodism	2,54E-01	0.7%
48573	Photoperiodism, flowering	5,04E-01	0.3%
15979	Photosynthesis	7,85E-01	0.3%
48827	Phyllome development	4,43E-01	1.4%
46148	Pigment biosynthetic process	2,36E-01	1.1%
42440	Pigment metabolic process	2,83E-01	1.1%
9828	Plant-type cell wall loosening	5,03E-01	0.3%
9827	Plant-type cell wall modification	5,46E-01	0.3%
9831	Plant-type cell wall modification involved in multidimensional cell growth	4,39E-01	0.3%
9664	plant-type cell wall organization	6,67E-01	0.3%
71669	Plant-type cell wall organization or biogenesis	8,22E-01	0.3%
9657	Plastid organization	5,03E-01	0.7%
9944	Polarity specification of adaxial/abaxial axis	3,55E-01	0.3%

9555	Pollen development	8,58E-01	0.3%
9846	Pollen germination	4,79E-01	0.3%
9865	Pollen tube adhesion	1,61E-01	0.3%
48868	Pollen tube development	2,83E-01	1.1%
9860	Pollen tube growth	2,05E-01	1.1%
10183	Pollen tube guidance	1,00E-01	0.7%
9875	Pollen-pistil interaction	4,79E-01	0.3%
9856	Pollination	2,71E-01	1.4%
6596	Polyamine biosynthetic process	3,21E-01	0.3%
6598	Polyamine catabolic process	1,61E-01	0.3%
6595	Polyamine metabolic process	1,45E-01	0.7%
19751	Polyol metabolic process	5,04E-01	0.3%
271	Polysaccharide biosynthetic process	7,41E-01	0.3%
272	Polysaccharide catabolic process	4,39E-01	0.3%
5976	Polysaccharide metabolic process	4,53E-01	1.1%
6779	Porphyrin biosynthetic process	1,59E-01	1.1%
6778	Porphyrin metabolic process	1,95E-01	1.1%
50918	Positive chemotaxis	1,00E-01	0.7%
45727	Positive n of translation	1,61E-01	0.3%
48518	Positive regulation of biological process	2,07E-01	2.2%
9891	Positive regulation of biosynthetic process	2,05E-01	1.1%
10647	Positive regulation of cell communication	4,15E-01	0.3%
31328	Positive regulation of cellular biosynthetic process	2,05E-01	1.1%
31325	Positive regulation of cellular metabolic process	2,36E-01	1.1%
48522	Positive regulation of cellular process	2,56E-01	1.4%
32270	Positive regulation of cellular protein metabolic process	1,95E-01	0.3%
80040	Positive regulation of cellular response to phosphate starvation	1,95E-01	0.3%
51094	Positive regulation of developmental process	3,21E-01	0.7%
9911	Positive regulation of flower development	2,34E-01	0.7%
10628	Positive regulation of gene expression	3,48E-01	0.7%
10557	Positive regulation of macromolecule biosynthetic process	1,99E-01	1.1%
10604	Positive regulation of macromolecule metabolic process	2,14E-01	1.1%
9893	Positive regulation of metabolic process	2,47E-01	1.1%
51173	Positive regulation of nitrogen compound metabolic process	3,60E-01	0.7%
45935	Positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	3,55E-01	0.7%
48582	Positive regulation of post-embryonic development	2,72E-01	0.7%
60148	Positive regulation of post-transcriptional gene silencing	1,42E-01	0.3%
51247	Positive regulation of protein metabolic process	1,95E-01	0.3%
32103	Positive regulation of response to external stimulus	1,95E-01	0.3%
32106	Positive regulation of response to extracellular stimulus	1,95E-01	0.3%
32109	Positive regulation of response to nutrient levels	1,95E-01	0.3%
48584	Positive regulation of response to stimulus	5,92E-01	0.3%
45941	Positive regulation of transcription	3,43E-01	0.7%
9791	Post-embryonic development	6,19E-02	8.2%
9886	Post-embryonic morphogenesis	2,54E-01	1.1%
48569	Post-embryonic organ development	6,19E-02	3.3%
48563	Post-embryonic organ morphogenesis	4,63E-01	0.3%
48528	Post-embryonic root development	3,50E-01	0.7%

10101	Post-embryonic root morphogenesis	4,39E-01	0.3%
6892	Post-Golgi vesicle-mediated transport	2,27E-01	0.3%
16441	Post-transcriptional gene silencing	7,54E-01	0.3%
10608	Post-transcriptional regulation of gene expression	2,36E-01	1.4%
43687	Post-translational protein modification	1,42E-01	8.2%
6813	Potassium ion transport	5,39E-01	0.3%
44238	Primary metabolic process	7,41E-02	33.7%
12501	Programed cell death	8,96E-01	0.3%
6471	Protein amino acid ADPribosylation	2,05E-01	0.3%
6470	Protein amino acid dephosphorylation	2,24E-03	2.6%
6486	Protein amino acid glycosylation	5,88E-01	0.3%
6493	Protein amino acid O-linked glycosylation	1,42E-01	0.3%
6468	Protein amino acid phosphorylation	5,47E-01	4.1%
30163	Protein catabolic process	4,08E-01	1.8%
6461	Protein complex assembly	1,61E-01	1.8%
70271	Protein complex biogenesis	1,61E-01	1.8%
338	Protein deneddylation	1,14E-01	0.7%
6457	Protein folding	3,61E-01	1.4%
8104	Protein localization	5,23E-01	1.8%
19538	Protein metabolic process	5,49E-01	14.6%
32446	Protein modification by small protein conjugation	5,09E-01	0.7%
70647	Protein modification by small protein conjugation or removal	2,31E-01	1.4%
70646	Protein modification by small protein removal	1,61E-01	0.7%
6464	Protein modification process	1,71E-01	8.6%
6605	Protein targeting	5,62E-01	0.7%
6623	Protein targeting to vacuole	3,67E-01	0.3%
6467	Protein thiol-disulfide exchange	2,27E-01	0.3%
15031	Protein transport	5,04E-01	1.8%
16567	Protein ubiquitination	4,79E-01	0.7%
43335	Protein unfolding	1,42E-01	0.3%
6508	Proteolysis	5,62E-01	2.9%
51603	Proteolysis involved in cellular protein catabolic process	5,07E-01	1.4%
10498	Proteosomal protein catabolic process	6,19E-02	1.1%
43161	Proteosomal ubiquitin-dependent protein catabolic process	6,19E-02	1.1%
43248	Proteosome assembly	1,95E-01	0.3%
15992	Proton transport	5,32E-01	0.3%
9954	Proximal/distal pattern formation	2,27E-01	0.3%
42278	Purine nucleoside metabolic process	3,74E-01	0.3%
9145	Purine nucleoside triphosphate biosynthetic process	3,48E-01	0.7%
9144	Purine nucleoside triphosphate metabolic process	3,48E-01	0.7%
6164	Purine nucleotide biosynthetic process	4,03E-01	0.7%
6163	Purine nucleotide metabolic process	2,36E-01	1.1%
46128	Purine ribonucleoside metabolic process	3,74E-01	0.3%
9206	Purine ribonucleoside triphosphate biosynthetic process	3,48E-01	0.7%
9205	Purine ribonucleoside triphosphate metabolic process	3,48E-01	0.7%
9152	Purine ribonucleotide byosynthetic process	3,75E-01	0.7%
9150	Purine ribonucleotide metabolic process	2,26E-01	1.1%
19363	Pyridine nucleotide biosynthetic process	3,21E-01	0.3%
19362	Pyridine nucleotide metabolic process	2,36E-01	0.7%

3002	Regionalization	2,88E-01	1.1%
9787	Regulation of abscisic acid mediated signaling pathway	4,86E-01	0.3%
90066	Regulation of anatomical structure size	5,10E-01	1.4%
31540	Regulation of anthocyanin biosynthetic process	3,11E-01	0.3%
31537	Regulation of anthocyanin metabolic process	3,48E-01	0.3%
50789	Regulation of biological process	4,41E-01	13.8%
65008	Regulation of biological quality	5,16E-01	2.9%
9889	Regulation of biosynthetic process	3,48E-01	8.6%
10646	Regulation of cell communication	4,86E-01	0.7%
45595	Regulation of cell differentiation	2,72E-01	0.7%
42127	Regulation of cell proliferation	4,39E-01	0.3%
8361	Regulation of cell size	5,09E-01	1.4%
31326	Regulation of cellular biosynthetic process	3,48E-01	8.6%
32535	Regulation of cellular component size	5,10E-01	1.4%
31323	Regulation of cellular metabolic process	4,39E-01	8.6%
50794	Regulation of cellular process	4,85E-01	11.9%
32268	Regulation of cellular protein metabolic process	1,14E-01	1.1%
80135	Regulation of cellular response to stress	3,48E-01	0.3%
42752	Regulation of circadian rhythm	3,67E-01	0.3%
31347	Regulation of defense response	6,20E-01	0.3%
50793	Regulation of developmental process	4,11E-01	1.8%
9962	Regulation of flavonoid biosynthetic process	3,48E-01	0.3%
9909	Regulation of flower development	5,06E-01	0.7%
10468	Regulation of gene expression	3,27E-01	9.3%
40029	Regulation of gene expression, epigenetic	6,35E-01	0.7%
60968	Regulation of gene silencing	1,61E-01	0.3%
32583	Regulation of gene-specific transcription	3,55E-01	0.3%
9937	Regulation of gibberelic acid mediated signaling pathway	3,43E-01	0.3%
10817	Regulation of hormone levels	8,09E-01	0.3%
10556	Regulation of macromolecule biosynthetic process	3,74E-01	8.2%
60255	Regulation of macromolecule metabolic process	3,48E-01	9.3%
19222	Regulation of metabolic process	3,82E-01	9.7%
51239	Regulation of multicellular organismal process	7,96E-01	0.7%
51171	Regulation of nitrogen compound metabolic process	5,88E-01	7.1%
19219	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	5,71E-01	7.1%
48580	Regulation of post-embryonic development	6,32E-01	0.7%
60147	Regulation of post-transcriptional gene silencing	1,42E-01	0.3%
80090	Regulation of primary metabolic process	3,93E-01	8.6%
51246	Regulation of protein metabolic process	1,45E-01	1.1%
32101	Regulation of response to external stimulus	2,05E-01	0.3%
32104	Regulation of response to extracellular stimulus	2,05E-01	0.3%
32107	Regulation of response to nutrient levels	2,05E-01	0.3%
48583	Regulation of response to stimulus	3,21E-01	1.4%
51252	Regulation of RNA metabolic process	4,39E-01	4.4%
43455	Regulation of secondary metabolic process	4,68E-01	0.3%
35466	Regulation of signaling pathway	4,71E-01	0.7%
45449	Regulation of transcription	5,32E-01	7.1%
6355	Regulation of transcription, DNA-dependent	4,39E-01	4.4%
6417	Regulation of translation	6,96E-02	1.1%

6446	Regulation of translational initiation	2,54E-01	0.3%
80134	Regulation to response to stress	4,15E-01	0.7%
3	Reproduction	6,19E-02	8.6%
48610	Reproductive cellular process	3,77E-01	1.1%
3006	Reproductive developmental process	6,19E-02	7.8%
22414	Reproductive process	6,19E-02	8.6%
48609	Reproductive process in a multicellular organism	1,93E-01	1.1%
48608	Reproductive structure development	1,00E-01	6.7%
22904	Respiratory electron transport chain	4,39E-01	0.3%
9628	Response to abiotic stimulus	1,45E-01	8.6%
9737	Response to abscisic acid stimulus	5,39E-01	1.4%
9646	Response to absence of light	1,42E-01	0.7%
10044	Response to aluminium ion	2,36E-01	0.3%
9733	Response to auxin stimulus	2,26E-01	2.6%
9617	Response to bacterium	6,25E-01	1.1%
9607	Response to biotic stimulus	5,88E-01	2.6%
9637	Response to blue light	5,62E-01	0.3%
46686	Response to cadmium ion	4,15E-01	1.8%
9743	Response to carbohydrate stimulus	8,97E-01	0.3%
42221	Response to chemical stimulus	1,42E-01	11.9%
9409	Response to cold	6,25E-01	1.1%
34097	Response to cytokinin stimulus	1,42E-01	0.3%
9735	Response to cytokinin stimulus	2,26E-01	1.1%
34285	Response to disaccharide stimulus	5,04E-01	0.3%
6974	Response to DNA damage stimulus	6,49E-01	0.7%
9719	Response to endogenous stimulus	2,05E-01	5.9%
9723	Response to ethylene stimulus	8,09E-01	0.3%
9605	Response to external stimulus	1,30E-01	2.9%
9991	Response to extracellular stimulus	2,69E-01	1.4%
10218	Response to far red light	5,27E-01	0.3%
9750	Response to fructose stimulus	2,71E-01	0.3%
9620	Response to fungus	8,70E-01	0.3%
9749	Response to glucose stimulus	4,11E-01	0.3%
9629	Response to gravity	5,39E-01	0.3%
9408	Response to heat	1,14E-01	2.2%
9746	Response to hexose stimulus	4,15E-01	0.3%
9644	Response to high light intensity	5,34E-01	0.3%
9725	Response to hormone stimulus	2,05E-01	5.6%
42542	Response to hydrogen peroxide	2,66E-01	0.7%
10035	Response to inorganic substance	3,48E-01	2.9%
9625	Response to insect	3,67E-01	0.3%
9753	Response to jasmonic acid stimulus	6,13E-01	0.7%
9642	Response to light intensity	2,28E-01	1.1%
9416	Response to light stimulus	1,42E-01	4.4%
10038	Response to metal ion	4,15E-01	2.2%
51788	Response to misfolded protein	1,95E-01	0.3%
34284	Response to monosaccharide stimulus	4,15E-01	0.3%
9624	Response to nematode	5,97E-01	0.3%
31667	Response to nutrient levels	3,74E-01	1.1%

10033	Response to organic substance	2,05E-01	7.1%
6970	Response to osmotic stress	5,92E-01	1.8%
51707	Response to other organism	6,67E-01	2.2%
6979	Response to oxidative stress	1,93E-01	2.6%
51789	Response to protein stimulus	3,21E-01	0.3%
9314	Response to radiation	1,42E-01	4.4%
302	Response to reactive oxygen species	2,05E-01	1.1%
10114	Response to red light	3,38E-01	0.7%
9639	Response to red or far red light	1,42E-01	2.2%
9751	Response to salicylic acid stimulus	8,31E-01	0.3%
9651	Response to salt stress	5,49E-01	1.8%
10269	Response to selenium ion	1,61E-01	0.3%
304	Response to singlet oxygen	2,54E-01	0.3%
42594	Response to starvation	3,36E-01	1.1%
50896	Response to stimulus	1,45E-01	19.4%
6950	Response to stress	3,48E-01	10.1%
9744	Response to sucrose stimulus	5,03E-01	0.3%
9266	Response to temperature stimulus	2,36E-01	2.9%
9615	Response to virus	2,72E-01	0.7%
9415	Response to water	3,88E-01	1.4%
9414	Response to water deprivation	3,71E-01	1.4%
9611	Response to wounding	5,78E-01	0.7%
48511	Rhythmic process	5,57E-01	0.3%
22618	Ribonucleoprotein complex assembly	2,88E-01	0.3%
22613	Ribonucleoprotein complex biogenesis	8,20E-01	0.3%
9119	Ribonucleoside metabolic process	4,53E-01	0.3%
9201	Ribonucleoside triphosphate biosynthetic process	3,48E-01	0.7%
9199	Ribonucleoside triphosphate metabolic process	3,48E-01	0.7%
9260	Ribonucleotide biosynthetic process	4,06E-01	0.7%
9259	Ribonucleotide metabolic process	2,47E-01	1.1%
32774	RNA biosynthetic process	2,05E-01	1.1%
16070	RNA metabolic process	3,48E-01	3.3%
6396	RNA processing	5,18E-01	1.8%
8380	RNA splicing	2,36E-01	1.1%
375	RNA splicing, via transesterification reactions	1,61E-01	1.1%
377	RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	1,61E-01	1.1%
48829	Root cap development	3,43E-01	0.3%
48364	Root development	3,43E-01	1.8%
10015	Root morphogenesis	7,44E-01	0.3%
22622	Root system development	3,43E-01	1.8%
31146	SCF-dependent proteosomal ubiquitin-dependent protein catabolic process	6,19E-02	0.7%
19748	Secondary metabolic process	3,82E-01	2.2%
48316	Seed development	4,42E-01	2.6%
10162	Seed dormancy	3,48E-01	0.3%
9845	Seed germination	5,34E-01	0.3%
10431	Seed maturation	1,61E-01	0.7%
90351	Seedling development	5,46E-01	0.3%
10149	Senescence	5,27E-01	0.3%
16144	S-glycoside biosynthetic process	4,68E-01	0.3%

16143	S-glycoside metabolic process	5,44E-01	0.3%
9641	Shade avoidance	2,88E-01	0.3%
48367	Shoot development	5,88E-01	1.4%
10016	Shoot morphogenesis	8,70E-01	0.3%
22621	Shoot system development	4,68E-01	1.8%
7165	Signal transduction	8,53E-01	1.4%
23060	Signal transmission	8,09E-01	1.8%
23052	Signaling	9,71E-01	2.6%
23033	Signaling pathway	9,51E-01	1.4%
23046	Signaling process	8,09E-01	1.8%
10387	Signalosome assemby	2,36E-01	0.3%
9647	Skotomorphogenesis	2,05E-01	0.3%
7264	Small GTPase mediated signal transduction	5,99E-01	0.3%
44283	Small molecule biosynthetic process	3,27E-01	4.1%
44282	Small molecule catabolic process	1,00E-01	2.6%
44281	Small molecule metabolic process	1,42E-01	9.3%
65001	Specification of axis polarity	3,67E-01	0.3%
10093	Specification of floral organ identity	3,11E-01	0.3%
48833	Specification of floral organ number	1,61E-01	0.3%
10092	Specification of organ identity	3,11E-01	0.3%
48832	Specification of organ number	1,61E-01	0.3%
8295	Spermidine biosynthetic process	2,05E-01	0.3%
8216	Spermidine metabolic process	2,05E-01	0.3%
6597	Spermine biosynthetic process	1,61E-01	0.3%
8215	Spermine metabolic process	1,61E-01	0.3%
6665	Sphingolipid metabolic process	3,82E-01	0.3%
245	Spliceosome assembly	2,36E-01	0.3%
48443	Stamen development	5,77E-01	0.3%
5982	Starch metabolic process	5,07E-01	0.3%
48864	Stem cell development	4,34E-01	0.3%
48863	Stem cell differentiation	4,39E-01	0.3%
19827	Stem cell maintenance	4,34E-01	0.3%
6694	Steroid biosynthetic process	5,10E-01	0.3%
8202	Steroid metabolic process	6,01E-01	0.3%
16126	Sterol biosynthetic process	4,15E-01	0.3%
16125	Sterol metabolic process	4,43E-01	0.3%
48480	Stigma development	1,61E-01	0.3%
10118	Stomatal movement	4,03E-01	0.3%
48479	Style development	1,95E-01	0.3%
6105	Succinate metabolic process	1,61E-01	0.3%
5986	Sucrose biosynthetic process	3,21E-01	0.3%
5987	Sucrose catabolic process	1,95E-01	0.3%
10131	Sucrose catabolic process, using invertase or sucrose synthase	1,95E-01	0.3%
5985	Sucrose metabolic process	1,61E-01	0.7%
103	Sulfate assimilation	3,48E-01	0.3%
44272	Sulfur compound biosynthetic process	7,03E-01	0.3%
6790	Sulfur metabolic process	4,79E-01	1.1%
7129	Synapsis	3,82E-01	0.3%
48731	System development	1,45E-01	5.9%

