Understanding DELLA in Wheat

Linking Genotype to Phenotype

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Abstract

DELLA proteins are members of a family of putative transcription factors and are involved in gibberellic acid (GA) signal transduction. Gain of function (GoF) mutant alleles, such as the well characterised Arabidopsis mutant *gibberellic acid insensitive* (*gai*), have reduced GA responsiveness, resulting in a dwarf phenotype. Arabidopsis DELLAs have been shown to enhance abiotic stress tolerance and play a role in disease response.

The 'Green Revolution' wheat semi-dwarfing alleles, *Rht-B1b* and *Rht-D1b*, are orthologues of Arabidopsis *gai* and are now used extensively in modern cultivars to reduce plant height. However, considering their importance in modern agriculture, very little work has been carried out on DELLA in crop species. To this end, this project set out to increase our understanding of DELLA (*Rht*) in wheat and barley.

A qRT-PCR assay has been developed to measure homoeologue specific expression of the wheat group 4 *Rht* genes (Chapter 2). I showed that temporal and spatial expression of each of the homoeologues was similar. I also showed that DELLA function is partially controlled at the transcriptional level in wheat; throughout development and in response to the environment. Additionally the assay enabled the identification of transcriptional differences of previously uncharacterised wheat *Rht* mutant alleles.

The, ultimately elusive, search for an additional *Rht* family member on wheat chromosome 7D was pursued in Chapter 3, because of the potential agronomic benefits of a novel source of dwarfing and the fundamental interest of GA mediated-growth control in a monocot crop species.

Wheat and barley lines differing in DELLA status were tested for relative resistance to a series of important cereal pathogens representing each of the three trophic lifestyles; biotroph, hemibiotroph and necrotroph (Chapter 4). It was demonstrated that wheat and barley GoF mutants generally confer increased resistance to necrotrophs and increased susceptibility to biotrophs compared to wild type and vice versa in loss-of-function barley mutants.

Reactive oxygen species (ROS) accumulation, and accordingly ROS-induced cell death, was reduced in DELLA GoF barley lines. Thus DELLA may function as a negative regulator of cell death through the modulation of ROS levels. This theme was developed further in Chapter 5 by assessing the effect of environment on the DELLA conferred biotroph-necrotroph trade-off. Exposing plants to a range of light treatments was shown to alter the DELLA conferred trade-off scenario possibly as a consequence of altered ROS homeostasis. Studying the interaction of environmental and pathogen-induced stress with agronomically important genes such as DELLA in crop plants is important in relation to maintaining and enhancing yields in a changing climate.

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Abbreviations

ANOVA	analysis of variance
BACs	bacterial artificial chromosomes
bp	base pair(s)
BLAST	Basic Local Alignment Search Tool
°C	degrees Celsius
CAAS	Chinese Academy of Agricultural Sciences
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CER	controlled environment room
cDNA	complementary DNA
Cm	centimorgans
cm	centimetre(s)
CS	Chinese Spring
CTAB	hexadecyltrimethylammonium bromide
C_t	cycle threshold
DON	deoxynivalenol
DNA	deoxyribonucleic acid
Et	ethylene
ESTs	expressed sequence tags
EEE	excess excitation energy
FHB	fusarium head blight
GoF	gain of function
GLM	general linear model
GA	gibberillic acid
GP	Golden Promise
GS	growth stage
HR	hypersensitive responce
JA	jasmonic acid
JIC	John Innes Centre
Kb	kilobase(s) (1×10^3 bp)
LoF	loss of function

Mb	mega-base(s) (1 x 10^6 bp)
M	mole(s)
mg	milligram(s)
mm	millimetre(s)
min	minute(s)
ml	millilitre(s)
NIAB	National Institute of Agricultural Botany
NBCI	National Center for Biotechnology Information
ng	nanogram(s)
NIL	near isogenic line
ORF	open reading frame
PAMP	pathogen associated molecular pattern
P	probability
PCR	polymerase chain reaction
PCD	progammed cell death
pH	hydrogen ion concentration
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
QTL	quantitative trait loci
RRes	Rothamsted Research
ROS	reactive oxygen species
Rht	*Reduced height*
RNA	ribonucleic acid
SA	salicylic acid
s	seconds
SNPs	single nucleotide polymorphisms
SSCP	single-stranded conformation polymorphism
SAA	systemic aquired acclimation
µg	microgram(s)
µl	microlitre(s)
µM	micromole(s)
WT	wildtype

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1. General Introduction

Fears of mass food shortages owing to an increasing world population were alleviated by unprecedented increases in wheat yields in the mid 20th century coined The Green Revolution. The Green Revolution was brought about by improvements in agricultural practice and the development and deployment of high yielding varieties in the developing and developed world. A reduction in stem height was key to the increase in yields. Stems of wild type wheat were not strong enough to support the weight of the heavy heads of the high yielding varieties and so fell over, referred to as lodging. In addition greater amounts of nitrogen fertiliser could be applied to dwarfed crops which only leads to an exacerbation of lodging in wild type crops. Introduction of the so-called dwarfing genes resulted in an increase in harvest index, that is, less assimilate invested in the stem in favour of the grain. Two of the main dwarfing genes utilised by worldwide wheat improvement programmes have been the semi-dwarfing 'Reduced height' genes *Rht-B1b* (also termed *Rht1*) and *Rht-D1b* (also termed *Rht2*). Due to their key role in the development of the high yielding varieties they have become known as the Green Revolution genes. It is now known that the wild type alleles of the semi-dwarfing genes encode DELLA proteins which function as negative regulators in gibberellic acid (GA) signal transduction (Peng *et al.*, 1999). In addition to developmental traits research in model plants has demonstrated that these genes have pleiotropic effects on abiotic and biotic stress tolerance/resistance. Now present in the majority of wheat cultivars, it is important to understand how growth is regulated and the positive and deleterious effects the semi-dwarfing alleles have on abiotic and biotic stress.

1.1. Gibberellin Biosynthesis

Gibberellins (GAs) are a large family of tetracyclic diterpenoid phytohormones which affect a broad range of growth and developmental processes throughout the plant life cycle from cell growth and division to the development of flowers, fruits and seeds (Hooley, 1994). Gibberellins were first identified from a fungus, *Gibberella fujikuroi*, which is the causative agent of bakanae (foolish seedling) disease in rice plants. The fungus causes a constitutive growth phenotype due to the secretion of GA. Since this discovery over one hundred gibberellins have been identified in plants (Richards *et al.*, 2001) but only a few of these, GA_1, GA_3, GA_4 and GA_7, are biologically active. The pathway leading to the biosynthesis of the bioactive GAs contains many steps but, for simplicity, can be reduced to six core enzymes. Copalyl diphosphate synthase (CPS) and kaurene synthase (KS) are involved in the early stages of the pathway in which geranylgeranyl diphosphate (GGPP) is converted to ent-kaurene, these reactions take place in the chloroplast and the enzymes involved are members of the diterpene cyclases. Kaurene oxidase (KO) and kaurenoic acid oxidase (KAO) are membrane associated cytochrome P450 monooxygenases and catalyse a series of

oxidations to produce GA_{12}. The final stages in the biosynthesis of bioactive GA take place in the cytoplasm and are catalysed by two 2-oxyglutarate-dependent dioxygenases (2ODD), GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox) which convert GA_{12}, via a series of intermediates, to the bioactive GAs. The first catabolic step of the bioactive GAs, resulting in their deactivation, is catalysed by a third 2ODD, GA 2-oxidase (GA2ox). A diagram of the steps of the GA biosynthetic pathway is shown in Fig. 1.1.

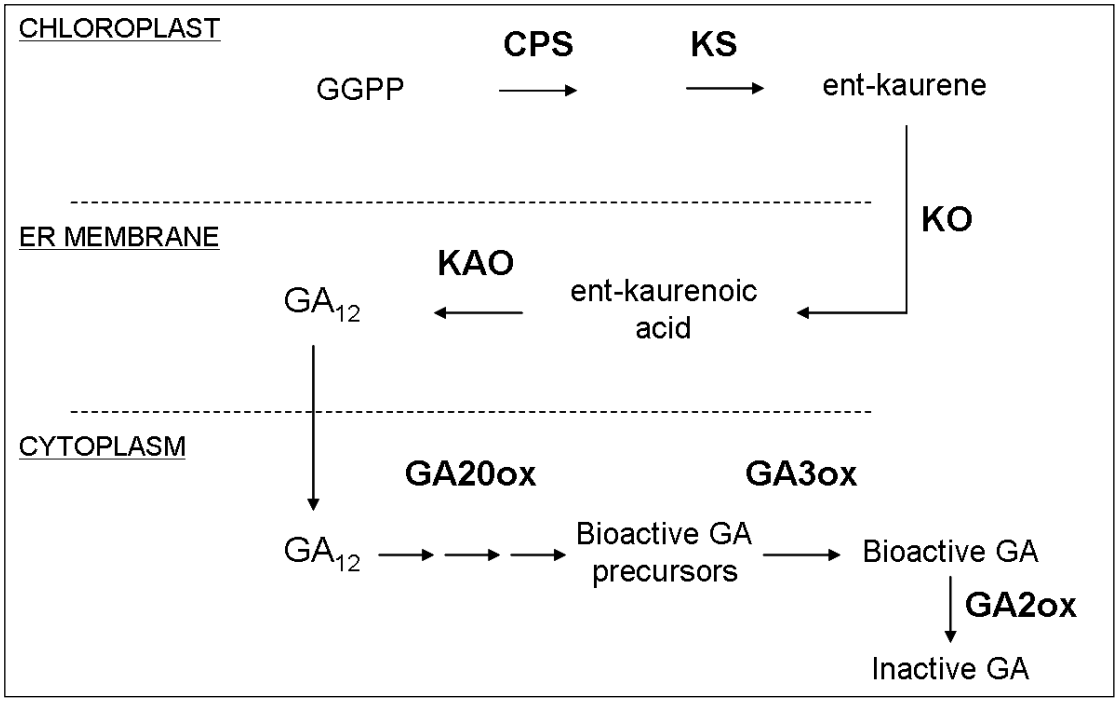

Figure 1.1 A simplified diagram of the GA biosynthetic pathway. CPS, copalyl diphosphate synthase; KS, kaurene synthase; KO, Kaurene oxidase; KAO, kaurenoic acid oxidase; GA20ox, GA 20-oxidase; GA3ox, GA 20-oxidase; GA2ox, GA 2-oxidase; GGPP, geranylgeranyl diphosphate. Modified from Sakamoto *et al.* (2004)

The identification and isolation of GA deficient dwarf mutants (and slender mutants in the case of defective GA2ox genes) across a range of plant species have helped to elucidate the components of the GA biosynthetic pathway (reviewed by Yamaguchi, 2008). Although GA deficient dwarf mutants have been isolated from many plant species the majority of the fundamental work has been carried out in Arabidopsis plants because of the availability of mutants along with the other attributes of using a model system such as rapid generation time and availability of the whole genome sequence. GA deficient mutants are unable to synthesise normal levels of GA because mutations in genes encoding enzymes required in the GA biosynthetic pathway result in a reduction or loss of function (LoF) of the enzymes. The more severe GA deficient mutants fail to germinate, but will germinate following exogenous application of bioactive GA. Once germinated the mutant plants exhibit darker green leaves than the wild type and have reduced fertility but these phenotypes can also be rescued to that of the wild type by the continued exogenous application of

bioactive GA. One of the most studied of the GA synthesis mutants is *ga1-3* of Arabidopsis. The GA deficiency of *ga1-3* is caused by a 5-kb deletion which abolishes the function of a gene (*GA1*; Sun *et al.*, 1992), encoding CPS, the enzyme responsible for the first committed step of GA biosynthesis. Since the introduction of *sd1* into high yielding rice cultivars which contributed to the yield increases of the rice Green Revolution Ashikari *et al* (2002) have shown that the *sd1* gene encoded a GA biosynthetic enzyme, GA20 oxidase.

GA biosynthesis is under the control of several regulatory mechanisms, important to coordinate growth and development in response to the environment. Photoperiod and hormone signals have been identified as influential factors in the control of GA metabolism. For example, PHYTOCHROME INTERACTING FACTOR 3-LIKE 5 (PIL5), a light-labile basic helix-loop-helix protein, inhibits seed germination by repressing GA biosynthetic genes, *GA3ox*, and activating the GA catabolic gene, *GA2ox* (Oh *et al*., 2004). Subsequently it has been demonstrated that PIL5 also directly regulate GA signalling components (Oh *et al*., 2007). The levels of GA and auxin have also been demonstrated to regulate GA metabolism. For example a study by Ross *et al.* (2000) presents evidence of auxin promoting the biosynthesis of GA_1 through an increase in *PsGA3ox* transcript levels in *Pisum sativum*. Application of GA was shown to auto regulate GA levels through negative feedback control, in *Pisum sativum* (Martin *et al.*, 1996).