42330	Taxis	1,00E-01	0.7%
16115	Terpenoid catabolic process	3,21E-01	0.3%
6721	Terpenoid metabolic process	7,20E-01	0.3%
33014	Tetrapyrrole biosynthetic process	1,61E-01	1.1%
33013	Tetrapyrrole metabolic process	2,03E-01	1.1%
9888	Tissue development	6,66E-01	1.1%
9407	Toxin catabolic process	5,46E-01	0.3%
9404	Toxin metabolic process	5,46E-01	0.3%
6350	Transcription	2,31E-01	1.4%
6383	Transcription from RNA polymerase III promoter	1,61E-01	0.3%
6352	Transcription initiation	2,72E-01	0.7%
6351	Transcription, DNA-dependent	2,03E-01	1.1%
41	Transition metal ion transport	5,62E-01	0.3%
6412	Translation	0	1.1%
55085	Transmembrane transport	6,71E-01	0.3%
6810	Transport	6,13E-01	6.7%
10026	Trichome differentiation	6,10E-01	0.3%
43039	tRNA aminoacylation	5,99E-01	0.3%
6418	tRNA aminoacylation for protein translation	5,97E-01	0.3%
6399	tRNAmetabolic process	7,41E-01	0.3%
9606	Tropism	5,53E-01	0.3%
35295	Tube development	2,83E-01	1.1%
160	Two-component signal transduction system (phosphorelay)	6,18E-01	0.3%
6511	Ubiquitin-dependent protein catabolic process	5,04E-01	1.4%
9826	Unidimensional cell growth	3,49E-01	1.4%
7034	Vacuolar transport	4,11E-01	0.3%
7033	Vacuole organization	3,43E-01	0.3%
10228	Vegetative to reproductive phase transition of meristem	4,43E-01	0.7%
16192	Vesicle-mediated transport	5,32E-01	1.1%
19079	Viral genome replication	2,05E-01	0.3%
19058	Viral infectious cycle	2,05E-01	0.3%
46786	Viral replication complex formation and maintenance	2,05E-01	0.3%
16032	Viral reproduction	2,71E-01	0.3%
22415	Viral reproductive process	2,71E-01	0.3%
9110	Vitamin biosynthetic process	5,97E-01	0.3%
6766	Vitamin metabolic process	6,13E-01	0.3%
42364	Water-soluble vitamin biosynthetic process	5,44E-01	0.3%
6767	Water-soluble vitamin metabolic process	5,62E-01	0.3%
45493	Xylan catabolic process	1,95E-01	0.3%
45491	Xylan metabolic process	3,43E-01	0.3%
10051	Xylem and phloem pattern formation	5,34E-01	0.3%

A.2 Microarray frequency analysis for molecular function

A.2.1 Transcripts simultaneously down-regulated in 2LD:7LD:11LD samples

Table A.5: Frequency analysis of the microarray results according to molecular function, of the simultaneously down-regulated transcripts in 2LD, 7 LD and 11 LD samples, using gene ontology information; performed using the Cytoscape v2.8.3 software platform (Cline *et al*, 2007) and BiNGO v2.44 plugin (Maere *et al*, 2005)

DOWN-REGULATED GENES (2LD:7LD:11LD)

GO-ID	MOLECULAR FUNCTION	p-val	Freq
10295	(+)-abscisic acid 8'-hydroxylase activity	2,69E-01	0.1%
16629	12-oxophytodienoate reductase activity	2,51E-01	0.1%
51537	2 iron, 2 sulfur cluster binding	1,67E-01	0.3%
43138	3'-5' DNA helicase activity	1,41E-01	0.1%
42781	3'-tRNA processing endoribonuclease activity	2,69E-01	0.1%
51539	4 iron, 4 sulfur cluster binding	2,88E-01	0.1%
3867	4-aminobutyrate transaminase activity	1,41E-01	0.1%
34387	4-aminobutyrate:pyruvate transaminase activity	1,41E-01	0.1%
10427	Abscisic acid binding	2,88E-01	0.1%
3984	Acetolactate synthase activity	2,51E-01	0.1%
16407	Acetyltransferase activity	6,13E-01	0.3%
3993	Acid phosphatase activity	7,82E-01	0.1%
16881	Acid-amino acid ligase activity	1,57E-03	3.3%
15172	Acidic amino acid transmembrane transporter activity	1,04E-01	0.3%
3779	Actin binding	2,50E-01	0.6%
51015	Actin filament binding	5,95E-02	0.3%
22804	Active transmembrane transporter activity	4,71E-01	2.4%
36	Acyl carrier activity	4,40E-01	0.1%
8415	Acyltransferase activity	2,51E-01	1.2%
30554	Adenyl nucleotide binding	4,02E-01	6.4%
32559	Adenyl ribonucleotide binding	3,16E-01	6.4%
70566	Adenylyltransferase activity	5,45E-01	0.1%
22858	Alanine transmembrane transporter activity	1,41E-01	0.1%
71771	Aldahyde decarbonylase activity	1,41E-01	0.1%
4038	Allantoinase activity	1,41E-01	0.1%
46556	Alpha-N-arabinofuranosidase activity	2,69E-01	0.1%
4044	Amidophosphoribosyltransferase activity	2,51E-01	0.1%
43176	Amine binding	1,41E-01	0.4%
5275	Amine transmembrane transporter activity	3,69E-02	1.1%
16597	Amino acid binding	1,41E-01	0.4%
15171	Amino acid transmembrane transporter activity	2,37E-02	1.1%
4812	Aminoacyl-tRNA ligase activity	8,91E-01	0.1%
16160	Amylase activity	5,28E-01	0.1%
8509	Anion transmembrane transporter activity	6,15E-01	0.4%
16209	Antioxidant activity	7,65E-01	0.4%
15297	Antiporter activity	5,82E-01	0.6%

15181	Argnine transmembrane transporter activity	2,69E-01	0.1%
15173	Aromatic amino acid transmembrane transporter activity	2,29E-01	0.1%
15105	Arsinite transmembrane transporter activity	2,51E-01	0.1%
4190	Aspartic-type endopeptidase activity	9,15E-01	0.1%
70001	Aspartic-type peptidase activity	9,15E-01	0.1%
3680	AT DNA binding	2,51E-01	0.1%
5524	ATP binding	3,11E-01	6.4%
16887	ATPase activity	7,33E-01	1.2%
42623	ATPase activity, coupled	6,86E-01	1.1%
43492	ATPase activity, coupled to movement of substances	7,65E-01	0.4%
42625	ATPase activity, coupled to transmembrane movement of ions	7,98E-01	0.1%
15662	ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism	7,33E-01	0.1%
42626	ATPase activity, coupled to transmembrane movement of substances	7,65E-01	0.4%
8026	ATP-dependent helicase activity	9,36E-01	0.1%
10011	Auxin binding	3,19E-01	0.1%
10329	Auxin efflux transmembrane transporter activity	2,69E-01	0.1%
15174	Basic amino acid transmembrane transporter activity	2,88E-01	0.1%
16161	Beta-amylase activity	3,84E-01	0.1%
4565	Beta-galactosidase activity	7,65E-01	0.1%
4566	Beta-glucuronidase activity	2,51E-01	0.1%
5488	Binding	3,50E-04	47.8%
34432	Bis(5'-adenosyl)-pentaphosphatase activity	2,51E-01	0.1%
5227	Calcium activated cation channel activity	1,41E-01	0.1%
5509	Calcium ion binding	4,25E-01	1.1%
15368	Calcium:cation antiporter activity	4,05E-01	0.1%
5432	Calcium:sodium antiporter activity	2,69E-01	0.1%
15269	Calcium-activated potassium channel activity	1,41E-01	0.1%
80087	Callose binding	8,57E-02	0.3%
5516	Calmodulin binding	8,79E-01	0.4%
4683	Calmodulin-dependent protein kinase activity	7,33E-01	0.1%
30246	Carbohydrate binding	5,93E-01	0.6%
19200	Carbohydrate kinase activity	7,55E-01	0.1%
16830	Carbon-carbon lyase activity	3,96E-01	0.6%
8805	Carbon-monoxide oxygenase activity	2,51E-01	0.1%
16835	Carbon-oxygen lyase activity	7,33E-01	0.4%
16846	Carbon-sulfur lyase activity	4,07E-01	0.3%
4091	Carboxylesterase activity	7,65E-01	1.2%
31406	Carboxylic acid binding	1,25E-01	0.6%
46943	Carboxylic acid transmembrane transporter activity	5,48E-02	1.1%
16831	Carboxy-lyase activity	5,82E-01	0.3%
4180	Carboxypeptidase activity	1,41E-01	0.8%
8808	Cardiolipin synthase activity	1,41E-01	0.1%
3824	Catalytic activity	6,61E-03	37.6%
43169	Cation binding	5,45E-01	9.1%
5261	Cation channel activity	3,76E-01	0.3%
8324	Cation transmembrane transporter activity	8,11E-01	1.2%
15491	Cation:cation antiporter activity	8,55E-01	0.1%
15326	Cationic amino acid transmembrane transporter activity	3,69E-01	0.1%
16759	Cellulose synthase activity	7,45E-01	0.1%

15267	Channel activity	5,28E-01	0.6%
3682	Chromatin binding	7,45E-01	0.1%
16859	Cis-trans isomerase activity	5,82E-01	0.3%
50502	Cis-zeatin O-beta-D-glucosyltransferase activity	2,88E-01	0.1%
50897	Cobalt ion binding	7,65E-01	0.1%
50662	Coenzyme binding	8,61E-01	0.4%
48037	Cofactor binding	7,73E-01	0.8%
5507	Copper ion binding	9,32E-01	0.4%
5375	Copper ion transmembrane transporter activity	2,42E-01	0.3%
15088	Copper uptake transmembrane transporter activity	2,29E-01	0.1%
8420	CTD phosphatase activity	2,69E-01	0.1%
30551	Cyclic nucleotide binding	6,56E-01	0.1%
30332	Cyclin binding	2,88E-01	0.1%
4693	Cyclin-dependent protein kinase activity	1,04E-01	0.6%
4861	Cyclin-dependent protein kinase inhibitor activity	3,69E-01	0.1%
16538	Cyclin-dependent protein kinase regulator activity	1,04E-01	0.6%
4197	Cysteine-type endopeptidase activity	7,95E-01	0.1%
8234	Cysteine-type peptidase activity	7,98E-01	0.3%
8092	Cytoskeletal protein binding	2,75E-01	0.8%
3684	Damage DNA binding	6,04E-01	0.1%
4536	Deoxyribonuclease activity	3,69E-01	0.1%
15082	Di-, tri-valent inorganic cation transmembrane transporter activity	5,98E-01	0.3%
8836	Diaminopimelate decarboxilase activity	2,29E-01	0.1%
42936	Dipeptide transporter activity	2,69E-01	0.1%
47334	Diphosphate-fructose-6-phosphate 1-phosphotransferase activity	2,69E-01	0.1%
15036	Disulfide oxidoreductase activity	4,07E-01	0.3%
3914	DNA (6-4) photolyase activity	1,41E-01	0.1%
3886	DNA (cytosine-5-)-methyltranferase activity	2,88E-01	0.1%
3677	DNA binding	3,81E-10	18.0%
3689	DNA clamp loader activity	5,95E-02	0.3%
3678	DNA helicase activity	5,45E-01	0.1%
19104	DNA N-glycosylate activity	5,08E-01	0.1%
3913	DNA photolyase activity	2,88E-01	0.1%
34061	DNA polymerase activity	1,94E-01	0.4%
3896	DNA primase activity	2,51E-01	0.1%
8094	DNA-dependent ATPase activity	2,25E-01	0.4%
3887	DNA-directed DNA polymerase activity	1,41E-01	0.4%
3899	DNA-directed RNA polymerase activity	3,69E-01	0.4%
9008	DNA-methyltransferase activity	2,88E-01	0.1%
3690	Double-stranded DNA binding	3,84E-01	0.3%
3725	Double-stranded RNA binding	2,51E-01	0.3%
8144	Drug binding	6,04E-01	0.1%
15238	Drug transmembrane transporter activity	5,93E-01	0.1%
8460	dTDP-glucose 4,6-dehydratase activity	1,41E-01	0.1%
4170	dUDP diphosphatase activity	1,41E-01	0.1%
15562	Efflux transmembrane transporter activity	4,24E-01	0.1%
9055	Electron carrier activity	7,65E-01	1.6%
4520	Endodeoxyribonuclease activity	2,69E-01	0.1%
4519	Endonuclease activity	1,00E-01	0.9%

16893	Endonuclease activity, active with either ribo or deoxyribonucleic acids and producing 5'-phosphomonoesters	3,76E-01	0.3%
4175	Endopeptidase activity	4,57E-01	1.4%
4521	Endoribonuclease activity	2,85E-01	0.4%
16891	Endoribonuclease activity, producing 5'-phosphomonoesters	3,69E-01	0.3%
4300	Enoyl-CoA hydratase activity	2,69E-01	0.1%
8047	Enzyme activator activity	8,88E-01	0.1%
19899	Enzyme binding	4,97E-01	0.3%
4857	Enzyme inhibitor activity	9,47E-01	0.3%
30234	Enzyme regulator activity	8,99E-01	0.9%
46923	ER retention sequence binding	2,29E-01	0.1%
51740	Ethylene binding	2,88E-01	0.1%
8238	Exopeptidase activity	2,51E-01	0.8%
9922	Fatty acid elongase activity	1,04E-01	0.3%
4312	Fatty acid synthase activity	2,19E-01	0.3%
15245	Fatty acid transporter activity	1,41E-01	0.1%
5528	FK506 binding	5,82E-01	0.1%
10181	FMN binding	5,40E-01	0.1%
9378	Four-way junction helicase activity	1,41E-01	0.1%
15925	Galactosidase activity	7,73E-01	0.1%
22836	Gated channel activity	3,76E-01	0.4%
4345	Glucose-6-phosphate dehydrogenase activity	3,19E-01	0.1%
46527	Glucosyltransferase activity	3,92E-01	0.8%
15020	Glucuronosyltransferase activity	3,84E-01	0.1%
16595	Glutamate binding	2,69E-01	0.1%
4367	Glycerol-3-phosphate dehydrogenase (NAD+) activity	2,69E-01	0.1%
4696	Glycogen synthase kinase 3 activity	8,57E-02	0.3%
5525	GTP binding	9,34E-01	0.4%
51020	GTPase binding	3,30E-01	0.3%
19001	Guanyl nucleotide binding	8,79E-01	0.6%
32561	Guanyl ribonucleotide binding	9,34E-01	0.4%
31072	Heat shock protein binding	7,95E-01	0.1%
4386	Helicase activity	9,36E-01	0.3%
20037	Heme binding	9,12E-01	0.8%
15334	High affinity oligopeptide transporter activity	1,41E-01	0.1%
42054	Histone methyltransferase activity	5,82E-01	0.1%
42800	Histone methyltransferase activity (H3-K4 specific)	2,29E-01	0.1%
18024	Histone-lysine N-methyltransferase activity	4,71E-01	0.1%
8898	Homocysteine S-methyltransferase activity	2,51E-01	0.1%
42562	Hormone binding	9,70E-02	0.4%
16787	Hydrolase activity	3,48E-01	12.0%
16820	Hydrolase activity, actin on acid anhydrides, catalyzing transmembrane movement of substances	7,73E-01	0.4%
16818	Hydrolase activity, actin on acid anhydrides, in phosphorus-containing anhydrides	7,73E-01	2.5%
16817	Hydrolase activity, acting on acid anhydrides	7,82E-01	2.5%
16810	Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	9,46E-01	0.1%
16812	Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in cyclic amides	3,19E-01	0.1%
16788	Hydrolase activity, acting on ester bonds	2,88E-01	4.6%
16798	Hydrolase activity, acting on glycosyl bonds	4,66E-01	2.0%
16799	Hydrolase activity, hydrolyzing N-glycosyl compounds	5,45E-01	0.1%

4553	Hydrolase activity, hydrolyzing O-glycosyl compounds	5,82E-01	1.7%
16836	Hydro-lyase activity	4,38E-01	0.4%
50734	Hydroxycinnamoyltransferase activity	2,69E-01	0.1%
42802	Identical protein binding	2,86E-01	0.8%
10279	Indole-3-acetic acid amido synthetase activity	3,19E-01	0.1%
15103	Inorganic anion transmembrane transporter activity	3,76E-01	0.4%
22890	Inorganic cation transmembrane transporter activity	9,60E-01	0.3%
4427	Inorganic diphosphatase activity	3,56E-01	0.1%
5217	Intracellular ligand-gated ion channel activity	5,82E-01	0.1%
5242	Inward rectifier potassium channel activity	3,84E-01	0.1%
43167	Ion binding	5,45E-01	9.1%
5216	Ion channel activity	5,30E-01	0.4%
22839	Ion gated channel activity	1,41E-01	0.1%
15075	Ion transmembrane transporter activity	7,95E-01	1.7%
5506	Iron ion binding	9,27E-01	0.9%
5381	Iron ion transmembrane transporter activity	3,56E-01	0.1%
51536	Iron-sulfur cluster binding	1,04E-01	0.6%
16853	Isomerase activity	7,09E-01	0.8%
19840	Isoprenoid binding	3,19E-01	0.1%
19209	Kinase activator activity	2,69E-01	0.1%
16301	Kinase activity	1,35E-02	8.0%
19210	Kinase inhibitor activity	3,69E-01	0.1%
19207	Kinase regulator activity	5,95E-02	0.8%
15180	L-alanine transmembrane transporter activity	1,41E-01	0.1%
15179	L-amino acid transmembrane transporter activity	3,84E-01	0.1%
5313	L-glutamate transmembrane transporter actvity	2,51E-01	0.1%
22834	Ligand-gated channel activity	3,56E-01	0.3%
15276	Ligand-gated ion channel activity	3,56E-01	0.3%
16874	Ligase activity	4,75E-02	3.5%
16876	Ligase activity, forming aminoacyl-tRNA and related compounds	8,91E-01	0.1%
16879	Ligase activity, forming carbon-nitrogen bonds	6,10E-03	3.3%
16875	Ligase activity, forming carbon-oxygen bonds	8,91E-01	0.1%
16298	Lipase activity	9,01E-01	0.3%
8289	Lipid binding	8,88E-01	0.4%
5319	Lipid transporter activity	2,42E-01	0.3%
15189	L-lysine transmembrane transporter activity	2,69E-01	0.1%
46577	Long-chain-alcohol oxidase activity	1,41E-01	0.1%
70529	L-tryptophan aminotransferase activity	2,51E-01	0.1%
50362	L-tryptophan:2-oxoglutarate aminotransferase activity	2,51E-01	0.1%
80097	L-tryptophan:pyruvate aminotransferase activity	2,51E-01	0.1%
16829	Lyase activity	6,52E-01	1.2%
16278	Lysine N-methyltransferase activity	4,71E-01	0.1%
5527	Macrolide binding	5,82E-01	0.1%
22884	Macromolecule transmembrane transporter activity	7,95E-01	0.1%
4708	MAP kinase kinase activity	4,24E-01	0.1%
51540	Metal cluster binding	1,04E-01	0.6%
46872	Metal ion binding	5,37E-01	8.6%
46873	Metal ion transmembrane transporter activity	4,71E-01	0.6%
4222	Metalloendopeptidase activity	7,45E-01	0.1%