1.2. **Gibberellin Signalling**

1.2.1. **GA perception**

It has been postulated that plants have both soluble and membrane-bound GA receptors (Gilroy & Jones, 1994; Ueguchi-Tanaka *et al.*, 2005). The *gid1 (giberellin insensitive dwarf 1)* mutant in rice exhibits a dwarf phenotype which cannot be rescued to wild type height with exogenous application of GA. Isolation of the gene responsible, by positional cloning, revealed that *GID1* encodes a hormone-sensitive lipase-like (HSL-like) protein. Loss of function (LoF) mutations of GID1 result in loss of GA responsiveness resulting in dwarf plants whilst the over expression of *GID1* in rice results in a "GA overdose" phenotype suggesting that the gene encodes a positive component of the GA signal transduction pathway. In addition yeast-2-hybrid studies demonstrate that GID1 interacts with the rice DELLA protein, SLR1 in a GA-dependent manner (Ueguchi-Tanaka *et al.*, 2007). Protein folding prediction software showed that multiple mutant alleles with disrupted GID1 function had mutations clustered around a putative GA binding pocket (Ueguchi-Tanaka *et al.*, 2007). Three orthologues of the *OsGID1* gene have been cloned from Arabidopsis, namely *AtGID1a*, *AtGID1b* and *AtGID1c* (Nakajima *et al.*, 2006). They have been shown to bind GA and interact with the Arabidopsis DELLAs in the presence of GA_4. Furthermore transgenic expression of *AtGID1* in the rice *gid1* mutant rescued the GA-insensitive phenotype. These studies

demonstrate that GID1 and orthologues function as GA receptors. Further understanding of GID1's function as a receptor was gained from determining its crystal structure in both rice (Shimada *et al.*, 2008) and Arabidopsis (Murase *et al.*, 2008) which is incorporated in the current GA signalling model (described at the end of this section in Fig. 1.2).

Plants encoding *gid1* are completely unresponsive to GA suggesting that GID1 is a solo receptor of GA in rice, however there is significant evidence for a membrane-localised GA receptor from experiments by Gilroy and Jones (1994). These experiments demonstrate that the microinjection of GA into aleurone cells did not elicit a GA response but extracellular GA did. In order to explain this it is suggested that GID1 may function as a receptor of intracellular GA in some cells while in others it may function, without binding GA, as a downstream signal transduction component of an, as yet unidentified, membrane bound receptor (Hartweck & Olszewski, 2006). The newly proposed model, suggesting that GID1 may function independently of GA would fit with the proposed early action of the heterotrimeric G-protein as another downstream signal transduction component of a membrane bound receptor. *Dwarf 1* of rice encodes the α-subunit of a heterotrimeric G-protein (Ashikari *et al.*, 1999). Rice plants carrying the *dwarf1* gene have reduced, but not abolished GA responsiveness, strengthening the suggestion that a second GA signalling pathway runs parallel to the GA dependent GID1 pathway (Ueguchi-Tanaka *et al.*, 2000).

1.2.2. **DELLA proteins are negative regulators of GA signalling**

DELLA proteins are negative regulators of the GA signalling pathway. The restraint on growth applied by DELLA is relieved by GA-mediated degradation of the protein thus relieving the DELLA mediated restraint on growth.

DELLA proteins are a sub family within a larger family known as the GRAS protein family. The GRAS proteins comprise of plant specific putative transcription factors. In addition to DELLA proteins other GRAS family members include *SCARECROW* (SCR; DiLaurenzio *et al.*, 1996) *LATERAL SUPPRESSOR* (LS; Schumacher *et al.*, 1999), *HAIRY MERISTEM* (HAM; Stuurman *et al.*, 2002), and *PHYTOCHROME A SIGNALLING* (PAT1; Bolle *et al.*, 2000).

Koorneef *et al.* (1985) identified a dwarf Arabidopsis plant with reduced apical dominance and seed germination, typical of a GA deficient mutant, however this plant did not respond to exogenous application of GA. For this reason the mutant was named *ga insensitive* (*gai*). GAI was cloned using insertional mutagenesis, which revealed it encodes a negative GA signal regulator (Peng *et al.*, 1997) The *gai-1* mutant allele identified by Koorneef *et al.* was determined to carry a 51bp deletion within the *GAI* open reading frame (ORF). This results in the encoded protein lacking 17 amino acid residues at the N- terminus within the DELLA motif, thus conferring GA insensitivity. The isolation of *GAI* was followed by the isolation of another DELLA member in the

Arabidopsis genome named *RGA* (for *repressor of ga1-3*; Silverstone *et al.*, 1998). So named and initially identified because the recessive *rga* mutant partially suppresses the *ga1-3* phenotype.

The RGA and GAI proteins show absolute conservation in two domains at their amino termini, the DELLA domain (disrupted in the *gai-1* allele) and the VHYNP domain, the former from which the DELLA gene family takes its name. The DELLA and VHYNP domains have been demonstrated to be required for interaction with GID1 which target DELLA proteins for degradation (Ueguchi-Tanaka *et al*., 2007). Since the isolation of GAI and RGA from Arabidopsis, three additional DELLA proteins have been identified in the Arabidopsis genome. The three proteins, which show homology to RGA, were named RGL 1, RGL2 and RGL3 (for RGA-like1, 2 and 3; Wen & Chang, 2002).

The importance of the two N-terminal domains (DELLA and VHYNP) of DELLA proteins in GA signal transduction is reinforced by their conservation in other plant species including monocots. Peng *et al.* (1999) isolated and molecularly characterised the 'Green Revolution' dwarfing alleles of hexaploid wheat, *Rht-B1b* and *Rht-D1b* and three mutant alleles at the *D8* locus of maize. The *Rht* (*Reduced-height)* locus of wheat and the *D8* (*Dwarf 8*) locus of maize both encode DELLA proteins. The mutant alleles at these loci are defective in the DELLA and/or the VHYNP domains. The *Rht-B1b* and *Rht-D1b* alleles have nucleotide substitutions that create stop codons within the DELLA domain. Other *Rht* mutant alleles have been identified and these will be discussed in section 1.3. The molecular characterisation of three maize dwarfing alleles (*D8-1*, *D8-2023* and *D8-Mpl*) shows that *D8-1* has a deletion within the DELLA conserved motif, similar to *gai*; *D8-2023* is caused by the absence of a section of the VHYNP domain and *D8-Mpl* is the result of a deletion of both of the conserved domains (Peng *et al*., 1999).

All of the DELLA proteins mentioned thus far have been identified as GA insensitive dwarfs resulting from gain of function (GoF) mutations. GoF DELLA proteins exhibit increased stability in the presence of GA resulting in a reduced sensitivity to bioactive GAs. In contrast, LoF mutants exhibit a constitutive GA response which is not affected by GA deficiency (Chandler & Robertson, 1999) or treatment with inhibitors of GA biosynthesis (Ikeda *et al.*, 2001). The LoF mutants, known as slender mutants, are characterised by their elongated internode length.

The first slender mutant protein to be characterised was *slr1* (*slender rice 1*; Ikeda *et al.*, 2001) from rice. The *slr1-1* mutant allele is caused by a LoF mutation in the rice orthologue of *GAI*, *SLENDER RICE 1* (*SLR1*). Slender mutants of barley have also been molecularly characterised, and the SLENDER 1 (SLN1) protein identified as a GAI orthologue (Chandler *et al.*, 2002). Three slender mutants have been identified in barley, designated *sln1a*, *sln1b* and *sln1c*. No mutation was found in the ORF of *sln1a*, it is thought that altered expression or translation causes the slender phenotype conferred by this gene. The *sln1b* and *sln1c* alleles have been associated with the

introduction of premature stop codons in the C-terminal region, which is associated with the repressor function of DELLA proteins. The GA constitutive response phenotype of the LoF mutants strengthens the proposed role of DELLAs as negative repressors of GA signalling. Chandler *et al.* (2002) also reported a barley GA insensitive dwarf mutant, M640. The mutated gene responsible for the dwarf phenotype mapped to the *SLN1* locus and so was designated *Sln1d*. The dwarf phenotype conferred by the *Sln1d* allele is caused by an amino acid substitution (G-to-E) very close to the DELLA motif (**DELLA**ALG to **DELLA**ALE). The M770 (*sln1c*) and M640 (*Sln1d*) mutant lines are available in the common background, Himalaya (Fig. 1.2), and provide useful tools to study the effect of DELLA in a monocotyledonous crop species.

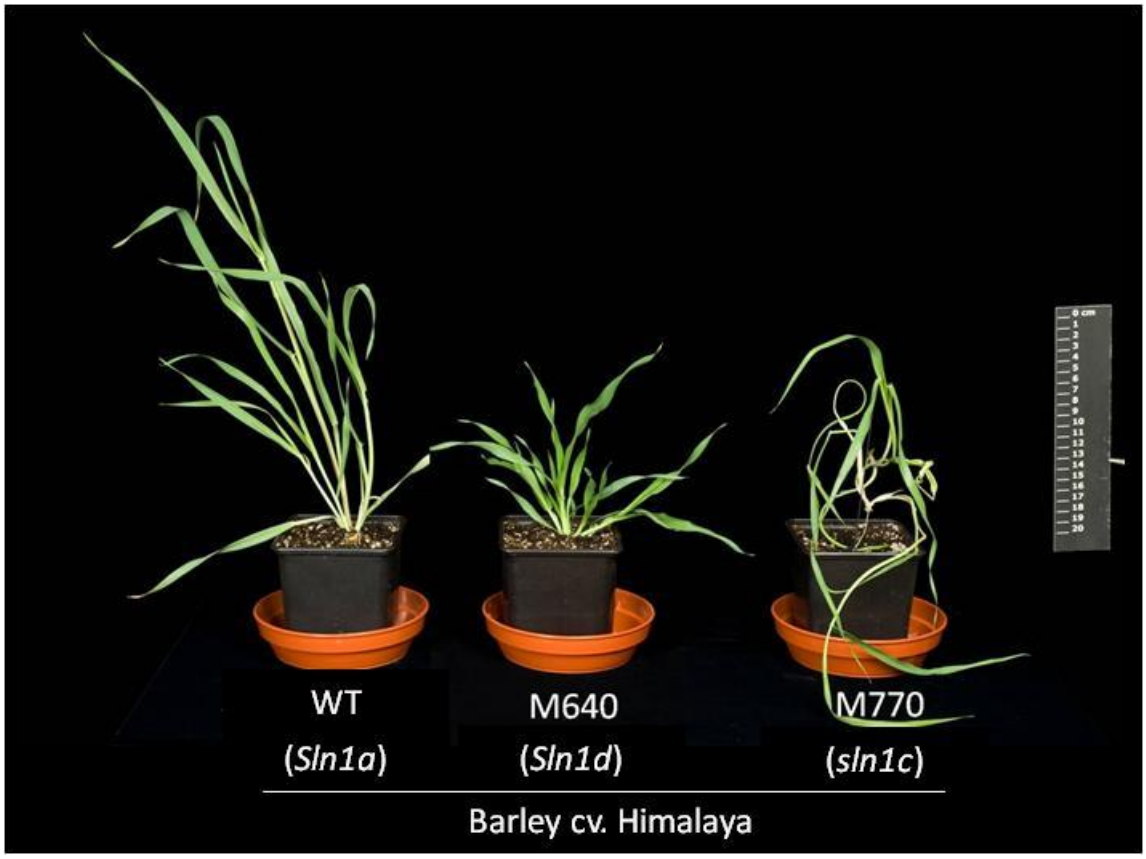

Figure 1.2. Barley cv. Himalaya lines allelic at the *Sln1* locus. The wildtype line (left), GoF dwarf line (centre) and LoF slender line (right) provide usful tools to investigate the effects of DELLA in monocotyledonous crop species. The allele designations are in parenthesis. Picture courtesy of Thomas Moore.

1.2.3. **DELLA redundancy and fine control**

Loss of function mutants have been artificially produced in Arabidopsis and wheat. Irradiation-induced mutagenesis of Arabidopsis carrying the *gai* gene resulted in a presumed LoF allele which conferred a revertant phenotype, indistinguishable from the wild type (Peng & Harberd, 1993).

Simirlarly Peng *et al.* (1999) used fast–neutron mutagenesis on wheat plants carrying the *Rht-B1b* allele and produced a novel allele named *Rht-B1g*. The new allele conferred a wild type phenotype caused by a deletion mutation that abolishes RHT-B1b function. A LoF mutation of an individual Arabidopsis or wheat DELLA protein does not confer a slender phenotype as seen in rice and barley due to a redundancy of function meaning that growth is still restrained. The Arabidopsis effect is due to four other genes in the gene family and wheat effect due to two other copies of the same gene in the polyploidy genome. In such cases, LoF mutations are only phenotypically identifiable when two or more of the DELLA encoding genes contain null mutations (e.g. in Arabidopsis both GAI and RGA function has to be abolished). A quadruple DELLA mutant has been generated in Arabidopsis which lacks four of the five DELLA proteins (Cheng *et al.*, 2004) and is used extensively in investigations into the effects of DELLA.