8237	Metallopeptidase activity	9,04E-01	0.1%
8168	Methyltransferase activity	5,28E-01	0.9%
3777	Microtubule motor activity	8,88E-01	0.1%
35198	miRNA binding	2,69E-01	0.1%
30983	Mismatched DNA binding	4,24E-01	0.1%
60089	Molecular transducer activity	3,96E-01	1.9%
33293	Monocarboxylic acid binding	4,61E-01	0.1%
4497	Monooxygenase activity	7,65E-01	1.1%
3774	Motor activity	9,27E-01	0.1%
16422	mRNA (2'-O-methyladenosine-N6-)-methyltransferase activity	1,41E-01	0.1%
8174	mRNA methyltransferase activity	1,41E-01	0.1%
8080	N-acetyltransferase activity	5,08E-01	0.3%
16410	N-acyltransferase activity	5,40E-01	0.3%
51287	NAD or NADH binding	6,15E-01	0.1%
19788	NEDD8 ligase activity	1,41E-01	0.1%
15175	Neutral amino acid transmembrane transporter activity	3,53E-02	0.4%
8940	Nitrate reductase activity	2,29E-01	0.1%
15112	Nitrate transmembrane transporter activity	4,71E-01	0.1%
8170	N-methyltransferase activity	3,56E-01	0.3%
4518	Nuclease activity	1,69E-01	1.2%
3676	Nucleic acid binding	3,80E-03	21.4%
15205	Nucleobase transmembrane transporter activity	6,45E-01	0.1%
15932	Nucleobase, nucleoside, nucleotide and nucleic acid transmembrane transporter activity	7,82E-01	0.1%
1882	Nucleoside binding	4,07E-01	6.4%
17111	Nucleoside-triphosphatase activity	9,32E-01	1.9%
47429	Nucleoside-triphosphate diphosphatase activity	2,29E-01	0.1%
166	Nucleotide binding	6,04E-01	8.6%
4551	Nucleotide diphosphatase activity	3,69E-01	0.1%
16779	Nucleotidyltransferase activity	2,69E-01	1.1%
8374	O-acyltransferase activity	6,81E-01	0.1%
50737	O-hydroxycinnamoyltransferase activity	1,41E-01	0.1%
15198	Oligopeptide transporter activity	2,69E-01	0.3%
5342	Organic acid transmembrane transporter activity	5,48E-02	1.1%
15271	Outward rectifier potassium channel activity	3,69E-01	0.1%
16491	Oxidoreductase activity	8,91E-01	4.6%
16614	Oxidoreductase activity, acting on CH-OH group of donors	6,14E-01	0.6%
16661	Oxidoreductase activity, acting on other nitrogenous compounds as donors	2,69E-01	0.1%
16705	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxigen	9,86E-01	0.1%
16709	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, NAD or NADH as one donor, and incorporation of one atom of oxygen	7,45E-01	0.1%
16684	Oxidoreductase activity, acting on peroxide as acceptor	8,26E-01	0.3%
16667	Oxidoreductase activity, acting on sulfur group of donors	4,85E-01	0.4%
16671	Oxidoreductase activity, acting on sulfur group of donors, disulfide as acceptor	6,45E-01	0.1%
16721	Oxidoreductase activity, acting on superoxide radicals as acceptor	3,84E-01	0.1%
16903	Oxidoreductase activity, acting on the aldehyde or oxo group donors	8,39E-01	0.1%
16622	Oxidoreductase activity, acting on the aldehyde or oxo group of donors, cytochrome as acceptor	2,51E-01	0.1%
16627	Oxidoreductase activity, acting on the CH-CH group of donors	6,45E-01	0.3%
16636	Oxidoreductase activity, acting on the CH-CH group of donors, iron-sulfur protein as acceptor	2,29E-01	0.1%
16628	Oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor	5,65E-01	0.1%

16616	Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	6,98E-01	0.4%
16899	Oxidoreductase activity, acting on the CH-OH group of donors, oxygen as acceptor	3,69E-01	0.1%
19825	Oxygen binding	7,82E-01	0.8%
22803	Passive transmembrane transporter activity	5,28E-01	0.6%
1871	Pattern binding	2,51E-01	0.3%
30599	Pectinesterase activity	9,27E-01	0.3%
8233	Peptidase activity	4,34E-01	2.4%
70011	Peptidase activity, acting on L-amino acid peptides	3,84E-01	2.4%
42277	Peptide binding	2,88E-01	0.3%
1653	Peptide receptor activity	2,69E-01	0.1%
15197	Peptide transporter activity	2,69E-01	0.3%
8113	Peptide-methionine-(S)-S-oxide reductase activity	4,71E-01	0.1%
3755	Peptidyl-prolyl cis-trans isomerase activity	5,82E-01	0.3%
4601	Peroxidase activity	8,26E-01	0.3%
16791	Phosphatase activity	5,82E-01	1.1%
8526	Phosphatidylinositol transporter activity	2,69E-01	0.1%
30572	Phosphatidyltransferase activity	2,51E-01	0.1%
8443	Phosphofructokinase activity	4,24E-01	0.1%
4620	Phospholipase activity	5,45E-01	0.3%
4629	Phospholipase C activity	2,85E-01	0.3%
5548	Phospholipid transporter activity	2,88E-01	0.1%
51219	Phosphoprotein binding	4,71E-01	0.1%
4721	Phosphoprotein phosphatase activity	3,76E-01	0.9%
8081	Phosphoric diester hydrolase activity	3,76E-01	0.4%
42578	Phosphoric ester hydrolase activity	4,25E-01	1.6%
16773	Phosphotransferase activity, alcohol group as acceptor	1,04E-01	6.1%
16780	Phosphotransferase activity, for other substituted phosphate groups	4,61E-01	0.1%
16775	Phosphotransferase activity, nitrogenous group as acceptor	5,45E-01	0.1%
47262	Polygalacturonate 4-alpha-galacturonosyltransferase activity	3,11E-01	0.3%
4650	Polygalacturonate activity	8,98E-01	0.1%
30247	Polysaccharide binding	2,51E-01	0.3%
5267	Potassium channel activity	2,92E-01	0.3%
15450	P-P-bond-hydrolysis-driven protein transmembrane transporter activity	7,95E-01	0.1%
15405	P-P-bond-hydrolysis-driven transmembrane transporter activity	7,82E-01	0.6%
15399	Primary active transmembrane transporter activity	7,82E-01	0.6%
5515	Protein binding	4,42E-02	13.5%
46983	Protein dimerization activity	5,94E-01	0.4%
15035	Protein disulfide oxidoreductase activity	3,19E-01	0.3%
46982	Protein heterodimerization activity	7,45E-01	0.1%
4673	Protein histidine kinase activity	5,45E-01	0.1%
42803	Protein homodimerization activity	2,88E-01	0.4%
30295	Protein kinase activator activity	2,51E-01	0.1%
4672	Protein kinase activity	3,69E-02	5.9%
4860	Protein kinase inhibitor activity	3,69E-01	0.1%
19887	Protein kinase regulator activity	5,65E-02	0.8%
8276	Protein methyltransferase activity	7,03E-01	0.1%
45309	Protein phosphorylated amino acid binding	4,71E-01	0.1%
4674	Protein serine/threonine kinase activity	2,87E-02	4.8%
30291	Protein serine/threonine kinase inhibitor activity	3,69E-01	0.1%

4722	Protein serine/threonine phosphatase activity	4,85E-01	0.6%
4712	Protein serine/threonine/tyrosine kinase activity	7,64E-01	0.1%
8320	Protein transmembrane transporter activity	7,95E-01	0.1%
8565	Protein transporter activity	4,82E-01	0.6%
4713	Protein tyrosine kinase activity	6,15E-02	1.9%
4725	Protein tyrosine phosphatase activity	4,71E-01	0.1%
8138	Protein tyrosine/serine/threonine phosphatase activity	4,40E-01	0.1%
33170	Protein-DNA loading ATPase activity	5,95E-02	0.3%
16279	Protein-lysine N-methyltransferase activity	4,71E-01	0.1%
70035	Purine NTP-dependent helicase activity	9,36E-01	0.1%
1883	Purine nucleoside binding	4,05E-01	6.4%
17076	Purine nucleotide binding	4,85E-01	7.0%
32555	Purine ribonucleotide binding	4,33E-01	6.9%
5345	Purine transmembrane transporter activity	5,93E-01	0.1%
30170	Pyrodoxal phosphate binding	4,57E-01	0.3%
16462	Pyrophosphatase activity	8,30E-01	2.4%
80045	Quercetin 3'-O-glucosyltransferase activity	2,51E-01	0.1%
80043	Quercetin 3-O-glucosyltransferase activity	2,88E-01	0.3%
80046	Quercetin 4'-O-glucosyltransferase activity	1,41E-01	0.3%
80044	Quercetin 7-O-glucosyltransferase activity	2,51E-01	0.3%
47205	Quinate O-hydroxycinnamoyltransferase activity	1,41E-01	0.1%
16854	Racemase and epimerase activity	1,79E-01	0.4%
16857	Racemase and epimerase activity, acting on carbohydrates and derivatives	1,41E-01	0.4%
8536	Ran GTPase binding	5,82E-01	0.1%
17016	Ras GTPase binding	5,93E-01	0.1%
4872	Receptor activity	7,98E-01	0.6%
51743	Red chlorophyll catabolite reductase activity	1,41E-01	0.1%
4540	Ribonuclease activity	3,86E-01	0.4%
32553	Ribonucleotide binding	4,33E-01	6.9%
3723	RNA binding	1	1.2%
339	RNA cap binding	2,69E-01	0.1%
8419	RNA lariat debranching enzyme activity	1,41E-01	0.1%
8173	RNA methyltransferase activity	1,30E-01	0.4%
34062	RNA polymerase activity	4,02E-01	0.4%
8757	S-adenosylemthionine-dependent methyltransferase activity	2,62E-01	0.8%
15291	Secondary active transmembrane transporter activity	9,32E-01	0.6%
43565	Sequence-specific DNA binding	5,19E-01	0.4%
17171	Serine hydrolase activity	1,06E-01	1.6%
4185	Serine-type carboxypeptidase activity	1,25E-01	0.8%
4252	Serine-type endopeptidase activity	2,56E-01	0.8%
70008	Serine-type exopeptidase activity	1,25E-01	0.8%
8236	Serine-type peptidase activity	1,06E-01	1.6%
47172	Shikimate O-hydroxycinnamoyltransferase activity	1,41E-01	0.1%
5048	Signal sequence binding	3,56E-01	0.1%
4871	Signal transducer activity	3,96E-01	1.9%
16752	Sinapoyltransferase activity	2,29E-01	0.1%
14	Single-stranded DNA specific endodeoxyribonuclease activity	2,51E-01	0.1%
35197	siRNA binding	5,95E-02	0.3%
19787	Small conjugating protein ligase activity	1,65E-03	3.2%

19783	Small conjugating protein-specific protease activity	6,68E-01	0.1%
31267	Small GTPase binding	5,93E-01	0.1%
8172	S-methyltransferase activity	3,84E-01	0.1%
5484	SNAP receptor activity	6,92E-01	0.1%
15298	Solute:cation antiporter activity	8,69E-01	0.1%
15300	Solute:solute antiporter activity	9,06E-01	0.1%
16566	Specific transcriptional repressor activity	3,69E-01	0.1%
10012	Steroid 22-alpha hydroxylase activity	1,41E-01	0.1%
8395	Steroid hydroxylase activity	2,51E-01	0.1%
3735	Structural constituent of ribosome	9,27E-01	0.9%
5198	Structural molecule activity	9,46E-01	1.2%
43566	Structure-specific DNA binding	4,85E-01	0.3%
22838	Substarte-specific channel activity	5,27E-01	0.6%
22891	Substrate-specific transmembrane transporter activity	4,66E-01	3.2%
22892	Substrate-specific transporter activity	2,40E-01	4.5%
5529	Sugar binding	8,91E-01	0.1%
4781	Sulfate adenyltransferase (ATP) activity	2,69E-01	0.1%
4779	Sulfate adenyltransferase activity	2,88E-01	0.1%
19789	SUMO ligase activity	4,42E-02	0.3%
4784	Superoxide dismutase activity	3,84E-01	0.1%
46906	Tetrapyrrole binding	9,32E-01	0.8%
16790	Thioester hydrolase activity	3,59E-01	0.6%
4298	Threonine-type endopeptidase activity	5,82E-01	0.1%
70003	Threonine-type peptidase activity	5,82E-01	0.1%
8483	Transaminase activity	5,08E-01	0.3%
16563	Transcription activator activity	2,51E-01	1.1%
3713	Transcription coactivator activity	2,69E-01	0.3%
3712	Transcription cofactor activity	3,30E-01	0.3%
3700	Transcription factor activity	7,12E-08	13.0%
8134	Transcription factor binding	4,07E-01	0.3%
30528	Transcription regulator activity	6,85E-08	14.3%
16564	Transcription repressor activity	8,44E-01	0.1%
16757	Transferae activity, transferring glycosyl groups	3,69E-01	2.2%
16740	Transferase activity	5,39E-03	14.6%
16769	Transferase activity, transfering nitrogenous groups	5,40E-01	0.3%
16741	Transferase activity, transfering one-carbon groups	5,37E-01	0.9%
16763	Transferase activity, transfering pentosyl groups	7,89E-01	0.1%
16746	Transferase activity, transferring acyl groups	3,19E-01	1.2%
16747	Transferase activity, transferring acyl groups other than amino-acyl groups	2,62E-01	1.2%
16744	Transferase activity, transferring aldehyde or ketonic groups	4,24E-01	0.1%
16758	Transferase activity, transferring hexosyl groups	3,69E-01	1.6%
16772	Transferase activity, transferring phosphorus-containing groups	5,39E-03	9.3%
46914	Transition metal ion binding	6,63E-01	6.9%
46915	Transition metal ion transmembrane transporter activity	4,07E-01	0.3%
8135	Translation factor activity, nucleic acid binding	9,65E-01	0.1%
3743	Translation initiation factor activity	9,26E-01	0.1%
45182	Translation regulator activity	2,88E-01	0.1%
90079	Translation regulator activity, nucleic acid binding	2,69E-01	0.1%
30371	Translation repressor activity	2,88E-01	0.1%

900	Translation repressor activity, nucleic acid binding	2,69E-01	0.1%
4888	Transmembrane receptor activity	9,32E-01	0.3%
22857	Transmembrane transporter activity	4,75E-01	3.8%
5215	Transporter activity	4,57E-01	5.1%
50403	Trans-zeatin O-beta-D-glucosyltransferase activity	2,88E-01	0.1%
42937	Tripeptide transporter activity	2,51E-01	0.1%
16423	tRNA (guanine) methyltransferase activity	2,69E-01	0.1%
4809	tRNA (guanine-N2-)-methyltransferase activity	2,51E-01	0.1%
8175	tRNA methyltransferase activity	2,88E-01	0.1%
156	Two-component response regulator activity	4,02E-01	0.3%
4221	Ubiquitin thiolesterase activity	2,51E-01	0.6%
4842	Ubiquitin-protein ligase activity	1,49E-02	2.7%
4843	Ubiquitin-specific protease activity	5,82E-01	0.1%
10489	UDP-4-keto-6-deoxy-glucose-3,5-epimerase activity	2,29E-01	0.1%
10490	UDP-4-keto-rhamnose-4-keto-reductase activity	2,29E-01	0.1%
50377	UDP-glucose 4,6-dehydratase activity	2,29E-01	0.1%
35251	UDP-glucosyltransferase activity	3,59E-01	0.6%
50378	UDP-glucuronate 4-epimerase activity	8,57E-02	0.3%
48040	UDP-glucuronate decarboxylase activity	3,56E-01	0.1%
8194	UDP-glycosyltransferase activity	2,51E-01	1.2%
10280	UDP-L-rhamnose synthase activity	2,51E-01	0.1%
51082	Unfolded protein binding	8,39E-01	0.1%
4844	Uracil DNA N-glycosylase activity	1,41E-01	0.1%
46422	Violaxanthin de-epoxidase activity	1,41E-01	0.1%
70279	Vitamin B6 binding	4,57E-01	0.3%
19842	Vitamin binding	5,45E-01	0.3%
22843	Voltage-gated cation channel activity	2,88E-01	0.3%
22832	Voltage-gated channel activity	4,25E-01	0.3%
5244	Voltage-gated ion channel activity	4,25E-01	0.3%
5249	Voltage-gated potassium channel activity	2,85E-01	0.3%
15250	Water channel activity	7,25E-01	0.1%
5372	Water transmembrane transporter activity	7,25E-01	0.1%
9044	Xylan 1,4-beta-xylosidase activity	3,19E-01	0.1%
16762	Xyloglucan:xyloglucosyl transferase activity	3,76E-01	0.3%
8270	Zinc ion binding	3,11E-01	5.4%

A.2.2 Transcripts simultaneously up-regulated in 2LD:7LD:11LD samples

Table A.6: Frequency analysis of the microarray results according to molecular function, of the simultaneously up-regulated transcripts in 2LD, 7 LD and 11 LD samples, using gene ontology information; performed using the Cytoscape v2.8.3 software platform (Cline *et al*, 2007) and BiNGO v2.44 plugin (Maere *et al*, 2005)

UP-REGULATED GENES (2LD:7LD:11LD)