As described, LoF mutants of barley and rice (recessive *sln* and *slr* alleles) confer a slender phenotype suggesting that SLN1 and SLR1 are the only DELLA proteins in their respective GA signalling pathways. This is consistent with database searches of the rice genome that suggest that rice only has one DELLA protein compared with the Arabidopsis genome which contains five DELLA proteins. However, a study carried out by Itoh *et al.* (2005) identified two genes, *SLR1-LIKE1* and *2* (*SLRL-1* and *-2*), that are highly homologous to *SLR1*. The SLRL proteins cluster within the DELLA protein clade despite the fact that they lack the DELLA and VHYNP motifs. Transgenic plants over-expressing SLRL1 exhibit a GA-insensitive dwarf phenotype (Itoh *et al.*, 2005), indicating that the repressor domain at the C-terminus is functioning in the overproduction condition and the protein does not degrade in response to GA treatment, presumably due to the lack of the DELLA and VHYNP motifs at the N-terminus. It is suggested that these GA non-degradable DELLAs function as a fine control mechanism preventing an excessive growth response to GA, which could be very costly to the plant. This fine control mechanism may also be present in Arabidopsis, it has been demonstrated that both RGL1 and GAI are less susceptible to GA-stimulated destabilisation than RGA (Fleck & Harberd, 2002; Wen & Chang, 2002).

1.2.4. **SPY - another negative regulator of GA signalling**

Mutations at the *spindly* (*spy*) locus of Arabidopsis plants suppress all the phenotypes caused by GA deficiency (Jacobsen & Olszewski, 1993) and partially suppress the DELLA GoF phenotype (Jacobsen *et al.*, 1996). *SPY* encodes an O-linked N-acetylglucosamine (GlcNAc) transferase (OGT; Jacobsen et al., 1996). OGTs have been shown to function in a variety of eukaryotes, in the transfer of GlcNAc monosaccharides to proteins containing regions rich in serine and threonine residues (Hanover *et al.*, 2005). The DELLA proteins contain sequences that are rich in serine and threonine residues and these are thought to act as target sites for OGT modification. GlcNAc modification has been shown to affect nuclear localisation, protein stability and/or activity of the

target proteins (Hanover *et al.*, 2005). Silverstone *et al.*, (2007) demonstrates that SPY negatively regulates the GA signal transduction pathway through OGT modification of the DELLA proteins directly increasing the activity of DELLA proteins.

1.2.5. **Multiple hormone signals affect DELLA function**

Various phytohormones in addition to GA are involved in the growth of plants, e.g. auxin, ethylene, cytokinin, abscisic acid and brassinosteroid. DELLA proteins have been implicated as an integrator of many of these hormone signalling inputs and it is suggested that the hormones may modulate the growth-repressing effects of DELLA proteins either directly or indirectly by acting on GA signalling and/or biosynthesis (as previously discussed).

Ethylene (ET), for example, was demonstrated to regulate Arabidopsis development by the modulation of DELLA proteins in a study by Achard *et al.* (2003). ET functions in many processes including the regulation of shoot and root growth and differentiation (Davies, 1995). The study demonstrated, using DELLA LoF mutants, that ET inhibited Arabidopsis root growth in a DELLA-dependant manner and that GFP-RGA was maintained in the root nuclei in ET treated plants. It is therefore suggested that ET enhances the resistance of DELLA to the destabilising effects of GA.

Auxin has also been demonstrated to modulate the gibberellin mediated degradation of DELLA proteins. Auxin is involved in a number of growth and developmental processes including shoot apical dominance and root elongation (Davies, 1995). Shoot tips are one of the main sites of auxin biosynthesis. In experiments carried out by Fu & Harberd (2003) the shoot apices of pRGA:GFP-RGA seedlings were removed resulting in reduced auxin levels. Following treatment with exogenous GA, intact pRGA:GFP-RGA seedlings exhibited a rapid disappearance of GFP-RGA however, GFP-RGA was still present in the decapitated seedlings after 4 hours of treatment with GA. Application of auxin to the site from which the shoot apex was removed resulted in the rapid GA-induced disappearance of GFP-RGA, as seen in intact seedlings. A specific inhibitor of auxin efflux, 1 N-naphthylphthalamic acid (NPA) applied to *ga1-3* plants inhibits the reversion of normal growth after the application of GA, while *ga1-3* plants lacking GAI and RGA reverted to near-normal growth. The study concluded that auxin functions in part through affecting the properties of DELLA proteins, and promotes the disappearance of DELLA in response to GA.

1.2.6. **F-box proteins – positive regulators of GA signalling**

F-box proteins function as part of an SCF complex. The role of SCF complexes is to catalyse the ubiquitination of proteins destined for proteasomal mediated degradation (Patton *et al*., 1998). The SCF complex consists of four main subunits, Skp, Cullin, F-box and a ring domain protein. The role of the F-box protein, as part of an SCF complex, is to recognise and interact with specific

protein substrates. F-box proteins have been implicated in the GA-dependent degradation of DELLA proteins through the characterisation of the rice LoF mutation, *gid2*, (*GA-insensitive dwarf 2*; Sasaki *et al.*, 2003). Sasaki and colleagues showed that the wild type *GID2* encodes an F-box protein that interacts with the rice Skp homologue. The *gid2* mutants accumulated DELLA, even after GA treatment, whilst DELLA in wild type plants was rapidly ubiquitinated and degraded (Sasaki *et al.*, 2003). Corroborating the involvement of 26S proteasome degradation of DELLA in the GA signal transduction pathway is the observation that a proteasome inhibitor, MG132, prevents GA-mediated degradation in barley (Fu *et al.*, 2002). The Arabidopsis homolog of GID2, SLEEPY1 (SLY1), has also been identified (McGinnis *et al.*, 2003). The identification of SLY1 along with other GID2 homologues in other plant species suggests that SCF complexes play an important role in GA signalling (Fig. 1.3).

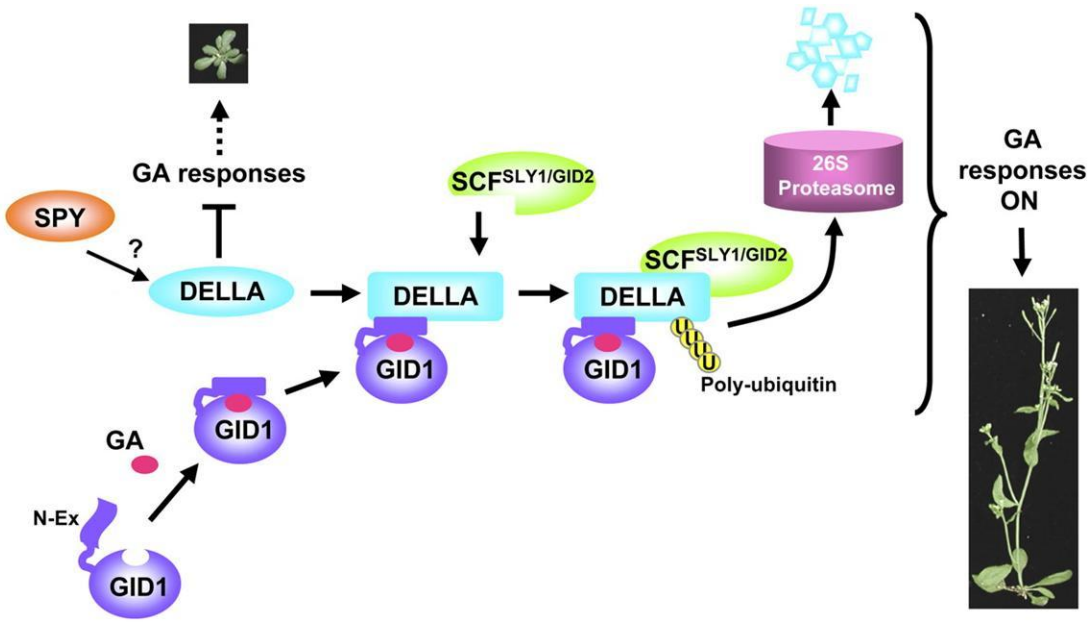

Figure 1.3 Current model for GA signalling in plants. In the absence of GA, DELLA regulates the transcription of downstream GA-responsive genes. Bioactive GA binding to the GA receptor, GID1, induces a conformational change allowing association with the N-terminal domain of the DELLA protein. The GA/GID1/DELLA complex interacts with the $SCF^{SLY1/GID2}$ complex resulting in the ubiquitination of DELLA leading to its degradation in the 26S proteasome, thus relieving DELLA induced growth restraint. SPY is a negative regulator of GA signalling and may activate DELLA by GlcNAc-modification. Figure adapted from Sun (2010)

1.2.7. **Downstream Events**

DELLAs are unlikely to directly bind to DNA to elicit its effects because no recognised DNA binding domain has been identified. However, DELLA have been shown to act as transcriptional regulators, controlling plant development by repressing the action/function of interacting transcription factors. A chromatin immuno-precipitation (ChIP) experiment suggests that

Arabidopsis RGA is able to bind to the promotors of early GA response genes, either directly or as part of a complex (Zentella *et al.*, 2007). Furthermore, two DELLA interacting proteins, PHYTOCHROME INTERACTING FACTOR 3 and 4 (PIF3 and PIF4), were simultaneously identified by two groups, Feng *et al.(2008)* and De Lucus *et al.* (2008). Both proteins are basic helix loop helix (bHLH) transcription factors involved in phytochrome mediated light signalling. DELLAs interaction with both proteins prevent their action. Upon GA mediated degradation of DELLA, PIF3 and PIF4 are released from restraint enabling the proteins to promote the expression of their target genes, including as yet unidentified genes promoting growth. Three additional bHLH transcription factors, PIF1, SPT, and PIL2 also interact with DELLA in yeast-2-hybrid assays (Gallego-Bartolome *et al.*, 2010). Although yet to be shown *in vivo* DELLA interaction with bHLH transcription factors may be a common mechanism in which DELLA regulates downstream gene expression.

1.3. **Wheat Dwarfing Genes**

Triticum aestivum, commonly known as bread wheat is a globally important crop ranked second in total production behind maize (The International Grains Council). The genome of bread wheat is hexaploid, consisting of three homeologous chromosome sets (A, B and D), contributed by three diploid progenitors. The dwarfing genes of the wheat Green Revolution are located at the *Reduced height* (*Rht*) locus on chromosomes 4BS and 4DS. As described previously, the wild type *RHT-1* genes are orthologues of Arabidopsis *GAI* and encode a DELLA protein which acts as a negative regulator in the GA signal transduction pathway, the mutant alleles at this locus have reduced sensitivity to GA resulting in a dwarf phenotype (Peng *et al.*, 1999).

Genes assigned the *Rht* symbol do not just include the GA-insensitive dwarfing genes on group 4 chromosomes, but all of the genes conferring an effect on plant height including the GA sensitive mutants such as *Rht 8* and *Rht 12*. The original accepted gene symbol for alleles conferring reduced height was *Rht* 'n' however with the discovery of new dwarfing alleles at the *Rht* loci on group 4 chromosomes this system became confusing and so was replaced by a new system put forward by Börner *et al.* (1996) which specifies homoeologue location and abides by the general rules set out for gene symbolisation in wheat. The new gene symbolisation is presented in Table 1.1 and resultant dwarf phenotype of most of the alleles is illustrated in figure 1.4.

Table 1.1 Gene nomenclature and origins of the GA-insensitive *Rht* alleles.

New gene symbols	Old gene symbols	Gene origin
Rht-B1a	*rht*	-
Rht-B1b	*Rht 1*	Norin 10
Rht-B1c	*Rht 3*	Carter G
Rht-B1d	*Rht 1 Saitama*	Saitama 27
Rht-B1e	*Rht Krasnodari*	Krasnodari
Rht-B1f	*Rht T. aethiopicum*	*T. aethiopicum*
Rht-D1a	*rht*	-
Rht-D1b	*Rht 2*	Norin 10
Rht-D1c	*Rht 10*	Ai-bian 1
Rht-D1d	*Rht Ai-bian*	Ai-bian 1a

Figure 1.4. The Phenotypes of *Rht-B1* and *Rht-D1* alleles in Mercia near isogenic lines (NILs). From left to right; *rht-tall* (wild-type), *Rht-B1d* (formally *Rht1 Saitana*), *Rht-B1b* (*Rht1*), *Rht-D1b* (*Rht2*), *Rht-D1d* (*Rht2 Ai-bian1a*), *Rht-B1e* (*Rht1 Bezostaya*), *Rht-B1c* (*Rht3*) and *Rht-D1c* (*Rht10*), all in the Mercia background. Picture courtesy of Tony Worland.

Interestingly no GA-insensitive mutant alleles of *Rht-A1* have been identified to date. In the Peng study (Peng *et al*., 1999) only *Rht-B1* and *Rht-D1* genes were cloned. In the absence of sequence or expression data for *Rht-A1* one can only speculate on why no *Rht-A1* mutant alleles have been identified. It could be that the gene is non-functional, that the gene is not expressed or expression is reduced so that any mutations present result in a phenotype too mild to identify, or that the expression profile in different tissues is markedly different from *Rht-B1* and *Rht-D1*.