GO-ID	MOLECULAR FUNCTION	p-val	Freq
47513	1,2-alpha-L-fucosidase activity	1,26E-01	0.1%
46509	1,2-diacylglycerol 3-beta-galactosyltransferase activity	1,98E-01	0.1%
3844	1,4-alpha-glucan branching enzyme activity	1,77E-01	0.1%
16629	12-oxophytodienoate reductase activity	1,98E-01	0.1%
9815	1-aminocyclopropane-1-carboxylate oxidase activity	4,32E-01	0.1%
16847	1-aminocyclopropane-1-carboxylate synthase activity	5,05E-01	0.1%
8660	1-aminocydopropane-1-carboxylate deaminase activity	1,26E-01	0.1%
285	1-phosphatidylinositol-3-phosphate 5-kinase activity	1,98E-01	0.1%
16308	1-phosphatidylinositol-4-phosphate 5-kinase activity	1,98E-01	0.2%
3842	1-pyrroline-5-carboxylate dehydrogenase activity	1,26E-01	0.1%
3854	3-beta-hydroxy-delta5-steroid dehydrogenase activity	3,13E-01	0.1%
4028	3-chloroallyl aldehyde dehydrogenase activity	9,83E-02	0.3%
3849	3-deoxy-7-phosphoheptulonate synthase activity	1,98E-01	0.1%
8442	3-hydroxyisobutyrate dehydrogenase activity	1,77E-01	0.1%
42781	3'-tRNA processing endoribonuclease activity	2,34E-01	0.1%
51539	4 iron, 4 sulfur cluster binding	2,65E-01	0.1%
4134	4-alpha-glucanotransferase activity	1,77E-01	0.1%
8696	4-amino-4-deoxychorismate lyase activity	1,26E-01	0.1%
46429	4-hydroxy-3-methylbut-2-en-1-yl-diphosphate synthase activity	1,77E-01	0.1%
30272	5-formyltetrahydrofolate cyclo-ligase activity	1,26E-01	0.1%
17168	5-oxoprolinase (ATP-hydrolizing) activity	1,26E-01	0.1%
80084	5S rDNA binding	1,26E-01	0.1%
8097	5S rRNA binding	1,98E-01	0.1%
3872	6-phosphofructokinase activity	3,13E-01	0.1%
45549	9-cis-epoxycarotenoid dioxygenase activity	3,13E-01	0.1%
3987	Acetate-CoA ligase activity	1,77E-01	0.1%
3984	Acetolactate synthase activity	1,98E-01	0.1%
3985	Acetyl-CoA C-acetyltransferase activity	1,26E-01	0.1%
3988	Acetyl-CoA C-acyltransferase activity	1,77E-01	0.1%
16407	Acetyltransferase activity	1,63E-01	0.6%
3993	Acid phosphatase activity	5,10E-01	0.2%
16881	Acid-amino acid ligase activity	7,63E-01	1.0%
16878	Acid-thiol ligase activity	2,10E-01	0.2%
10309	Acireductone dioxygenase [iron(II)-requiring] activity	7,63E-02	0.2%
3994	Aconitate hydratase activity	1,98E-01	0.1%
3779	Actin binding	3,83E-01	0.3%
22804	Active transmembrane transporter activity	1,34E-02	4.0%
45300	Acyl-[acyl-carrier-protein] desaturase activity	3,13E-01	0.1%
62	Acyl-CoA binding	2,93E-01	0.1%
3995	Acyl-CoA dehydrogenase activity	7,63E-02	0.2%

16411	Acylglycerol O-acyltransferase activity	4,10E-01	0.1%
8415	Acyltransferase activity	1,42E-01	1.3%
4000	Adenosine deaminase activity	1,77E-01	0.1%
4001	Adenosine kinase activity	1,77E-01	0.1%
30554	Adenyl nucleotide binding	1,30E-01	7.2%
32559	Adenyl ribonucleotide binding	1,26E-01	7.1%
47632	Agmatine deiminase activity	1,26E-01	0.1%
8453	Alanine-glyoxylate transaminase activity	2,93E-01	0.1%
4022	Alcohol dehydrogenase (NAD) activity	3,39E-01	0.1%
15665	Alcohol transmembrane transporter activity	3,13E-01	0.1%
4029	Aldehyde dehydrogenase (NAD) activity	2,93E-01	0.1%
16832	Aldehyde-lyase activity	4,73E-01	0.1%
4033	Aldo-keto reductase activity	3,62E-01	0.1%
4555	Alpha, alpha-trehalase activity	1,26E-01	0.1%
4556	Alpha-amylase activity	3,39E-01	0.1%
4560	Alpha-L-fucosidase activity	1,98E-01	0.1%
42887	Amide transmembrane transporter activity	2,93E-01	0.1%
43176	Amine binding	6,30E-01	0.1%
5275	Amine transmembrane transporter activity	4,60E-01	0.3%
16597	Amino acid binding	6,20E-01	0.1%
19202	Amino acid kinase activity	3,39E-01	0.1%
15171	Amino acid transmembrane transporter activity	6,48E-01	0.2%
4045	Aminoacyl-tRNA hydrolase activity	1,26E-01	0.2%
4046	Aminocylase activity	1,98E-01	0.1%
16160	Amylase activity	3,56E-02	0.5%
5253	Anion channel activity	1,98E-01	0.2%
8509	Anion transmembrane transporter activity	7,05E-01	0.3%
42895	Antibiotic transporter activity	2,65E-01	0.1%
16209	Antioxidant activity	6,76E-01	0.5%
15297	Antiporter activity	6,79E-01	0.5%
8060	ARF GTPase activator activity	4,55E-01	0.1%
8793	Aromatic-amino-acid:2-oxoglutarate aminotransferase activity	1,77E-01	0.1%
4067	Asparaginase activity	2,34E-01	0.1%
4190	Aspartic-type endopeptidase activity	5,58E-01	0.3%
70001	Aspartic-type peptidase activity	5,58E-01	0.3%
5524	ATP binding	1,26E-01	7.1%
16887	ATPase activity	2,21E-02	2.8%
42623	ATPase activity, coupled	2,49E-02	2.4%
43492	ATPase activity, coupled to movement of substances	1,34E-02	1.6%
42625	ATPase activity, coupled to transmembrane movement of ions	2,17E-02	0.9%
15662	ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism	2,09E-01	0.3%
42626	ATPase activity, coupled to transmembrane movement of substances	1,34E-02	1.6%
8026	ATP-dependent helicase activity	4,85E-01	0.5%
4176	ATP-dependent peptidase activity	6,20E-01	0.1%
9672	Auxin:hydrogen symporter activity	4,73E-01	0.1%
19187	Beta-1,4-mannosyltransferase activity	1,98E-01	0.1%
16161	Beta-amylase activity	4,68E-02	0.3%
4564	Beta-fructofuranosidase activity	2,93E-01	0.1%
3837	Beta-ureidopropionase activity	1,26E-01	0.1%
5488	Binding	9,60E-02	43.1%
4075	Biotin carboxylase activity	1,77E-01	0.1%

16453	C-acetyltransferase activity	1,26E-01	0.1%
16408	C-acyltransferase activity	1,26E-01	0.2%
5262	Calcium channel activity	1,98E-01	0.1%
10857	Calcium -dependent protein kinase activity	1,77E-01	0.1%
5509	Calcium ion binding	3,05E-01	1.1%
15085	Calcium ion transmembrane transporter activity	5,25E-01	0.1%
9931	Calcium-dependent protein serine/threonine kinase activity	1,77E-01	0.1%
5388	Calcium-transporting ATPase activity	5,05E-01	0.1%
5516	Calmodulin binding	3,92E-01	0.9%
4683	Calmodulin-dependent protein kinase activity	7,55E-01	0.1%
30246	Carbohydrate binding	2,51E-01	0.9%
19200	Carbohydrate kinase activity	8,07E-02	0.6%
19203	Carbohydrate phosphatase activity	3,89E-01	0.1%
15144	Carbohydrate transmembrane transporter activity	1,26E-01	1.0%
4089	Carbonate dehydratase activity	5,43E-01	0.1%
16830	Carbon-carbon lyase activity	1,98E-01	0.7%
8805	Carbon-monoxide oxygenase activity	1,98E-01	0.1%
16835	Carbon-oxygen lyase activity	6,30E-01	0.5%
16846	Carbon-sulfur lyase activity	7,63E-02	0.6%
4091	Carboxylesterase activity	9,76E-01	0.7%
31406	Carboxylic acid binding	4,30E-01	0.2%
46943	Carboxylic acid transmembrane transporter activity	7,59E-01	0.2%
16831	Carboxy-lyase activity	2,11E-01	0.5%
10436	Carotenoid dioxygenase activity	3,13E-01	0.1%
3824	Catalitic activity	7,58E-10	42.7%
43169	Cation binding	1,79E-01	10.4%
5261	Cation channel activity	3,83E-01	0.2%
8324	Cation transmembrane transporter activity	1,34E-02	3.2%
15491	Cation:cation antiporter activity	8,80E-01	0.1%
5402	Cation:sugar symporter activity	1,26E-01	0.9%
19829	Cation-transporting ATPase activity	4,96E-02	0.6%
16759	Cellulose synthase activity	7,67E-01	0.1%
45430	Chalcone isomerase activity	2,34E-01	0.1%
15267	Channel activity	1,98E-01	0.9%
51087	Chaperone binding	2,34E-01	0.1%
5254	Chloride channel activity	3,13E-01	0.1%
16168	Chlorophyll binding	7,63E-02	0.5%
10290	Chlorophyll catabolite transmembrane transporter activity	1,26E-01	0.1%
4105	Choline-phosphate cytidylyltransferase activity	1,77E-01	0.1%
16859	Cis-trans isomerase activity	9,83E-02	0.7%
4108	Citrate (Si)-synthase activity	2,65E-01	0.1%
16421	CoA carboxylase activity	1,26E-01	0.2%
16405	CoA-ligase activity	1,98E-01	0.2%
50897	Cobalt ion binding	1,07E-03	1.1%
50662	Coenzyme binding	6,70E-01	0.6%
48037	Cofactor binding	4,90E-01	1.0%
5507	Copper ion binding	2,42E-01	1.3%
5375	Copper ion transmembrane transporter activity	1,98E-01	0.2%
9975	Cyclase activity	4,73E-01	0.1%
30551	Cyclic nucleotide binding	6,78E-01	0.1%
4693	Cyclin-dependent protein kinase activity	3,83E-01	0.2%

16882	Cyclo-ligase activity	1,77E-01	0.1%
31071	Cysteine desulfurase activity	1,77E-01	0.1%
4197	Cysteine-type endopeptidase activity	8,21E-01	0.1%
4869	Cysteine-type endopeptidase inhibitor activity	3,62E-01	0.1%
8234	Cysteine-type peptidase activity	8,56E-01	0.2%
4127	Cytidylate kinase activity	1,26E-01	0.1%
70567	Cytidylyltransferase activity	4,55E-01	0.1%
8092	Cytoskeletal protein binding	3,13E-01	0.6%
3684	Damage DNA binding	6,30E-01	0.1%
19148	D-cysteine desulfhydrase activity	1,26E-01	0.1%
19213	Deacetylase activity	6,20E-01	0.1%
19239	Deaminase activity	1,98E-01	0.2%
17084	Delta-1-pyrroline-5-carboxylate synthetase activity	1,26E-01	0.1%
15082	Di-, tri-valent inorganic cation transmembrane transporter activity	1,10E-01	0.7%
4144	Diacylglycerol O-acyltransferase activity	1,98E-01	0.1%
30523	Dihydrolipoamide S-acyltransferase activity	2,93E-01	0.1%
4148	Dihydrolipoyl dehydrogenase activity	1,98E-01	0.1%
4742	Dihydrolipoyllysine-residue acetyltransferase activity	2,65E-01	0.1%
4161	Dimethylallyltranstransferase activity	2,34E-01	0.1%
51213	Dioxygenase activity	1,58E-01	0.3%
16778	Diphosphotransferase activity	4,10E-01	0.1%
15036	Disulfide oxidoreductase activity	1,26E-01	0.5%
8301	DNA bending activity	1,77E-01	0.1%
3677	DNA binding	1,98E-01	10.5%
3910	DNA ligase (ATP) activity	2,65E-01	0.1%
3909	DNA ligase activity	2,65E-01	0.1%
19104	DNA N-glycosylate activity	5,05E-01	0.1%
44212	DNA regulatory region binding	2,34E-01	0.1%
3918	DNA topoisomerase (ATP-hydrolyzing) activity	5,49E-02	0.3%
3916	DNA topoisomerase activity	1,21E-01	0.3%
3906	DNA-(apurinic or apyrimidinic site) lyase activity	2,34E-01	0.1%
8094	DNA-dependent ATPase activity	7,11E-01	0.1%
3899	DNA-directed RNA polymerase activity	6,42E-01	0.2%
3690	Double-stranded DNA binding	3,92E-01	0.2%
10385	Double-stranded methylated DNA binding	2,34E-01	0.1%
3725	Double-stranded RNA binding	5,05E-01	0.1%
8144	Drug binding	6,30E-01	0.1%
15238	Drug transmembrane transporter activity	6,20E-01	0.1%
15307	Drug:hydrogen antiporter activity	1,77E-01	0.1%
9055	Electron carrier activity	9,91E-01	0.9%
31176	Endo-1,4-beta-xylanase activity	2,65E-01	0.1%
4519	Endonuclease activity	9,17E-01	0.1%
16893	Endonuclease activity, active with either ribo or deoxyribonucleic acids and producing 5'-phosphomonoesters	7,20E-01	0.1%
4175	Endopeptidase activity	6,20E-01	1.1%
4866	Endopeptidase inhibitor activity	7,72E-01	0.1%
61135	Endopeptidase regulator activity	7,72E-01	0.1%
4521	Endoribonuclease activity	8,19E-01	0.1%
16891	Endoribonuclease activity, producing 5'-phosphomonoesters	7,11E-01	0.1%
8047	Enzyme activator activity	1,77E-01	0.6%
4857	Enzyme inhibitor activity	9,85E-01	0.2%

30234	Enzyme regulator activity	6,74E-01	1.3%
51740	Ethylene binding	2,65E-01	0.1%
8238	Exopeptidase activity	9,51E-01	0.1%
50660	FAD binding	9,30E-01	0.1%
4310	Farnesyl-diphosphate farnesyltransferase activity	1,77E-01	0.1%
4311	Farnesyltranstransferase	4,10E-01	0.1%
5504	Fatty acid binding	2,93E-01	0.1%
15245	Fatty acid transporter activity	1,26E-01	0.1%
8937	Ferredoxin reductase activity	1,98E-01	0.1%
30385	Ferredoxin:thioredoxin reductase activity	1,77E-01	0.1%
4325	Ferrochelatase activity	1,98E-01	0.1%
5528	FK506 binding	6,09E-01	0.1%
10181	FMN binding	5,43E-01	0.1%
4329	Formate-tetrahydrofolate ligase activity	1,98E-01	0.1%
8865	Fructokinase activity	1,98E-01	0.1%
4332	Fructose-bisphosphate aldolase activity	3,13E-01	0.1%
15928	Fucosidase activity	2,34E-01	0.1%
8417	Fucosyltransferase activity	4,73E-01	0.1%
4335	Galactokinase activity	1,14E-01	0.2%
8378	Galactosyltransferase activity	4,73E-01	0.1%
22836	Gated channel activity	4,08E-01	0.3%
5092	GDP-dissociation inhibitor activity	3,13E-01	0.1%
16251	General RNA polymerase II transcription factor activity	2,65E-01	0.1%
16767	Geranylgeranyl-diphosphate geranylgeranyl transferase activity	1,26E-01	0.1%
4337	Geranyltransferase activity	5,20E-02	0.2%
45544	Gibberellin 20-oxidase activity	2,65E-01	0.1%
4340	Glucokinase actvity	1,98E-01	0.1%
4345	Glucose-6-phosphate dehydrogenase activity	2,93E-01	0.1%
4347	Glucose-6-phosphate isomerase activity	1,77E-01	0.1%
15152	Glucose-6-phosphate transmembrane transporter activity	1,98E-01	0.1%
46527	Glucosyltransferase activity	6,48E-01	0.5%
4349	Glutamate 5-kinase activity	1,26E-01	0.1%
4350	Glutamate 5-semialdehyde dehydrogenase activity	1,26E-01	0.1%
16595	Glutamate binding	2,34E-01	0.1%
4359	Glutaminase activity	1,26E-01	0.1%
4360	Glutamine-fructose-6-phosphate transaminase (isomerizing) activity	1,26E-01	0.1%
43295	Glutathione binding	3,89E-01	0.1%
15038	Glutathione disulfide oxidoreductase activity	3,13E-01	0.1%
4602	Glutathione peroxidase activity	3,62E-01	0.1%
15431	Glutathione S-conjugate-exporting ATPase activity	1,26E-01	0.1%
4364	Glutathione transferase activity	1,79E-01	0.5%
4362	Glutathione-disulfide reductase activity	1,77E-01	0.1%
8886	Glyceraldehyde-3-phosphate dehydrogenase (NADP+) activity	1,26E-01	0.1%
8943	Glyceraldehyde-3-phosphate dehydrogenase activity	3,39E-01	0.1%
4370	Glycerol kinase activity	1,26E-01	0.1%
4368	Glycerol-3-phosphate dehydrogenase activity	1,26E-01	0.1%
8889	Glycerophosphodiester phosphodiesterase activity	4,32E-01	0.1%
4133	Glycogen debranching enzyme activity	9,91E-02	0.2%
8020	G-protein coupled photoreceptor activity	7,63E-02	0.2%
4930	G-protein coupled receptor activity	1,14E-01	0.2%
8083	Growth factor activity	3,13E-01	0.1%

5525	GTP binding	8,51E-01	0.6%
8728	GTP diphosphokinase activity	1,98E-01	0.1%
5096	GTPase activator activity	1,02E-01	0.6%
3924	GTPase activity	7,17E-01	0.2%
30695	GTPase regulator activity	1,27E-01	0.7%
19001	Guanyl nucleotide binding	8,58E-01	0.6%
32561	Guanyl ribonucleotide binding	8,51E-01	0.6%
31072	Heat shock protein binding	3,05E-01	0.3%
4386	Helicase activity	3,58E-01	0.9%
20037	Heme binding	1	0.1%
10297	Heteroglycan binding	1,77E-01	0.1%
4395	Hexaprenyldihydroxybenzoate methyltransferase activity	1,26E-01	0.1%
4396	Hexokinase activity	2,93E-01	0.1%
15119	Hexose phosphate transmembrane transporter activity	1,98E-01	0.1%
15149	Hexose transmembrane transporter activity	3,62E-01	0.1%
9927	Histidine phosphotransfer kinase activity	3,62E-01	0.1%
4402	Histone acetyltransferase activity	4,89E-01	0.1%
42393	Histone binding	5,25E-01	0.1%
4407	Histone deacetylase activity	5,43E-01	0.1%
42054	Histone methyltransferase activity	2,69E-01	0.2%
46974	Histone methyltransferase activity (H3-K9 specific)	1,26E-01	0.1%
18024	Histone-lysine N-methyltransferase activity	1,92E-01	0.2%
8898	Homocysteine S-methyltransferase activity	1,98E-01	0.1%
42562	Hormone binding	5,05E-01	0.1%
15078	Hydrogen ion transmembrane transporter activity	3,14E-01	0.6%
46933	Hydrogen ion transporting ATP synthase activity, rotational mechanism	4,32E-01	0.1%
9678	Hydrogen-translocating pyrophosphatase activity	2,65E-01	0.1%
16787	Hydrolase activity	9,83E-02	13.5%
16818	Hydrolase activity, actin on acid anhydrides, in phosphorus-containing anhydrides	1,26E-01	4.0%
16817	Hydrolase activity, acting on acid anhydrides	1,26E-01	4.0%
16820	Hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances	1,34E-02	1.6%
16810	Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	9,99E-03	1.5%
16812	Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in cyclic amides	2,93E-01	0.1%
16814	Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in cyclic amidines	5,58E-01	0.1%
16811	Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amides	1,01E-01	0.7%
16813	Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amidines	2,09E-02	0.3%
16788	Hydrolase activity, acting on ester bonds	3,30E-01	4.2%
16798	Hydrolase activity, acting on glycosyl bonds	5,77E-01	1.8%
16799	Hydrolase activity, hydrolyzing N-glycosyl compounds	5,58E-01	0.1%
4553	Hydrolase activity, hydrolyzing O-glycosyl compounds	5,78E-01	1.6%
16836	Hydro-lyase activity	4,89E-01	0.3%
50734	Hydroxycinnamoyltransferase activity	2,34E-01	0.1%
42802	Identical protein binding	9,74E-01	0.1%
15103	Inorganic anion transmembrane transporter activity	8,87E-01	0.1%
22890	Inorganic cation transmembrane transporter activity	7,84E-02	1.6%
4428	Inositol or phosphatidylinositol kinase activity	2,84E-01	0.3%
4437	Inositol or phosphatidylinositol phosphatase activity	1,98E-01	0.3%
16872	Intramolecular lyase activity	3,62E-01	0.1%
16860	Intramolecular oxidoreductase activity	1,74E-01	0.5%
16861	Intramolecular oxidoreductase activity, interconverting aldoses and ketoses	1,98E-01	0.2%
16862	Intramolecular oxidoreductase activity, interconverting keto- and enol-groups	4,89E-01	0.1%

16864	Intramolecular oxidoreductase activity, transposing S-S bonds	4,89E-01	0.1%
16866	Intramolecular transferase activity	1,98E-01	0.3%
16868	Intramolecular transferase activity, phosphotransferases	1,26E-01	0.2%
50486	Intramolecular transferase activity, transferring hydroxy groups	1,98E-01	0.1%
43167	Ion binding	1,79E-01	10.4%
5216	Ion channel activity	2,42E-01	0.6%
15075	Ion transmembrane transporter activity	2,40E-02	3.7%
5506	Iron ion binding	1	0.3%
51536	Iron-sulfur cluster binding	3,92E-01	0.2%
19156	Isoamylase activity	1,98E-01	0.1%
8909	Isochorismate synthase activity	1,77E-01	0.1%
4449	Isocitrate dehydrogenase (NAD+) activity	2,65E-01	0.1%
4448	Isocitrate dehydrogenase activity	3,39E-01	0.1%
16853	Isomerase activity	1,07E-03	2.6%
8470	Isovaleryl-CoA dehydrogenase activity	1,26E-01	0.1%
16301	Kinase activity	1,05E-01	6.7%
19207	Kinase regulator activity	7,93E-01	0.1%
4462	Lactoylglutathione lyase activity	1,26E-01	0.2%
9702	L-arabinokinase activity	1,26E-01	0.1%
4069	L-aspartate:2-oxoglutarate aminotransferase activity	2,42E-02	0.3%
32791	Lead ion binding	1,77E-01	0.1%
16874	Ligase activity	4,29E-01	2.0%
16885	Ligase activity, forming carbon-carbon bonds	1,26E-01	0.2%
16879	Ligase activity, forming carbon-nitrogen bonds	4,71E-01	1.5%
16877	Ligase activity, forming carbon-sulfur bonds	2,69E-01	0.2%
16886	Ligase activity, forming phosphoric ester bonds	2,93E-01	0.1%
16298	Lipase activity	9,43E-01	0.2%
8289	Lipid binding	3,01E-01	1.0%
1727	Lipid kinase activity	2,69E-01	0.2%
5319	Lipid transporter activity	4,89E-01	0.1%
16992	Lipoate synthase activity	1,98E-01	0.1%
16979	Lipoate-protein ligase activity	1,98E-01	0.1%
16829	Lyase activity	1,26E-01	2.0%
45435	Lycopene epsilon cyclase activity	1,26E-01	0.1%
4468	Lysine N-acetyltransferase activity	4,89E-01	0.1%
16278	Lysine N-methyltransferase activity	1,92E-01	0.2%
5527	Macrolide binding	6,09E-01	0.1%
22884	Macromolecule transmembrane transporter activity	8,22E-01	0.1%
287	Magnesium ion binding	6,57E-01	0.2%
15095	Magnesium ion transmembrane transporter activity	1,26E-01	0.2%
4473	Malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+) activity	7,63E-02	0.2%
16615	Malate dehydrogenase activity	1,98E-01	0.2%
4470	Malic enzyme activity	1,26E-01	0.2%
30145	Manganese ion binding	5,58E-01	0.1%
5384	Manganese ion transmembrane transporter activity	3,13E-01	0.1%
10486	Manganese:hydrogen antiporter activity	1,26E-01	0.1%
51753	Mannan synthase activity	1,98E-01	0.1%
30	Mannosyltransferase activity	3,39E-01	0.1%
33549	MAP kinase phosphatase activity	1,98E-01	0.1%
51540	Metal cluster binding	3,92E-01	0.2%
46872	Metal ion binding	1,63E-01	10.0%

46873	Metal ion transmembrane transporter activity	8,68E-02	1.1%
51139	Metal ion:hydrogen antiporter activity	2,65E-01	0.1%
8237	Metallopeptidase activity	7,59E-01	0.2%
35064	Methylated histone residue binding	3,89E-01	0.1%
8327	methyl-CpG binding	1,98E-01	0.2%
10428	Methyl-CpNpG binding	2,34E-01	0.1%
10429	Methyl-CpNpN binding	2,34E-01	0.1%
4485	Methylcrotonoyl-CoA carboxylase activity	2,42E-02	0.2%
8168	Methyltransferase activity	7,86E-01	0.6%
8017	Microtubule bindind	7,20E-01	0.1%
3777	Microtubule motor activity	9,11E-01	0.1%
60089	Molecular transducer activity	9,17E-01	1.0%
30151	Molybdenum ion binding	1,98E-01	0.1%
33293	Monocarboxylic acid binding	4,55E-01	0.1%
16656	Monodehydroascorbate reductase (NADH) activity	2,34E-01	0.1%
15145	Monosaccharide transmembrane transporter activity	4,55E-01	0.1%
15077	Monovalent inorganic cation transmembrane transporter activity	1,98E-01	0.9%
3774	Motor activity	6,15E-01	0.3%
3729	mRNA binding	5,95E-01	0.1%
5365	Myo-inositol transmembrane transporter activity	2,34E-01	0.1%
5366	myo-inositol:hydrogen synporter activity	1,98E-01	0.1%
8080	N-acetyltransferase activity	3,05E-01	0.3%
16410	N-acyltransferase activity	3,35E-01	0.3%
51287	NAD or NADH binding	6,42E-01	0.1%
3951	NAD+ kinase activity	1,98E-01	0.1%
50126	N-carbamoylputrescine amidase activity	1,26E-01	0.1%
4516	Nicotinate phosphoribosyltransferase activity	1,77E-01	0.1%
8170	N-methyltransferase activity	3,46E-01	0.2%
4518	Nuclease activity	8,75E-01	0.3%
3676	Nucleic acid binding	6,74E-01	15.3%
15932	Nucleobase, nucleoside, nucleotide and nucleic acid transmembrane transporter activity	8,12E-01	0.1%
19205	Nucleobase, nucleoside, nucleotide kinase activity	3,13E-01	0.2%
1882	Nucleoside binding	1,26E-01	7.4%
19206	Nucleoside kinase activity	2,65E-01	0.1%
5337	Nucleoside transmembrane transporter activity	4,32E-01	0.1%
17111	Nucleoside-triphosphatase activity	1,21E-01	4.0%
60589	Nucleoside-triphosphatase regulator activity	1,43E-01	0.7%
166	Nucleotide binding	6,76E-02	11.3%
19201	Nucleotide kinase activity	4,55E-01	0.1%
5338	Nucleotide-sugar transmembrane transporter activity	3,13E-01	0.1%
16779	Nucleotidyltransferase activity	7,83E-01	0.5%
8374	O-acyltransferase activity	1,15E-01	0.5%
50737	O-hydroxycinnamoyltransferase activity	1,26E-01	0.1%
8242	Omega peptidase activity	1,98E-01	0.1%
5342	Organic acid transmembrane transporter activity	7,59E-01	0.2%
4587	Ornithine-oxo-acid transaminase activity	1,26E-01	0.1%
15271	Outward rectifier potassium channel activity	3,39E-01	0.1%
16491	Oxidoreductase activity	1,98E-01	6.6%
16702	Oxidoreductase activity, actin on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen	1,32E-01	0.3%
16645	Oxidoreductase activity, actin on the CH-NH group of donors	6,30E-01	0.1%

16725	Oxidoreductase activity, acting on CH or CH2 groups	3,13E-01	0.1%
16728	Oxidoreductase activity, acting on CH or CH2 groups, disulfide as acceptor	2,65E-01	0.1%
16614	Oxidoreductase activity, acting on CH-OH group of donors	1,26E-01	1.1%
16730	Oxidoreductase activity, acting on iron-sulfur proteins as donors	2,65E-01	0.1%
16731	Oxidoreductase activity, acting on iron-sulfur proteins as donors, NAD or NADP as acceptor	1,98E-01	0.1%
16652	Oxidoreductase activity, acting on NADH or NADPH, NAD or NADP as acceptor	1,14E-01	0.2%
16655	Oxidoreductase activity, acting on NADH or NADPH, quitone or similar compound as acceptor	5,58E-01	0.1%
16661	Oxidoreductase activity, acting on other nitrogenous compounds as donors	2,34E-01	0.1%
16663	Oxidoreductase activity, acting on other nitrogenous compounds as donors, oxygen as acceptor	1,26E-01	0.1%
16705	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxigen	7,83E-01	0.5%
16706	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxigen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxigen into both donors	4,89E-01	0.3%
16717	Oxidoreductase activity, acting on paired donors, with oxidation of a pair of donors resulting in the reduction of molecular oxygen to two molecules of water	4,32E-01	0.1%
16684	Oxidoreductase activity, acting on peroxide as acceptor	8,76E-01	0.2%
16701	Oxidoreductase activity, acting on single donors with incorporation of molecular oxygen	1,77E-01	0.3%
16667	Oxidoreductase activity, acting on sulfur group of donors	1,10E-01	0.9%
16668	Oxidoreductase activity, acting on sulfur group of donors, NAD or NADP as acceptor	1,26E-01	0.2%
16670	Oxidoreductase activity, acting on sulfur group of donors, oxigen as acceptor	9,91E-02	0.2%
16903	Oxidoreductase activity, acting on the aldehyde or oxo group donors	1,34E-02	1.0%
16622	Oxidoreductase activity, acting on the aldehyde or oxo group of donors, cytochrome as acceptor	1,98E-01	0.1%
16624	Oxidoreductase activity, acting on the aldehyde or oxo group of donors, disulfide as acceptor	1,77E-01	0.2%
16620	Oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	7,29E-02	0.6%
16627	Oxidoreductase activity, acting on the CH-CH group of donors	1,77E-01	0.6%
16628	Oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor	2,50E-01	0.2%
16646	Oxidoreductase activity, acting on the CH-NH group of donors, NAD or NADP as acceptor	3,89E-01	0.1%
16638	Oxidoreductase activity, acting on the CH-NH2 group of donors	8,22E-01	0.1%
16641	Oxidoreductase activity, acting on the CH-NH2 group of donors, oxigen as acceptor	7,61E-01	0.1%
16616	Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	1,26E-01	1.0%
16651	Oxidoreductase ativity, acting on NADH or NADPH	1,58E-01	0.6%
16833	Oxo-acid-lyase activity	4,55E-01	0.1%
22803	Passive transmembrane transporter activity	1,98E-01	0.9%
1871	Pattern binding	2,10E-01	0.2%
30599	Pectinesterase activity	9,98E-01	0.1%
8233	Peptidase activity	8,19E-01	1.6%
70011	Peptidase activity, acting on L-amino acid peptides	7,74E-01	1.6%
30414	Peptidase inhibitor activity	8,08E-01	0.1%
61134	Peptidase regulator activity	8,08E-01	0.1%
42277	Peptide binding	2,85E-01	0.2%
15037	Peptide disulfide oxidoreductase activity	3,13E-01	0.1%
3755	Peptidyl-prolyl cis-trans isomerase activity	9,60E-02	0.7%
4601	Peroxidase activity	8,76E-01	0.2%
5052	Peroxisome matrix targeting signal-1 binding	1,26E-01	0.1%
268	Peroxisome targeting sequence binding	1,77E-01	0.1%
8195	Phospatidate phosphatase activity	2,65E-01	0.1%
16791	Phosphatase activity	4,90E-03	2.7%
15114	Phosphate transmembrane transporter activity	5,43E-01	0.1%
80096	Phosphatidate-sterol O-acyltransferase activity	1,26E-01	0.1%
70300	Phosphatidic acid binding	1,26E-01	0.1%
4607	Phosphatidylcholine-sterol O-acyltransferase activity	1,14E-01	0.2%

8429	Phosphatidylethanolamine binding	3,13E-01	0.1%
80095	Phosphatidylethanolamine-sterol O-acyltransferase activity	1,26E-01	0.1%
34593	Phosphatidylinositol bisphosphate phosphatase activity	3,62E-01	0.1%
16307	Phosphatidylinositol phosphate kinase activity	1,98E-01	0.2%
4439	Phosphatidylinositol-4,5-biphosphate 5-phosphatase activity	3,62E-01	0.1%
4609	Phosphatidylserine decarboxylase activity	1,98E-01	0.1%
30572	Phosphatidyltransferase activity	1,98E-01	0.1%
4612	Phosphoenolpyruvate carboxykinase (ATP) activity	1,77E-01	0.1%
4611	Phosphoenolpyruvate carboxykinase activity	1,26E-01	0.2%
8964	Phosphoenolpyruvate carboxylase activity	2,65E-01	0.1%
8443	Phosphofructokinase activity	4,10E-01	0.1%
4614	Phosphoglucomutase activity	1,26E-01	0.1%
4616	Phosphogluconate dehydrogenase (decarboxylating) activity	3,62E-01	0.1%
34595	Phosphogluconate dehydrogenase (decarboxylating) activity	3,62E-01	0.1%
4618	Phosphoglycerate kinase activity	1,98E-01	0.1%
4619	Phosphoglycerate mutase activity	1,77E-01	0.1%
8967	Phosphoglycolate phosphatase activity	2,93E-01	0.1%
35091	Phosphoinositide binding	1,63E-01	0.5%
5543	Phospholipid binding	7,67E-02	0.9%
4632	Phosphopantothenate-cysteine ligase activity	1,26E-01	0.1%
51219	Phosphoprotein binding	4,73E-01	0.1%
4721	Phosphoprotein phosphatase activity	2,49E-02	1.6%
4634	Phosphopyruvate hydratase activity	1,98E-01	0.1%
8081	Phosphoric diester hydrolase activity	8,87E-01	0.1%
42578	Phosphoric ester hydrolase activity	1,34E-02	2.8%
4645	Phosphorylase activity	2,34E-01	0.1%
16773	Phosphotransferase activity, alcohol group as acceptor	1,75E-01	5.3%
16774	Phosphotransferase activity, carboxyl group as acceptor	1,42E-01	0.2%
16780	Phosphotransferase activity, for other substituted phosphate groups	4,55E-01	0.1%
16775	Phosphotransferase activity, nitrogenous group as acceptor	4,96E-02	0.5%
16776	Phosphotransferase activity, phosphate group as acceptor	5,77E-01	0.1%
9881	Photoreceptor activity	1,26E-01	0.2%
46905	Phytoene synthase activity	1,26E-01	0.1%
15166	Polyol transmembrane transporter activity	2,93E-01	0.1%
10420	Polyprenyldihydroxybenzoate methyltransferase activity	1,26E-01	0.1%
30247	Polysaccharide binding	2,10E-01	0.2%
5267	Potassium channel activity	6,30E-01	0.1%
22820	Potassium ion symporter activity	1,26E-01	0.1%
15079	Potassium ion transmembrane transporter activity	2,65E-01	0.2%
9674	Potassium:sodium symporter activity	1,26E-01	0.1%
15450	P-P-bond-hydrolysis-driven protein transmembrane transporter activity	8,22E-01	0.1%
15405	P-P-bond-hydrolysis-driven transmembrane transporter activity	2,09E-02	1.9%
4659	Prenyltransferase activity	1,77E-01	0.3%
4665	Prephenate dehydrogenase (NADP+) activity	1,77E-01	0.1%
15399	Primary active transmembrane movement of ions	2,09E-02	1.9%
10843	Promoter binding	2,34E-01	0.1%
5515	Protein binding	2,99E-01	10.9%
33558	Protein deacetylase activity	5,43E-01	0.1%
46983	Protein dimerization activity	1,98E-01	0.7%
3756	Protein disulfide isomerase activity	4,89E-01	0.1%
15035	Protein disulfide oxidoreductase activity	3,13E-01	0.2%

46982	Protein heterodimerization activity	7,63E-02	0.6%
4673	Protein histidine kinase activity	4,96E-02	0.5%
42803	Protein homodimerization activity	8,22E-01	0.1%
4672	Protein kinase activity	3,13E-01	4.1%
19887	Protein kinase regulator activity	7,80E-01	0.1%
8276	Protein methyltransferase activity	3,83E-01	0.2%
45309	Protein phosphorylated amino acid binding	4,73E-01	0.1%
43621	Protein self-association	1,77E-01	0.1%
4674	Protein serine/threonine kinase activity	6,30E-01	2.6%
4722	Protein serine/threonine phosphatase activity	9,83E-02	1.1%
4712	Protein serine/threonine/tyrosine kinase activity	1,30E-01	0.5%
8320	Protein transmembrane transporter activity	8,22E-01	0.1%
8565	Protein transporter activity	3,89E-01	0.6%
4713	Protein tyrosine kinase activity	9,63E-01	0.3%
4725	Protein tyrosine phosphatase activity	4,73E-01	0.1%
8138	Protein tyrosine/serine/threonine phosphatase activity	7,63E-02	0.3%
16279	Protein-lysine N-methyltransferase activity	1,92E-01	0.2%
46961	Proton-transporting ATPase activity, rotational mechanism	2,09E-02	0.5%
70035	Purine NTP-dependent helicase activity	4,85E-01	0.5%
1883	Purine nucleoside binding	1,32E-01	7.2%
17076	Purine nucleotide binding	1,92E-01	7.9%
32555	Purine ribonucleotide binding	1,59E-01	7.8%
30170	Pyrodoxal phosphate binding	2,64E-01	0.3%
16462	Pyrophosphatase activity	1,26E-01	4.0%
4739	Pyruvate dehydrogenase (acetyl-transferring) activity	1,14E-01	0.2%
4740	Pyruvate dehydrogenase (acetyl-transferring) kinase activity	1,26E-01	0.1%
4738	Pyruvate dehydrogenase activity	1,14E-01	0.2%
47205	Quinate O-hydroxycinnamoyltransferase activity	1,26E-01	0.1%
5093	Rab GDP-dissociation inhibitor activity	2,34E-01	0.1%
5097	Rab GTPase activator activity	7,63E-02	0.5%
16854	Racemase and epimerase activity	3,39E-01	0.2%
16857	Racemase and epimerase activity, acting on carbohydrates and derivatives	2,85E-01	0.2%
70283	Radical SAM enzyme activity	2,34E-01	0.1%
5099	Ras GTPase activator activity	1,14E-01	0.5%
182	rDNA binding	1,77E-01	0.1%
4872	Receptor activity	9,58E-01	0.3%
5102	Receptor binding	7,72E-01	0.1%
9883	Red or far-red light photoreceptor activity	5,20E-02	0.2%
4540	Ribonuclease activity	8,95E-01	0.1%
32549	Ribonucleoside binding	2,34E-01	0.1%
32553	Ribonucleotide binding	1,59E-01	7.8%
4750	Ribulose-phosphate 3-epimerase activity	1,98E-01	0.1%
3723	RNA binding	1	1.8%
34062	RNA polymerase activity	6,88E-01	0.2%
3702	RNA polymerase II transcription factor activity	7,11E-01	0.1%
19843	rRNA binding	2,65E-01	0.1%
16418	S-acetyltransferase activity	2,65E-01	0.1%
16417	S-acyltransferase activity	3,39E-01	0.1%
8757	S-adenosylemthionine-dependent methyltransferase activity	2,73E-01	0.6%
15291	Secondary active transmembrane transporter activity	1,88E-01	1.6%
8430	Selenium binding	3,13E-01	0.1%