Plant breeders have seen the potential for yield increases associated with shorter crops for many years. As a result genes conferring small height reductions have been accumulated. However, with the discovery of the semi-dwarfing alleles this enabled breeders to achieve greater height reduction with a single, dominant gene. Japanese breeders who realised this potential, developed two wheat cultivars, Akakomugi and Daruma, both of which have been utilised in worldwide breeding programmes and become the main source of the semi-dwarf characteristics seen in most of the modern high yielding wheat varieties.

Akakomugi is an important source of *Rht 8*. Located on the short arm of chromosome 2D (Korzun *et al.*, 1998), *Rht 8* confers a GA sensitive phenotype. Although *Rht8* dwarfing genes have not been agronomically important in the past they are becoming more so. GA biosynthesis mutant plants confer a reduced final height without affecting coleoptile length or seedling vigour, in contrast to the GA insensitive mutants (Rebetzke *et al.*, 1999). The shorter coleoptile length and reduced seedling vigour of GA insensitive mutants is not a problem in favourable environments where seeds can be sown relatively shallow but, it does result in reduced seedling establishment in dry regions where seeds need to be sown deeper. Therefore *Rht 8* is now being introduced into wheat varieties required for drier environments as a replacement for the GA insensitive dwarfing genes (Rebetzke & Richards, 2000).

Daruma was crossed with high yielding American varieties by Japanese breeders to produce Norin 10 in the early part of the 20th century. Norin 10 became the main source of the *Rht-B1b* and *Rht-D1b* semi-dwarfing genes and at present more than 70% of the current commercial wheat cultivars are derived from a Norin 10 progenitor (Evans, 1998). The semi-dwarfing genes were introduced in the UK through the Plant Breeding Institute (PBI) programme in 1964. *Rht-D1b* is the semi-dwarfing allele favoured by UK breeding programmes while mainland Europe favour the *Rht-B1b* semi-dwarfing allele. Other GA-insensitive dwarfing alleles at the *Rht-1* locus have been identified (Table 1.1) that confer differing severities of dwarfism. For example, *Rht-B1c* and *Rht-D1c*, as their names suggest are allelic to *Rht-B1b* and *Rht-D1b* on the B and D genomes respectively. The *Rht-B1c* and *Rht-D1c* alleles confer severe dwarf phenotypes due to increased GA-insensitivity compared to Norin 10 derived dwarfing alleles. These alleles have not been utilised in commercial cultivars to date.

The most characterised of the dwarfing alleles at the *Rht-1* loci are *Rht-B1b* and *Rht-D1b* on account of their economic importance. As mentioned previously, these are GoF alleles caused by nucleotide substitutions that create stop codons within the DELLA domain (Peng *et al*., 1999). In *Rht-B1b*, a C is substituted by a T which converts the glutamine residue at position 64 (Q64; CGA) into a translational stop codon (TGA). Similarly, in *Rht-D1b*, a G is substituted by a T converting the Glycine residue at position 61 (G61; GGA) into a translational stop codon (Fig. 1.5). The similarities of the mutations are reflected in the phenotype which is also very similar (Fig. 1.4). The mutant alleles are semi-dominant and so cause dwarfism even in the presence of the two wild type homoeologues. The semi-dominant effects conferred by the mutant alleles suggest that they encode an active product. The authors of the Peng study (1999) suggested that either the short N-terminal peptide fragments confer the mutant phenotype or that the ribosomal complex reinitiates translation at one of the methionine residues which follow the stop codon, thereby resulting in an N-terminally truncated product which lacks the DELLA domain. Since the N-terminal DELLA motif has been shown to be required for GA-dependent targeting of these proteins for degradation it is predicted that the C-terminal truncated product would act as a GA insensitive, constitutive repressor of GA mediated signalling. Since the isolation and sequencing of the *Rht* semi-dwarfing alleles, a group (Ellis *et al.*, 2002) developed a set of PCR-based markers which are capable of discriminating between mutant *Rht-B1b* and *Rht-D1b* and their respective wild type alleles, *Rht-B1a* and *Rht-D1a*. Described as "perfect markers" they have been designed to discriminate on the basis of their specificity to the base pair change of the mutant alleles.

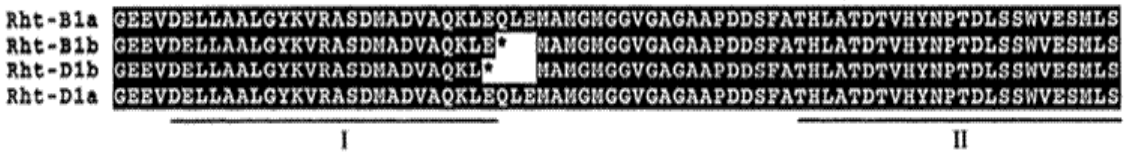

Figure 1.5 The amino acid sequence of wild type (top and bottom) and mutant (middle) semi-dwarfing genes *Rht-B1b* and *Rht-D1b*. An asterisk symbolises a stop codon. I and II are the unique conserved domains of the DELLA proteins, DELLA and VHYNP. Figure modified from Peng *et al.*, 1999.

1.4. **Plants are exposed to a multitude of stresses**

Throughout their lifespan plants are exposed to numerous stresses. Although the levels of stress are controlled as much as is economically viable in an agricultural setting, for example by irrigation or fungicide application, crops still have many adversities with which to contend. Stress can be of two general types; abiotic and biotic. Abiotic stress refers to adverse, non-living environmental factors such as drought, extremes in temperature, salinity, high light and UV-radiation. Abscisic acid (ABA) has been demonstrated to be an important hormone in controlling abiotic stress responses (reviewed by Zhu, 2002). Biotic stress includes all pathogenic living organisms such as bacteria,

viruses, fungi and oomycetes. Biotic stress originating from pathogens can be broadly categorised into two classes depending on their nutritional lifestyle; biotroph and necrotroph. Biotrophs derive nutrients from living cells whilst necrotrophs kill host cells in order to derive energy (Lewis, 1973). In addition a subclass of biotophic pathogens are referred to as hemibiotrophic, which require an initial biotrophic phase before switching to necrotrophy to complete their life cycle (Perfect & Green, 2001). The host response to the two main classes of pathogen are distinct to cope with the contrasting challenges posed. The host defence against biotrophs is controlled by the salicylic acid (SA) dependent pathway whilst necrotroph defence is controlled by an ethylene/jasmonic acid (ET/JA) dependent pathway (Glazebrook, 2005). Common responses have been identified in the plant to abiotic and biotic stresses. For example, analysis of genome array data of the effects of abiotic and biotic stresses identified a cluster of genes co-regulated by both forms of stress in a study by Ma & Bohnert (2007) who propose that these genes constitute a universal stress response transcriptome. Reactive oxygen species related transcripts were highly represented in the universal stress response transcriptome (Ma & Bohnert, 2007) and have been the subject of much investigation in abiotic and biotic stresses (reviewed by Miller *et al.*, 2010; Torres, 2010 respectivley).