9000	Selenocysteine lyase activity	1,77E-01	0.1%
43565	Sequence-specific DNA binding	1,20E-01	0.9%
17171	Serine hydrolase activity	8,21E-01	0.5%
4252	Serine-type endopeptidase activity	4,23E-01	0.5%
8236	Serine-type peptidase activity	8,21E-01	0.5%
47172	Shikimate O-hydroxycinnamoyltransferase activity	1,26E-01	0.1%
5048	Signal sequence binding	3,13E-01	0.1%
4871	Signal transducer activity	9,17E-01	1.0%
3727	Single-stranded RNA binding	6,42E-01	0.1%
19787	Small conjugating protein ligase activity	8,09E-01	0.9%
19783	Small conjugating protein-specific protease activity	6,89E-01	0.1%
5083	Small GTPase regulator activity	1,26E-01	0.7%
8172	S-methyltransferase activity	3,62E-01	0.1%
5484	SNAP receptor activity	7,11E-01	0.1%
15298	Solute:cation antiporter activity	6,67E-01	0.2%
15294	Solute:cation symporter activity	1,26E-01	1.0%
15299	Solute:hydrogen antiporter activity	5,95E-01	0.2%
15295	Solute:hydrogen symporter activity	1,26E-01	0.9%
15300	Solute:solute antiporter activity	7,63E-01	0.2%
16566	Specific transcriptional repressor activity	3,39E-01	0.1%
4766	Spermidine synthase activity	1,98E-01	0.1%
16768	Spermine synthase activity	1,77E-01	0.1%
9011	Starch synthase activity	1,77E-01	0.1%
4768	Stearoyl-CoA 9-desaturase activity	1,26E-01	0.1%
16229	Steroid dehydrogenase activity	4,10E-01	0.1%
33764	Steroid dehydrogenase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	3,13E-01	0.1%
5199	Structural constituent of cell wall	7,67E-01	0.1%
5200	Structural constituent of cytoskeleton	3,62E-01	0.2%
3735	Structural constituent of ribosome	1	0.3%
5198	Structural molecule activity	9,85E-01	1.1%
43566	Structure-specific DNA binding	5,23E-01	0.2%
22838	Substarte-specific channel activity	1,98E-01	0.9%
22891	Substrate-specific transmembrane transporter activity	2,44E-02	4.8%
22892	Substrate-specific transporter activity	2,26E-02	5.4%
104	Succinate dehydrogenase activity	3,39E-01	0.1%
4776	Succinate-CoA ligase (GTP-forming) activity	1,98E-01	0.1%
4774	Succinate-CoA ligase activity	1,98E-01	0.1%
16157	Sucrose synthase activity	2,93E-01	0.1%
50307	Sucrose-phosphatase activity	2,34E-01	0.1%
46524	Sucrose-phosphate synthase activity	2,34E-01	0.1%
5529	Sugar binding	7,22E-01	0.2%
51119	Sugar transmembrane transporter activity	1,10E-01	1.0%
5351	Sugar:hydrogen symporter activity	1,26E-01	0.9%
8482	Sulfite oxidase activity	1,26E-01	0.1%
16783	Sulfur transferase activity	1,42E-01	0.2%
15293	Symporter activity	1,14E-01	1.1%
42162	Telomeric DNA binding	3,62E-01	0.1%
8493	Tetracycline transporter activity	2,65E-01	0.1%
15520	Tetracycline:hydrogen antiporter activity	1,77E-01	0.1%
46906	Tetrapyrrole binding	9,84E-01	0.6%
15927	Thelase activity	1,26E-01	0.1%

16790	Thioester hydrolase activity	8,10E-01	0.2%
16972	Thiol oxidase activity	1,98E-01	0.1%
8483	Transaminase activity	2,09E-02	0.9%
16563	Transcription activator activity	6,76E-02	1.4%
3700	Transcription factor activity	4,90E-01	6.6%
30528	Transcription regulator activity	3,39E-01	7.9%
16564	Transcription repressor activity	2,11E-01	0.5%
16757	Transferae activity, transferring glycosyl groups	1,91E-01	2.4%
16740	Transferase activity	4,90E-03	14.4%
16769	Transferase activity, transfering nitrogenous groups	1,34E-02	1.0%
16741	Transferase activity, transfering one-carbon groups	7,93E-01	0.6%
16763	Transferase activity, transfering pentosyl groups	2,93E-01	0.3%
16746	Transferase activity, transferring acyl groups	1,77E-01	1.4%
16747	Transferase activity, transferring acyl groups other than amino-acyl groups	1,77E-01	1.3%
46912	Transferase activity, transferring acyl groups, acyl groups converted into alkyl on transfer	5,05E-01	0.1%
16744	Transferase activity, transferring aldehyde or ketonic groups	1,63E-01	0.2%
16765	Transferase activity, transferring alkyl or aryl (other than methyl) groups	1,05E-01	1.1%
16758	Transferase activity, transferring hexosyl groups	3,93E-01	1.4%
16772	Transferase activity, transferring phosphorus-containing groups	1,14E-01	7.5%
16782	Transferase activity, transferring sulfur-containing groups	3,46E-01	0.2%
46914	Transition metal ion binding	2,65E-01	7.9%
46915	Transition metal ion transmembrane transporter activity	2,18E-01	0.3%
4802	Transketolase activity	1,98E-01	0.1%
3746	Translation elongation factor activity	6,78E-01	0.1%
8135	Translation factor activity, nucleic acid binding	6,42E-01	0.5%
3743	Translation initiation factor activity	9,51E-01	0.1%
3747	Translation release factor activity	1,77E-01	0.2%
16149	Translation release factor activity, codon specific	2,34E-01	0.1%
8079	Translation termination factor activity	1,77E-01	0.2%
4888	Transmembrane receptor activity	9,74E-01	0.2%
22857	Transmembrane transporter activity	2,17E-02	5.7%
5215	Transporter activity	9,99E-03	7.6%
4805	Trehalose-phosphatase activity	3,13E-01	0.1%
4806	Triglyceride lipase activity	4,90E-01	0.2%
8251	tRNA-specific adenosine deaminase activity	1,26E-01	0.1%
15631	Tubulin binding	7,72E-01	0.1%
156	Two-component response regulator activity	4,16E-01	0.2%
155	Two-component sensor activity	2,34E-01	0.1%
4221	Ubiquitin thiolesterase activity	6,42E-01	0.2%
4842	Ubiquitin-protein ligase activity	8,75E-01	0.7%
4843	Ubiquitin-specific protease activity	6,09E-01	0.1%
35250	UDP-galactosyltransferase activity	2,93E-01	0.1%
3978	UDP-glucose 4-epimerase activity	3,39E-01	0.1%
35251	UDP-glucosyltransferase activity	8,10E-01	0.2%
8194	UDP-glycosyltransferase activity	9,34E-01	0.3%
51082	Unfolded protein binding	6,20E-01	0.2%
4845	Uracil phosphoribosyltransferase activity	1,14E-01	0.2%
4846	Urate oxidase activity	1,26E-01	0.1%
15204	Urea transmembrane transporter activity	2,93E-01	0.1%
71522	Ureidoglycine aminohydrolase activity	1,26E-01	0.1%
4848	Ureidoglycolate hydrolase activity	1,26E-01	0.1%

9041	Uridylate kinase activity	1,77E-01	0.1%
70279	Vitamin B6 binding	2,64E-01	0.3%
19842	Vitamin binding	3,39E-01	0.3%
8308	Voltage-gated anion channel activity	4,89E-01	0.1%
5245	Voltage-gated calcium channel activity	1,26E-01	0.1%
22843	Voltage-gated cation channel activity	2,85E-01	0.2%
22832	Voltage-gated channel activity	2,34E-01	0.3%
5247	Voltage-gated chloride channel activity	3,13E-01	0.1%
5244	Voltage-gated ion channel activity	2,34E-01	0.3%
5249	Voltage-gated potassium channel activity	6,09E-01	0.1%
15250	Water channel activity	4,06E-01	0.2%
5372	Water transmembrane transporter activity	4,06E-01	0.2%
9045	Xylose isomerase activity	1,26E-01	0.1%
8270	Zinc ion binding	1,98E-01	5.5%

A.2.3 Transcripts simultaneously down-regulated in 7LD:11LD samples

Table A.7: Frequency analysis of the microarray results according to molecular function, of the simultaneously down-regulated transcripts in 7 LD and 11 LD samples, using gene ontology information; performed using the Cytoscape v2.8.3 software platform (Cline *et al*, 2007) and BiNGO v2.44 plugin (Maere *et al*, 2005)

DOWN-REGULATED (7LD:11LD)

GO-ID	MOLECULAR FUNCTION	p-val	Freq
3849	3-deoxy-7-phosphoheptulonate synthase activity	1,72E-01	0.3%
16784	3-mercaptopyruvate sulfurtransferase activity	1,61E-01	0.3%
16207	4-coumarate-CoA ligase activity	2,52E-01	0.3%
17108	5'-flap endonuclease activity	1,41E-01	0.3%
42084	5-methyltetrahydrofolate-dependent methyltransferase activity	1,72E-01	0.3%
3871	5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase activity	1,72E-01	0.3%
42085	5-methyltetrahydropteroyltri-L-glutamate-dependent methyltransferase activity	1,72E-01	0.3%
18454	Acetoacetyl-CoA reductase activiy	1,41E-01	0.3%
16407	Acetyltransferase activity	3,19E-01	0.7%
3993	Acid phosphatase activity	5,16E-01	0.3%
16881	Acid-amino acid ligase activity	6,67E-01	1.1%
16878	Acid-thiol ligase activity	3,41E-01	0.3%
3779	Actin binding	2,89E-01	0.7%
3785	Actin monomer binding	1,72E-01	0.3%
22804	Active transmembrane transporter activity	1,17E-01	5.1%
36	Acyl carrier activity	2,89E-01	0.3%
8415	Acyltransferase activity	2,61E-01	1.5%
30554	Adenyl nucleotide binding	2,02E-01	8.3%
32559	Adenyl ribonucleotide binding	1,73E-01	8.3%
4040	Amidase activity	2,02E-01	0.3%
5275	Amine transmembrane transporter activity	3,35E-01	0.7%
15171	Amino acid transmembrane transporter activity	3,04E-01	0.7%
8509	Anion transmembrane transporter activity	4,69E-01	0.7%
15296	Anion:cation symporter activity	1,72E-01	0.3%
4048	Anthranilate phosphoribosyltransferase activity	1,61E-01	0.3%
16209	Antioxidant activity	7,97E-01	0.3%
15297	Antiporter activity	2,02E-01	1.5%
15173	Aromatic amino acid transmembrane transporter activity	1,61E-01	0.3%
4190	Aspartic-type endopeptidase activity	6,63E-01	0.3%
70001	Aspartic-type peptidase activity	6,63E-01	0.3%
5524	ATP binding	1,72E-01	8.3%
16887	ATPase activity	5,76E-01	1.5%
42623	ATPase activity, coupled	6,47E-01	1.1%
43492	ATPase activity, coupled to movement of substances	3,36E-01	1.1%

42625	ATPase activity, coupled to transmembrane movement of ions	5,32E-01	0.3%
15662	ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism	4,78E-01	0.3%
42626	ATPase activity, coupled to transmembrane movement of substances	3,36E-01	1.1%
19187	Beta-1,4-mannosyltransferase activity	1,72E-01	0.3%
4565	Beta-galactosidase activity	4,98E-01	0.3%
5488	Binding	4,14E-02	50.3%
16408	C-acyltransferase activity	2,45E-01	0.3%
42409	Caffeoyl-CoA O-methyltransferase activity	1,72E-01	0.3%
5509	Calcium ion binding	9,20E-01	0.3%
5516	Calmodulin binding	1,41E-01	2.3%
19200	Carbihydrate kinase activity	4,89E-01	0.3%
15144	Carbohydrate transmembrane transporter activity	7,29E-01	0.3%
16830	Carbon-carbon lyase activity	4,46E-01	0.7%
16884	Carbon-nitrogen ligase activity, with glutamine as amido-N-donor	2,89E-01	0.3%
16835	Carbon-oxygen lyase activity	7,80E-01	0.3%
4091	Carboxylesterase activity	9,19E-01	0.7%
46943	Carboxylic acid transmembrane transporter activity	3,57E-01	0.7%
16831	Carboxy-lyase activity	2,89E-01	0.7%
4180	Carboxypeptidase activity	5,82E-01	0.3%
3824	Catalytic activity	1,23E-01	39.2%
43169	Cation binding	9,95E-01	5.5%
8324	Cation transmembrane transporter activity	6,44E-01	1.5%
15491	Cation:cation antiporter activity	5,82E-01	0.3%
15377	Cation:chloride symporter activity	1,61E-01	0.3%
5402	Cation:sugar symporter activity	6,88E-01	0.3%
16759	Cellulose synthase activity	4,83E-01	0.3%
15267	Channel activity	4,98E-01	0.7%
16168	Chlorophyll binding	3,99E-01	0.3%
16405	CoA-ligase activity	3,36E-01	0.3%
50662	Coenzyme binding	6,25E-01	0.7%
48037	Cofactor binding	5,32E-01	1.1%
5507	Copper ion binding	9,25E-01	0.3%
4124	Cysteine synthase activity	2,52E-01	0.3%
8234	Cysteine-type peptidase activity	7,12E-01	0.3%
19139	Cytokinin dehydrogenase activiy	2,36E-01	0.3%
8092	Cytoskeletal protein binding	4,51E-01	0.7%
4536	Deoxyribonuclease activity	2,45E-01	0.3%
17050	D-erythro-sphingosine kinase activity	1,72E-01	0.3%
15082	Di-, tri-valent inorganic cation transmembrane transporter activity	5,89E-01	0.3%
4143	Diacylglycerol kinase activity	2,82E-01	0.3%
51213	Dioxygenase activity	4,14E-01	0.3%
42936	Dipeptide transporter activity	1,77E-01	0.3%
16778	Diphosphotransferase activity	2,82E-01	0.3%
3677	DNA binding	1,17E-01	14.6%

3678	DNA helicase activity	3,58E-01	0.3%
3899	DNA-directed RNA polymerase activity	5,82E-01	0.3%
15238	Drug transmembrane transporter activity	3,99E-01	0.3%
9055	Electron carrier activity	9,70E-01	0.7%
4520	Endodeoxyribonuclease activity	1,77E-01	0.3%
16888	Endodeoxyribonuclease activity, producing 5'-phosphomonoesters	1,41E-01	0.3%
4519	Endonuclease activity	3,37E-01	0.7%
16893	Endonuclease activity, active with either ribo or deoxyribonucleic acids and producing 5'-phosphomonoesters	4,72E-01	0.3%
4175	Endopeptidase activity	6,58E-01	1.1%
8238	Exopeptidase activity	6,67E-01	0.3%
80132	Fatty acid alpha-hydroxylase activity	1,61E-01	0.3%
4321	Fatty-acyl-CoA synthase activity	1,61E-01	0.3%
48256	Flap endonuclease activity	1,41E-01	0.3%
16711	Flavonoid 3'-monooxygenase activity	1,41E-01	0.3%
8865	Fructokinase activity	1,72E-01	0.3%
15925	Galactosidase activity	5,02E-01	0.3%
22836	Gated channel activity	5,89E-01	0.3%
45543	Gibberellin 2-beta-dioxygenase activity	2,36E-01	0.3%
4340	Glucokinase actvity	1,72E-01	0.3%
46527	Glucosyltransferase activity	3,21E-01	1.1%
4364	Glutathione transferase activity	5,32E-01	0.3%
4375	Glycine dehydrogenase (decarboxylating) activity	2,02E-01	0.3%
5525	GTP binding	2,36E-01	1.9%
3924	GTPase activity	6,31E-01	0.3%
19001	Guanyl nucleotide binding	2,44E-01	1.9%
32561	Guanyl ribonucleotide binding	2,36E-01	1.9%
4386	Helicase activity	2,51E-01	1.5%
20037	Heme binding	9,81E-01	0.3%
4396	Hexokinase activity	2,13E-01	0.3%
15334	High affinity oligopeptide transporter activity	1,41E-01	0.3%
9927	Histidine phosphotransfer kinase activity	2,52E-01	0.3%
15078	Hydrogen ion transmembrane transporter activity	7,15E-01	0.3%
16787	Hydrolase activity	6,49E-01	10.7%
16820	Hydrolase activity, actin on acid anhydrides, catalyzing transmembrane movement of substances	3,41E-01	1.1%
16818	Hydrolase activity, actin on acid anhydrides, in phosphorus-containing anhydrides	3,49E-01	3.9%
16817	Hydrolase activity, acting on acid anhydrides	3,57E-01	3.9%
16810	Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	7,28E-01	0.3%
16811	Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amides	5,82E-01	0.3%
16788	Hydrolase activity, acting on ester bonds	6,78E-01	3.5%
16798	Hydrolase activity, acting on glycosyl bonds	9,96E-01	0.3%
4553	Hydrolase activity, hydrolyzing O-glycosyl compounds	9,96E-01	0.3%
42802	Identical protein binding	7,15E-01	0.3%
15103	Inorganic anion transmembrane transporter activity	5,89E-01	0.3%

22890	Inorganic cation transmembrane transporter activity	6,67E-01	0.7%
4434	Inositol or phosphatidylinositol phosphodiesterase activity	2,13E-01	0.3%
5217	Intracellular ligand-gated ion channel activity	3,93E-01	0.3%
16866	Intramolecular transferase activity	4,78E-01	0.3%
43167	Ion binding	9,95E-01	5.5%
5216	Ion channel activity	6,72E-01	0.3%
15075	Ion transmembrane transporter activity	5,32E-01	2.3%
5506	Iron ion binding	9,21E-01	0.7%
51536	Iron-sulfur cluster binding	4,76E-01	0.3%
16853	Isomerase activity	9,19E-01	0.3%
45703	Ketoreductase activity	1,61E-01	0.3%
16301	Kinase activity	2,82E-01	6.7%
8471	Laccase activity	3,36E-01	0.3%
22834	Ligand-gated channel activity	4,46E-01	0.3%
15276	Ligand-gated ion channel activity	4,46E-01	0.3%
16874	Ligase activity	6,01E-01	1.9%
16879	Ligase activity, forming carbon-nitrogen bonds	5,82E-01	1.5%
16877	Ligase activity, forming carbon-sulfur bonds	3,93E-01	0.3%
16298	Lipase activity	5,32E-01	0.7%
8289	Lipid binding	8,88E-01	0.3%
1727	Lipid kinase activity	3,93E-01	0.3%
16165	Lipoxygenase activity	2,45E-01	0.3%
16829	Lyase activity	2,66E-01	2.3%
22884	Macromolecule transmembrane transporter activity	2,51E-01	0.7%
287	Magnesium ion binding	1,34E-01	1.5%
51753	Mannan synthase activity	1,72E-01	0.3%
30	Mannosyltransferase activity	2,45E-01	0.3%
51540	Metal cluster binding	4,76E-01	0.3%
46872	Metal ion binding	9,95E-01	5.1%
46873	Metal ion transmembrane transporter activity	4,78E-01	0.7%
4222	Metalloendopeptidase activity	4,83E-01	0.3%
8237	Metallopeptidase activity	6,47E-01	0.3%
8705	Methionine synthase activity	1,72E-01	0.3%
8168	Methyltransferase activity	1,61E-01	2.3%
60089	Molecular transducer activity	7,85E-01	1.1%
4497	Monooxygenase activity	9,81E-01	0.3%
5451	Monovalent cation:hydrogen antiporter activity	5,16E-01	0.3%
15077	Monovalent inorganic cation transmembrane transporter activity	7,80E-01	0.3%
8080	N-acetyltransferase activity	5,32E-01	0.3%
16410	N-acyltransferase activity	5,54E-01	0.3%
51287	NAD or NADH binding	4,14E-01	0.3%
15175	Neutral amino acid transmembrane transporter activity	2,52E-01	0.3%
4518	Nuclease activity	3,70E-01	1.1%
3676	Nucleic acid binding	1,72E-01	20.2%

1882	Nucleoside binding	2,02E-01	8.3%
17111	Nucleoside-triphosphatase activity	3,20E-01	3.9%
166	Nucleotide binding	1,14E-01	14.6%
16779	Nucleotidyltransferase activity	8,53E-01	0.3%
15198	Oligopeptide transporter activity	1,61E-01	0.7%
42389	Omega-3 fatty acid desaturase activity	1,77E-01	0.3%
8171	O-methyltransferase activity	4,64E-01	0.3%
5342	Organic acid transmembrane transporter activity	3,57E-01	0.7%
17077	Oxidative phosphorylation uncoupler activity	1,77E-01	0.3%
16491	Oxidoreductase activity	4,78E-01	6.3%
16702	Oxidoreductase activity, actin on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen	3,99E-01	0.3%
16645	Oxidoreductase activity, actin on the CH-NH group of donors	4,09E-01	0.3%
16614	Oxidoreductase activity, acting on CH-OH group of donors	5,60E-01	0.7%
16682	Oxidoreductase activity, acting on diphenols and related substances as donors, oxygen as acceptor	4,38E-01	0.3%
16705	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxigen	1,41E-01	2.3%
16706	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxigen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxigen into both donors	1,77E-01	1.1%
16709	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, NAD or NADH as one donor, and incorporation of one atom of oxygen	4,83E-01	0.3%
16717	Oxidoreductase activity, acting on paired donors, with oxidation of a pair of donors resulting in the reduction of molecular oxygen to two molecules of water	2,89E-01	0.3%
16684	Oxidoreductase activity, acting on peroxide as acceptor	7,26E-01	0.3%
16701	Oxidoreductase activity, acting on single donors with incorporation of molecular oxygen	4,46E-01	0.3%
16638	Oxidoreductase activity, acting on the CH-NH2 group of donors	5,32E-01	0.3%
16642	Oxidoreductase activity, acting on the CH-NH2 group of donors, disulfide as acceptor	2,02E-01	0.3%
16616	Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	4,92E-01	0.7%
16679	Oxydoreductase activity, acting on diphenols and related substances as donors	4,78E-01	0.3%
19825	Oxygen binding	9,38E-01	0.3%
22803	Passive transmembrane transporter activity	4,98E-01	0.7%
8233	Peptidase activity	6,67E-01	1.9%
70011	Peptidase activity, acting on L-amino acid peptides	6,44E-01	1.9%
15197	Peptide transporter activity	1,61E-01	0.7%
4601	Peroxidase activity	7,26E-01	0.3%
16791	Phosphatase activity	7,81E-01	0.7%
4435	Phosphoinoivitysitide phospholipase C act	2,13E-01	0.3%
4620	Phospholipase activity	5,57E-01	0.3%
4629	Phospholipase C activity	3,93E-01	0.3%
8081	Phosphoric diester hydrolase activity	3,05E-01	0.7%
42578	Phosphoric ester hydrolase activity	5,32E-01	1.5%
16773	Phosphotransferase activity, alcohol group as acceptor	2,05E-01	6.3%
8266	Poly(U) RNA binding	3,41E-01	0.3%
47262	Polygalacturonate 4-alpha-galacturonosyltransferase activity	4,14E-01	0.3%
8187	Poly-pyrimidine tract binding	3,41E-01	0.3%
15450	P-P-bond-hydrolysis-driven protein transmembrane transporter activity	2,51E-01	0.7%

15405	P-P-bond-hydrolysis-driven transmembrane transporter activity	1,77E-01	1.9%
15399	Primary active transmembrane transporter activity	1,77E-01	1.9%
5515	Protein binding	4,73E-01	11.1%
46983	Protein dimerization activity	7,12E-01	0.3%
46982	Protein heterodimerization activity	4,83E-01	0.3%
4672	Protein kinase activity	2,23E-01	5.5%
4674	Protein serine/threonine kinase activity	2,27E-01	4.3%
8320	Protein transmembrane transporter activity	2,51E-01	0.7%
8565	Protein transporter activity	4,83E-01	0.7%
4713	Protein tyrosine kinase activity	4,84E-01	1.1%
9982	Pseudouridine synthase activity	1,61E-01	0.3%
1883	Purine nucleoside binding	2,02E-01	8.3%
17076	Purine nucleotide binding	1,61E-01	10.3%
32555	Purine ribonucleotide binding	1,41E-01	10.3%
30170	Pyrodoxal phosphate binding	4,98E-01	0.3%
16462	Pyrophosphatase activity	3,47E-01	3.9%
4740	Pyruvate dehydrogenase (acetyl-transferring) kinase activity	1,41E-01	0.3%
80043	Quercetin 3-O-glucosyltransferase activity	3,99E-01	0.3%
32553	Ribonucleotide binding	1,41E-01	10.3%
4749	Ribose phosphate diphosphokinase activity	1,77E-01	0.3%
3723	RNA binding	9,81E-01	2.3%
8173	RNA methyltransferase activity	1,61E-01	0.7%
34062	RNA polymerase activity	6,09E-01	0.3%
3702	RNA polymerase II transcription factor activity	4,64E-01	0.3%
8757	S-adenosylemthionine-dependent methyltransferase activity	4,29E-01	0.7%
15291	Secondary active transmembrane transporter activity	2,02E-01	2.3%
43565	Sequence-specific DNA binding	6,67E-01	0.3%
17171	Serine hydrolase activity	6,31E-01	0.7%
4185	Serine-type carboxypeptidase activity	5,74E-01	0.3%
4252	Serine-type endopeptidase activity	6,88E-01	0.3%
70008	Serine-type exopeptidase activity	5,74E-01	0.3%
8236	Serine-type peptidase activity	6,31E-01	0.7%
4871	Signal transducer activity	7,85E-01	1.1%
16752	Sinapoyltransferase activity	1,61E-01	0.3%
3697	Single-stranded DNA binding	3,49E-01	0.3%
3727	Single-stranded RNA binding	4,14E-01	0.3%
35197	siRNA binding	1,77E-01	0.3%
19787	Small conjugating protein ligase activity	6,44E-01	1.1%
19783	Small conjugating protein-specific protease activity	4,46E-01	0.3%
8172	S-methyltransferase activity	2,52E-01	0.3%
15081	Sodium ion transmembrane transporter activity	3,28E-01	0.3%
15385	Sodium:hydrogen antiporter activity	4,78E-01	0.3%
8511	Sodium:potassium:chloride symporter activity	1,41E-01	0.3%
15298	Solute:cation antiporter activity	5,94E-01	0.3%

15294	Solute:cation symporter activity	4,98E-01	0.7%
15299	Solute:hydrogen antiporter activity	5,57E-01	0.3%
15295	Solute:hydrogen symporter activity	6,88E-01	0.3%
15300	Solute:solute antiporter activity	6,49E-01	0.3%
16566	Specific transcriptional repressor activity	2,45E-01	0.3%
8481	Sphinganine kinase activity	1,41E-01	0.3%
5199	Structural constituent of cell wall	2,08E-01	0.7%
5200	Structural constituent of cytoskeleton	1,77E-01	0.7%
3735	Structural constituent of ribosome	4,78E-01	1.9%
5198	Structural molecule activity	1,73E-01	3.9%
43566	Structure-specific DNA binding	5,23E-01	0.3%
22838	Substarte-specific channel activity	4,98E-01	0.7%
22891	Substrate-specific transmembrane transporter activity	2,68E-01	4.3%
22892	Substrate-specific transporter activity	1,77E-01	5.5%
16157	Sucrose synthase activity	2,13E-01	0.3%
51119	Sugar transmembrane transporter activity	7,12E-01	0.3%
5351	Sugar:hydrogen symporter activity	6,88E-01	0.3%
16783	Sulfur transferase activity	2,69E-01	0.3%
15293	Symporter activity	4,98E-01	0.7%
46906	Tetrapyrrole binding	8,88E-01	0.7%
30976	Thiamin pyrophosphate bnding	2,13E-01	0.3%
4792	Thiosulfate sulfurtransferase activity	1,77E-01	0.3%
16563	Transcription activator activity	8,14E-01	0.3%
3700	Transcription factor activity	1,61E-01	9.9%
30528	Transcription regulator activity	1,41E-01	11.5%
16564	Transcription repressor activity	1,61E-01	1.1%
16757	Transferae activity, transferring glycosyl groups	3,21E-01	2.7%
16740	Transferase activity	1,14E-01	16.2%
16741	Transferase activity, transfering one-carbon groups	1,61E-01	2.3%
16763	Transferase activity, transfering pentosyl groups	2,45E-01	0.7%
16746	Transferase activity, transferring acyl groups	3,37E-01	1.5%
16747	Transferase activity, transferring acyl groups other than amino-acyl groups	2,82E-01	1.5%
16765	Transferase activity, transferring alkyl or aryl (other than methyl) groups	4,95E-01	0.7%
16758	Transferase activity, transferring hexosyl groups	3,49E-01	1.9%
16772	Transferase activity, transferring phosphorus-containing groups	2,89E-01	7.5%
16782	Transferase activity, transferring sulfur-containing groups	4,46E-01	0.3%
46914	Transition metal ion binding	1.000	3.1%
46915	Transition metal ion transmembrane transporter activity	4,83E-01	0.3%
3746	Translation elongation factor activity	4,46E-01	0.3%
8135	Translation factor activity, nucleic acid binding	3,20E-01	1.1%
3743	Translation initiation factor activity	6,69E-01	0.3%
45182	Translation regulator activity	2,02E-01	0.3%
90079	Translation regulator activity, nucleic acid binding	1,77E-01	0.3%
3747	Translation release factor activity	3,05E-01	0.3%

30371	Translation repressor activity	2,02E-01	0.3%
900	Translation repressor activity, nucleic acid binding	1,77E-01	0.3%
8079	Translation termination factor activity	3,05E-01	0.3%
22857	Transmembrane transporter activity	1,41E-01	6.7%
5215	Transporter activity	1,14E-01	9.1%
4806	Triglyceride lipase activity	5,02E-01	0.3%
42937	Tripeptide transporter activity	1,72E-01	0.3%
156	Two-component response regulator activity	4,78E-01	0.3%
4842	Ubiquitin-protein ligase activity	6,31E-01	1.1%
4843	Ubiquitin-specific protease activity	3,93E-01	0.3%
35251	UDP-glucosyltransferase activity	4,08E-01	0.7%
8194	UDP-glycosyltransferase activity	4,46E-01	1.1%
70279	Vitamin B6 binding	4,98E-01	0.3%
19842	Vitamin binding	2,72E-01	0.7%
15250	Water channel activity	4,78E-01	0.3%
5372	Water transmembrane transporter activity	4,78E-01	0.3%
42285	Xylosyltransferase activity	2,82E-01	0.3%
8270	Zinc ion binding	9,96E-01	1.9%
5385	Zinc ion transmembrane transporter activity	3,19E-01	0.3%

A.2.4 Transcripts simultaneously up-regulated in 7LD:11LD samples

Table A.8: Frequency analysis of the microarray results according to molecular function, of the simultaneously up-regulated transcripts in 7 LD and 11 LD samples, using gene ontology information; performed using the Cytoscape v2.8.3 software platform (Cline *et al*, 2007) and BiNGO v2.44 plugin (Maere *et al*, 2005)

UP-REGULATED (7LD:11LD)

GO-ID	MOLECULAR FUNCTION	p-val	Freq
46509	1,2-diacylglycerol 3-beta-galactosyltransferase activity	2,46E-01	0.3%
285	1-phosphatidylinositol-3-phosphate 5-kinase activity	2,46E-01	0.3%
16308	1-phosphatidylinositol-4-phosphate 5-kinase activity	1,78E-01	0.6%
4028	3-chloroallyl aldehyde dehydrogenase activity	3,56E-01	0.3%
80023	3R-hydroxyacyl-CoA dehydratase activity	1,64E-01	0.3%
3867	4-aminobutyrate transaminase activity	1,64E-01	0.3%
34387	4-aminobutyrate:pyruvate transaminase activity	1,64E-01	0.3%
80081	4-methylumbeliferyl-beta-D-glucopyranoside beta-glucosidase activity	1,64E-01	0.3%
8312	7S RNA binding	3,02E-01	0.3%
10427	Abscisic acid binding	2,63E-01	0.3%
3987	Acetate-CoA ligase activity	2,22E-01	0.3%
3993	Acid phosphatase activity	3,02E-01	0.6%
16881	Acid-amino acid ligase activity	2,80E-01	2.3%
16878	Acid-thiol ligase activity	3,77E-01	0.3%
22804	Active transmembrane transporter activity	8,04E-01	1.6%
16291	Acyl-CoA thioesterase activity	3,52E-01	0.3%
4014	Adenosylmethionine decarboxylase activity	2,46E-01	0.3%
30554	Adenyl nucleotide binding	5,26E-01	6.2%
32559	Adenyl ribonucleotide binding	5,26E-01	5.9%
9824	Adenylate dimethylallyltransferase activity	2,97E-01	0.3%
47627	Adenylylsulfatase activity	2,63E-01	0.3%
70566	Adenylyltransferase activity	4,02E-01	0.3%
4029	Aldehyde dehydrogenase (NAD) activity	2,80E-01	0.3%
4561	Alpha-N-acetyglucosaminidase activity	1,64E-01	0.3%
46556	Alpha-N-arabinofuranosidase activity	2,46E-01	0.3%
43176	Amine binding	4,40E-01	0.3%
8131	Amine oxidase activity	4,23E-01	0.3%
16597	Amino acid binding	4,33E-01	0.3%
4812	Aminoacyl-tRNA ligase activity	6,81E-01	0.3%
9940	Amino-terminal vacuolar sorting propeptide binding	1,64E-01	0.3%
16208	AMP binding	2,46E-01	0.3%
47668	Amygdalin beta-glucosidase activity	1,64E-01	0.3%
16209	Antioxidant activity	8,39E-01	0.3%
15297	Antiporter activity	8,39E-01	0.3%
4066	Asparagine synthase (glutamine-hydrolyzing) activity	2,46E-01	0.3%
5524	ATP binding	5,92E-01	5.5%
16887	ATPase activity	4,21E-01	1.9%
42623	ATPase activity, coupled	5,73E-01	1.3%

43492	ATPase activity, coupled to movement of substances	6,00E-01	0.6%
42625	ATPase activity, coupled to transmembrane movement of ions	5,77E-01	0.3%
42626	ATPase activity, coupled to transmembrane movement of substances	6,00E-01	0.6%
51117	ATPase binding	1,64E-01	0.3%
4176	ATP-dependent peptidase activity	2,46E-01	0.6%
4564	Beta-fructofuranosidase activity	2,80E-01	0.3%
80083	Beta-gentiobiose beta-glucosidase activity	1,64E-01	0.3%
8422	Beta-glucosidase activity	3,52E-01	0.3%
5488	Binding	2,46E-01	44.0%
9882	Blue light photoreceptor activity	2,63E-01	0.3%
5509	Calcium ion binding	7,82E-01	0.6%
15368	Calcium:cation antiporter activity	3,16E-01	0.3%
15369	Calcium:hydrogenantiporter activity	2,46E-01	0.3%
5516	Calmodulin binding	8,91E-01	0.3%
30246	Carbohydrate binding	8,44E-01	0.3%
15144	Carbohydrate transmembrane transporter activity	5,26E-01	0.6%
16830	Carbon-carbon lyase activity	7,65E-01	0.3%
16884	Carbon-nitrogen ligase activity, with glutamine as amido-N-donor	3,52E-01	0.3%
16835	Carbon-oxygen lyase activity	8,21E-01	0.3%
4091	Carboxylesterase activity	5,70E-01	1.6%
31406	Carboxylic acid binding	2,81E-01	0.6%
16831	Carboxy-lyase activity	6,21E-01	0.3%
4180	Carboxypeptidase activity	3,56E-01	0.6%
3824	Catalytic activity	4,74E-03	42.4%
43169	Cation binding	4,59E-01	9.8%
5261	Cation channel activity	5,00E-01	0.3%
8324	Cation transmembrane transporter activity	2,46E-01	2.9%
15491	Cation:cation antiporter activity	6,29E-01	0.3%
5402	Cation:sugar symporter activity	4,81E-01	0.6%
19829	Cation-transporting ATPase activity	4,95E-01	0.3%
80079	Cellobiose glucosidase activity	1,64E-01	0.3%
45430	Chalcone isomerase activity	2,46E-01	0.3%
15267	Channel activity	5,70E-01	0.6%
3682	Chromatin binding	5,26E-01	0.3%
16621	Cinnamoyl-CoA reductase activity	2,22E-01	0.3%
16859	Cis-trans isomerase activity	6,26E-01	0.3%
4129	Citochrome- C oxidase activity	3,56E-01	0.3%
16289	CoA hydrolase activity	3,56E-01	0.3%
16405	CoA-ligase activity	3,65E-01	0.3%
50897	Cobalt ion binding	2,93E-01	0.6%
50662	Coenzyme binding	8,84E-01	0.3%
48037	Cofactor binding	8,07E-01	0.6%
50269	Coniferyl-aldehyde dehydrogenase activity	1,64E-01	0.3%
5507	Copper ion binding	6,00E-01	0.9%
5375	Copper ion transmembrane transporter activity	3,57E-01	0.3%
8420	CTD phosphatase activity	2,46E-01	0.3%
30551	Cyclic nucleotide binding	4,73E-01	0.3%
4869	Cysteine-type endopeptidase inhibitor activity	1,64E-01	0.6%
8092	Cytoskeletal protein binding	7,71E-01	0.3%

15082	Di-, tri-valent inorganic cation transmembrane transporter activity	3,57E-01	0.6%
51213	Dioxygenase activity	4,52E-01	0.3%
15036	Disulfide oxidoreductase activity	5,26E-01	0.3%
3677	DNA binding	1,64E-01	13.1%
19104	DNA N-glycosylate activity	3,65E-01	0.3%
3918	DNA topoisomerase (ATP-hydrolyzing) activity	3,16E-01	0.3%
3916	DNA topoisomerase activity	3,77E-01	0.3%
3899	DNA-directed RNA polymerase activity	3,56E-01	0.6%
4500	Dopamine beta-monooxygenase activity	2,22E-01	0.3%
9055	Electron carrier activity	8,21E-01	1.3%
4519	Endonuclease activity	6,81E-01	0.3%
4866	Endopeptidase inhibitor activity	2,83E-01	0.6%
61135	Endopeptidase regulator activity	2,83E-01	0.6%
8047	Enzyme activator activity	6,74E-01	0.3%
19899	Enzyme binding	3,12E-01	0.6%
4857	Enzyme inhibitor activity	5,26E-01	0.9%
30234	Enzyme regulator activity	6,83E-01	1.3%
80082	Esculin beta-glucosidase activity	1,64E-01	0.3%
8238	Exopeptidase activity	4,54E-01	0.6%
50660	FAD binding	6,98E-01	0.3%
4325	Ferrochelatase activity	2,46E-01	0.3%
8378	Galactosyltransferase activity	3,56E-01	0.3%
22836	Gated channel activity	6,40E-01	0.3%
45543	Gibberellin 2-beta-dioxygenase activity	2,83E-01	0.3%
15926	Glucosidase activity	3,57E-01	0.3%
46527	Glucosyltransferase activity	8,28E-01	0.3%
4364	Glutathione transferase activity	5,77E-01	0.3%
5525	GTP binding	8,04E-01	0.6%
5096	GTPase activator activity	5,56E-01	0.3%
51020	GTPase binding	4,69E-01	0.3%
30695	GTPase regulator activity	7,04E-01	0.3%
19001	Guanyl nucleotide binding	8,05E-01	0.6%
32561	Guanyl ribonucleotide binding	8,04E-01	0.6%
31072	Heat shock protein binding	3,16E-01	0.6%
20037	Heme binding	7,71E-01	0.9%
15002	Heme-copper terminal oxidase activity	3,56E-01	0.3%
15929	Hexosaminidase activity	2,46E-01	0.3%
42054	Histone methyltransferase activity	4,33E-01	0.3%
42562	Hormone binding	3,65E-01	0.3%
15078	Hydrogen ion transmembrane transporter activity	3,32E-01	0.9%
16787	Hydrolase activity	2,63E-01	13.8%
16820	Hydrolase activity, actin on acid anhydrides, catalyzing transmembrane movement of substances	6,12E-01	0.6%
16818	Hydrolase activity, actin on acid anhydrides, in phosphorus-containing anhydrides	4,23E-01	3.6%
16817	Hydrolase activity, acting on acid anhydrides	3,56E-01	3.9%
16819	Hydrolase activity, acting on acid anhydrides, in sulfonyl-containing anhydrides	2,63E-01	0.3%
16788	Hydrolase activity, acting on ester bonds	3,56E-01	4.9%
16798	Hydrolase activity, acting on glycosyl bonds	2,46E-01	3.2%
16799	Hydrolase activity, hydrolyzing N-glycosyl compounds	4,02E-01	0.3%
4553	Hydrolase activity, hydrolyzing O-glycosyl compounds	2,63E-01	2.9%

16836	Hydro-lyase activity	6,83E-01	0.3%
42282	Hydroxymethylglutaryl-CoA reductase activity	2,22E-01	0.3%
42802	Identical protein binding	5,20E-01	0.6%
22890	Inorganic cation transmembrane transporter activity	2,83E-01	1.6%
4427	Inorganic diphosphatase activity	2,83E-01	0.3%
4428	Inositol or phosphatidylinositol kinase activity	3,02E-01	0.6%
16872	Intramolecular lyase activity	3,02E-01	0.3%
16860	Intramolecular oxidoreductase activity	3,02E-01	0.6%
16861	Intramolecular oxidoreductase activity, interconverting aldoses and ketoses	3,57E-01	0.3%
16862	Intramolecular oxidoreductase activity, interconverting keto- and enol-groups	3,57E-01	0.3%
16864	Intramolecular oxidoreductase activity, transposing S-S bonds	3,57E-01	0.3%
16866	Intramolecular transferase activity	5,19E-01	0.3%
50486	Intramolecular transferase activity, transferring hydroxy groups	2,46E-01	0.3%
43167	Ion binding	4,59E-01	9.8%
5216	Ion channel activity	4,61E-01	0.6%
15075	Ion transmembrane transporter activity	2,89E-01	3.2%
5506	Iron ion binding	5,73E-01	1.6%
16853	Isomerase activity	1,64E-01	2.3%
19840	Isoprenoid binding	2,80E-01	0.3%
16301	Kinase activity	3,02E-01	6.5%
16874	Ligase activity	2,22E-01	3.6%
16876	Ligase activity, forming aminoacyl-tRNA and related compounds	6,81E-01	0.3%
16879	Ligase activity, forming carbon-nitrogen bonds	2,63E-01	2.6%
16875	Ligase activity, forming carbon-oxygen bonds	6,81E-01	0.3%
16877	Ligase activity, forming carbon-sulfur bonds	4,33E-01	0.3%
16298	Lipase activity	4,13E-01	0.9%
8289	Lipid binding	3,57E-01	1.3%
1727	Lipid kinase activity	2,46E-01	0.6%
16165	Lipoxygenase activity	2,97E-01	0.3%
16829	Lyase activity	6,37E-01	1.3%
15095	Magnesium ion transmembrane transporter activity	3,02E-01	0.3%
48529	Magnesium-protoporphyrin IX monomethyl ester (oxidative) cyclase activity	1,64E-01	0.3%
15923	Mannosidase activity	2,97E-01	0.3%
4708	MAP kinase kinase activity	3,36E-01	0.3%
46872	Metal ion binding	5,26E-01	8.8%
46873	Metal ion transmembrane transporter activity	5,39E-01	0.6%
51139	Metal ion:hydrogen antiporter activity	2,63E-01	0.3%
8237	Metallopeptidase activity	7,02E-01	0.3%
8168	Methyltransferase activity	9,19E-01	0.3%
8017	Microtubule bindind	5,00E-01	0.3%
35198	miRNA binding	2,46E-01	0.3%
60089	Molecular transducer activity	4,77E-01	1.9%
30151	Molybdenum ion binding	2,46E-01	0.3%
33293	Monocarboxylic acid binding	3,56E-01	0.3%
4497	Monooxygenase activity	6,29E-01	1.3%
15077	Monovalent inorganic cation transmembrane transporter activity	3,75E-01	0.9%
3950	NAD+ ADP-ribosyltransferase activity	2,97E-01	0.3%
3951	NAD+ kinase activity	2,46E-01	0.3%
3954	NADH dehydrogenase activity	4,02E-01	0.3%

42736	NADH kinase activity	2,22E-01	0.3%
4518	Nuclease activity	8,59E-01	0.3%
3676	Nucleic acid binding	4,33E-01	17.1%
1882	Nucleoside binding	5,26E-01	6.2%
17111	Nucleoside-triphosphatase activity	4,73E-01	3.2%
60589	Nucleoside-triphosphatase regulator activity	7,14E-01	0.3%
166	Nucleotide binding	3,57E-01	10.1%
16779	Nucleotidyltransferase activity	2,46E-01	1.6%
15271	Outward rectifier potassium channel activity	2,97E-01	0.3%
16491	Oxidoreductase activity	3,56E-01	6.9%
16702	Oxidoreductase activity, actin on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen	4,33E-01	0.3%
16645	Oxidoreductase activity, actin on the CH-NH group of donors	4,40E-01	0.3%
16614	Oxidoreductase activity, acting on CH-OH group of donors	8,53E-01	0.3%
16647	Oxidoreductase activity, acting on CH-OH group of donors, oxygen as acceptor	2,46E-01	0.3%
16681	Oxidoreductase activity, acting on diphenols and related substances as donors, cytochrome as acceptor	2,83E-01	0.3%
16675	Oxidoreductase activity, acting on heme group of donors	3,56E-01	0.3%
16676	Oxidoreductase activity, acting on heme group of donors, oxygen as acceptor	3,56E-01	0.3%
50664	Oxidoreductase activity, acting on NADH or NADPH, with oxygen as acceptor	3,02E-01	0.3%
16705	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxigen	3,32E-01	1.3%
16706	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxigen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxigen into both donors	4,08E-01	0.6%
16709	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, NAD or NADH as one donor, and incorporation of one atom of oxygen	5,26E-01	0.3%
16715	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, reduced ascorbate as one donor, and incorporation of one atom of oxigen	2,22E-01	0.3%
16684	Oxidoreductase activity, acting on peroxide as acceptor	7,87E-01	0.3%
16701	Oxidoreductase activity, acting on single donors with incorporation of molecular oxygen	4,73E-01	0.3%
16667	Oxidoreductase activity, acting on sulfur group of donors	7,13E-01	0.3%
16903	Oxidoreductase activity, acting on the aldehyde or oxo group donors	2,46E-01	0.9%
16624	Oxidoreductase activity, acting on the aldehyde or oxo group of donors, disulfide as acceptor	3,56E-01	0.3%
16620	Oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	2,80E-01	0.6%
16638	Oxidoreductase activity, acting on the CH-NH2 group of donors	5,73E-01	0.3%
16641	Oxidoreductase activity, acting on the CH-NH2 group of donors, oxigen as acceptor	5,26E-01	0.3%
16616	Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	8,07E-01	0.3%
16651	Oxidoreductase ativity, acting on NADH or NADPH	3,61E-01	0.6%
4591	Oxoglutarate dehydrogenase (succinyl-transferring) activity	2,22E-01	0.3%
16679	Oxydoreductase activity, acting on diphenols and related substances as donors	5,25E-01	0.3%
19825	Oxygen binding	8,12E-01	0.6%
4592	Pantoate-beta-alanine ligase activity	1,64E-01	0.3%
4594	Pantothenate kinase activity	2,46E-01	0.3%
22803	Passive transmembrane transporter activity	5,70E-01	0.6%
30599	Pectinesterase activity	8,61E-01	0.3%
8233	Peptidase activity	6,76E-01	1.9%
70011	Peptidase activity, acting on L-amino acid peptides	8,58E-01	1.3%
30414	Peptidase inhibitor activity	3,02E-01	0.6%
61134	Peptidase regulator activity	3,02E-01	0.6%
42277	Peptide binding	4,33E-01	0.3%

3755	Peptidyl-prolyl cis-trans isomerase activity	6,21E-01	0.3%
4601	Peroxidase activity	7,87E-01	0.3%
4826	Phenylalanine-tRNA ligase activity	2,83E-01	0.3%
16791	Phosphatase activity	1,64E-01	2.6%
8429	Phosphatidylethanolamine binding	2,83E-01	0.3%
16307	Phosphatidylinositol phosphate kinase activity	1,90E-01	0.6%
35091	Phosphoinositide binding	3,02E-01	0.6%
8970	Phospholipase A1 activity	2,97E-01	0.3%
4623	Phospholipase A2 activity	2,63E-01	0.3%
4620	Phospholipase activity	3,52E-01	0.6%
5543	Phospholipid binding	2,63E-01	0.9%
51219	Phosphoprotein binding	3,56E-01	0.3%
4721	Phosphoprotein phosphatase activity	1,64E-01	1.9%
42578	Phosphoric ester hydrolase activity	2,46E-01	2.6%
16773	Phosphotransferase activity, alcohol group as acceptor	2,81E-01	5.9%
16775	Phosphotransferase activity, nitrogenous group as acceptor	4,02E-01	0.3%
9881	Photoreceptor activity	3,02E-01	0.3%
48244	Phytanoyl-CoA dioxygenase activity	1,64E-01	0.3%
46592	Polyamine oxidase activity	2,46E-01	0.3%
47262	Polygalacturonate 4-alpha-galacturonosyltransferase activity	4,52E-01	0.3%
5267	Potassium channel activity	4,40E-01	0.3%
15405	P-P-bond-hydrolysis-driven transmembrane transporter activity	7,36E-01	0.6%
15399	Primary active transmembrane transporter activity	7,37E-01	0.6%
5515	Protein binding	1,90E-01	13.8%
46983	Protein dimerization activity	1,64E-01	1.6%
3756	Protein disulfide isomerase activity	3,57E-01	0.3%
15035	Protein disulfide oxidoreductase activity	4,59E-01	0.3%
46982	Protein heterodimerization activity	2,83E-01	0.6%
4673	Protein histidine kinase activity	4,02E-01	0.3%
42803	Protein homodimerization activity	3,16E-01	0.6%
4672	Protein kinase activity	3,75E-01	4.6%
8276	Protein methyltransferase activity	5,00E-01	0.3%
45309	Protein phosphorylated amino acid binding	3,56E-01	0.3%
4674	Protein serine/threonine kinase activity	6,44E-01	2.6%
4722	Protein serine/threonine phosphatase activity	1,67E-01	1.6%
4712	Protein serine/threonine/tyrosine kinase activity	1,64E-01	0.9%
8565	Protein transporter activity	8,04E-01	0.3%
4713	Protein tyrosine kinase activity	7,65E-01	0.6%
4725	Protein tyrosine phosphatase activity	3,56E-01	0.3%
8138	Protein tyrosine/serine/threonine phosphatase activity	3,52E-01	0.3%
8476	Protein-tyrosine sulfotransferase activity	1,64E-01	0.3%
46961	Proton-transporting ATPase activity, rotational mechanism	3,56E-01	0.3%
1883	Purine nucleoside binding	5,26E-01	6.2%
17076	Purine nucleotide binding	5,70E-01	6.9%
32555	Purine ribonucleotide binding	5,70E-01	6.5%
30170	Pyrodoxal phosphate binding	5,51E-01	0.3%
16462	Pyrophosphatase activity	4,22E-01	3.6%
16854	Racemase and epimerase activity	4,73E-01	0.3%
16857	Racemase and epimerase activity, acting on carbohydrates and derivatives	4,33E-01	0.3%

8536	Ran GTPase binding	4,23E-01	0.3%
5099	Ras GTPase activator activity	4,81E-01	0.3%
17016	Ras GTPase binding	4,33E-01	0.3%
4872	Receptor activity	7,57E-01	0.6%
5100	Rho GTPase activator activity	2,46E-01	0.3%
32553	Ribonucleotide binding	5,70E-01	6.5%
47345	Ribose-5-phosphate adenyltransferase activity	1,64E-01	0.3%
4751	Ribose-5-phosphate isomerase activity	2,46E-01	0.3%
3723	RNA binding	9,74E-01	2.3%
34062	RNA polymerase activity	2,46E-01	0.9%
3702	RNA polymerase II transcription factor activity	4,95E-01	0.3%
3968	RNA-directed RNA polymerase activity	2,80E-01	0.3%
15291	Secondary active transmembrane transporter activity	7,14E-01	0.9%
8430	Selenium binding	2,83E-01	0.3%
43565	Sequence-specific DNA binding	7,27E-01	0.3%
17171	Serine hydrolase activity	7,04E-01	0.6%
4185	Serine-type carboxypeptidase activity	3,56E-01	0.6%
4867	Serine-type endopeptidase inhibitor activity	4,40E-01	0.3%
70008	Serine-type exopeptidase activity	3,56E-01	0.6%
8236	Serine-type peptidase activity	7,04E-01	0.6%
16987	Sigma factor activity	2,80E-01	0.3%
5048	Signal sequence binding	2,83E-01	0.3%
4871	Signal transducer activity	4,77E-01	1.9%
19787	Small conjugating protein ligase activity	3,25E-01	1.9%
31267	Small GTPase binding	4,33E-01	0.3%
5083	Small GTPase regulator activity	6,81E-01	0.3%
5484	SNAP receptor activity	4,95E-01	0.3%
15298	Solute:cation antiporter activity	6,44E-01	0.3%
15294	Solute:cation symporter activity	5,70E-01	0.6%
15299	Solute:hydrogen antiporter activity	6,00E-01	0.3%
15295	Solute:hydrogen symporter activity	4,81E-01	0.6%
15300	Solute:solute antiporter activity	7,04E-01	0.3%
3735	Structural constituent of ribosome	9,42E-01	0.6%
5198	Structural molecule activity	8,79E-01	1.3%
22838	Substarte-specific channel activity	5,70E-01	0.6%
22891	Substrate-specific transmembrane transporter activity	5,18E-01	3.2%
22892	Substrate-specific transporter activity	5,53E-01	3.6%
16157	Sucrose synthase activity	2,80E-01	0.3%
51119	Sugar transmembrane transporter activity	5,13E-01	0.6%
5351	Sugar:hydrogen symporter activity	4,81E-01	0.6%
8146	Sulfotransferase activity	4,02E-01	0.3%
16783	Sulfur transferase activity	3,16E-01	0.3%
15293	Symporter activity	5,70E-01	0.6%
17025	TATA-binding protein binding	1,64E-01	0.3%
46906	Tetrapyrrole binding	8,04E-01	0.9%
16790	Thioester hydrolase activity	7,35E-01	0.3%
4792	Thiosulfate sulfurtransferase activity	2,46E-01	0.3%
8483	Transaminase activity	5,73E-01	0.3%
16563	Transcription activator activity	4,33E-01	0.9%

3700	Transcription factor activity	1,64E-01	10.1%
16986	Transcription initiation factor activity	3,65E-01	0.3%
30528	Transcription regulator activity	1,64E-01	10.8%
16564	Transcription repressor activity	3,56E-01	0.6%
16757	Transferae activity, transferring glycosyl groups	5,61E-01	1.9%
16740	Transferase activity	2,63E-01	12.8%
16769	Transferase activity, transfering nitrogenous groups	5,99E-01	0.3%
16741	Transferase activity, transfering one-carbon groups	9,19E-01	0.3%
16763	Transferase activity, transfering pentosyl groups	5,70E-01	0.3%
16765	Transferase activity, transferring alkyl or aryl (other than methyl) groups	3,57E-01	0.9%
16758	Transferase activity, transferring hexosyl groups	7,61E-01	0.9%
16772	Transferase activity, transferring phosphorus-containing groups	2,46E-01	8.2%
16782	Transferase activity, transferring sulfur-containing groups	2,50E-01	0.6%
46914	Transition metal ion binding	4,02E-01	8.2%
46915	Transition metal ion transmembrane transporter activity	5,26E-01	0.3%
8135	Translation factor activity, nucleic acid binding	8,27E-01	0.3%
3743	Translation initiation factor activity	7,30E-01	0.3%
4888	Transmembrane receptor activity	8,74E-01	0.3%
22857	Transmembrane transporter activity	5,99E-01	3.6%
5215	Transporter activity	5,00E-01	5.2%
4806	Triglyceride lipase activity	5,56E-01	0.3%
15631	Tubulin binding	5,26E-01	0.3%
156	Two-component response regulator activity	5,25E-01	0.3%
155	Two-component sensor activity	2,46E-01	0.3%
8121	Ubiquinol-cytochrome-C reductase activity	2,83E-01	0.3%
4842	Ubiquitin-protein ligase activity	3,08E-01	1.9%
35250	UDP-galactosyltransferase activity	2,80E-01	0.3%
3978	UDP-glucose 4-epimerase activity	2,97E-01	0.3%
35251	UDP-glucosyltransferase activity	7,35E-01	0.3%
8194	UDP-glycosyltransferase activity	5,26E-01	0.9%
10209	Vacuolar sorting signal binding	2,22E-01	0.3%
70279	Vitamin B6 binding	5,51E-01	0.3%
19842	Vitamin binding	6,00E-01	0.3%
22843	Voltage-gated cation channel activity	4,33E-01	0.3%
22832	Voltage-gated channel activity	5,26E-01	0.3%
5244	Voltage-gated ion channel activity	5,26E-01	0.3%
5249	Voltage-gated potassium channel activity	4,33E-01	0.3%
9044	Xylan 1,4-beta-xylosidase activity	2,80E-01	0.3%
8270	Zinc ion binding	4,71E-01	5.2%
5385	Zinc ion transmembrane transporter activity	3,56E-01	0.3%