1.4.1. **Reactive oxygen species**

Reactive oxygen species (ROS) are partially reduced intermediates of atmospheric oxygen (O_2) such as hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl radical ($HO^{\cdot}$). At certain levels these molecules are cytotoxic, causing oxidative damage to DNA, proteins and lipids. However ROS are also involved in versatile cellular processes including signal transduction and modulating gene expression in response to biotic and abiotic stress. ROS are generated as a normal by-product of respiration and photosynthesis. Steady state levels of ROS generated in this way are maintained by both enzymatic and non-enzymatic detoxification mechanisms. Abiotic and biotic stress can perturb the equilibrium between ROS generation and detoxification systems leading to oxidative stress, the severity and duration of the stress determines whether the cell survives and acclimates or experiences oxidative damage and cell death.

1.4.2. **ROS production and detoxification**

Sources of ROS production are localised to particular organelles. The photosynthetic electron transport (PET) chain, made up of photosystems I and II (PSI and II) is a major source of ROS in the chloroplast, and the respiratory electron transport (RET) chain also produces significant amounts of ROS in the mitochondria. These organelles produce considerable amounts of ROS during normal metabolism and ROS production is elevated during periods of adversity. In addition to the metabolic accumulation of ROS, the plant cell can also actively generate ROS under stress

conditions via ROS producing enzymes such as NADPH oxidases. Plasma membrane localised NADPH oxidases are homologs of the mammalian gp91^{phox} and as such are also known as respiratory burst homologs (Rboh). The Arabidopsis genome encodes 10 Rboh proteins (Torres *et al.*, 1998; Torres & Dangl, 2005), of which *At*RbohC/RHD2, *At*RbohD and *At*RbohF are the best characterised. The proteins form an electron transport chain that spans the plasma membrane which, using NADPH as an electron donor and O_2 as an electron acceptor, generates extracellular O_2^- which is rapidly converted to H_2O_2 (Torres *et al.*, 1998). Other apoplastic localised ROS producing enzymes include oxalate oxidases and amine oxidases (Mittler, 2002).

ROS detoxification is important in order to maintain ROS homeostasis and avoid oxidative damage. As with ROS generating systems, the associated scavenging systems are present in almost all cellular compartments. ROS detoxification is carried out by both enzymatic and non-enzymatic ROS scavenging systems (reviewed by Apel & Hirt, 2004). Superoxide dismutase (SOD) enzymes catalyse the conversion of O_2^- to H_2O_2. SOD associate with metal cofactors and are localised to different cellular compartments based on the particular metal cofactor; Cu/Zn SOD are localised to the cytosol and chloroplast, Fe SOD to the chloroplast and Mn SOD to the mitochondria (Foyer & Noctor, 2005). H_2O_2 scavenging enzymes include ascorbate peroxidises (APX), which have wide spread distribution in the cell and catalase (CAT), which is present in large quantities in peroxisomes (Willekens *et al.*, 1997). The non-enzymatic antioxidants ascorbate (AsA) and glutathione (GSH) are the two major cellular redox buffers. The ascorbate/glutathione cycle is present throughout the cell, and is particularly important in the chloroplast (Foyer *et al.*, 1994). The cycle removes H_2O_2 and consists of ascorbate and glutathione and four enzymes; ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase and glutathione reductase (Asada, 1999).

1.4.3. **ROS involved in signalling**

The elevated ROS levels resulting from abiotic adversity and active generation of ROS in response to biotic stress have been shown to influence the expression of a large number of genes (Neill *et al.*, 2002). This suggests that cells have evolved strategies to utilise these biological signals to initiate specific downstream processes. The specific outcome is partly dependent on hormone signals and the localisation of ROS accumulation. Retrograde signalling refers to the communication between the organelles and the nucleus of a cell necessary to programme the transcriptome for particular responses. H_2O_2, the most stable of the ROS species, is unlikely to be the signalling molecule *per se* due to the highly reducing environment of the cytosol, through which the signal is transmitted. Thus H_2O_2, and other ROS likely transmit their signal through the oxidation of signalling components. The components of the ROS signal transduction pathway are not yet fully understood in plants but better characterised in other systems. Studies in bacteria and yeast demonstrate that

transmembrane histidine kinases can function as redox sensors (Whistler *et al.*, 1998; Singh, 2000) triggering downstream processes such as MAP kinase cascades, calmodulin kinases and expression/activation of transcription factors. MAPK3 and 6 have been identified as important signal tranducers during oxidative stress in Arabidopsis (Jonak *et al.*, 2002) however little is known about the activation or downstream targets of these pathways. Also demonstrated in bacteria is the direct oxidation of transcription factors by H_2O_2, thereby affecting their DNA binding affinity (Lee *et al.*, 2009). Another signalling mechanism may be associated with the redox balance of the cell which is influenced by the ratios of reduced: oxidised forms of low molecular weight antioxidants such as ascorbate and glutathione generated by the aforementioned ascorbate/glutathione cycle (Foyer & Noctor, 2003; Kiddle *et al.*, 2003). Moller & Sweetlove (2010) propose that peptides deriving from ROS induced proteolytic breakdown may provide a source specific signal necessary to regulate source specific genes which have been demonstrated to be induced in addition to general oxidative stress response markers (Gadjev *et al.*, 2006).

Chloroplasts may have a central role as signal initiators to abiotic and biotic stress responses in plants, performing reprogramming of the nuclear transcriptome via retrograde signalling and initiating NADPH oxidases and possibly mitochondria to propagate the ROS signal (Fig.1.6; Zurbriggen *et al.*, 2010). For example, in a study by Joo *et al.* (2005) the biphasic oxidative burst caused by ozone treatment was measured over a time course. The early phase of ROS accumulation was confined to the chloroplast of guard cells and this was followed by a second burst of extracellular ROS production, which was dependent on NADPH oxidase activity, that subsequently spread to adjacent cells.

Flavodoxins are electron shuttle proteins present in photosynthetic microorganisms, but not in plants. Tobacco plants which express a plastid-targeted flavodoxin specifically prevents ROS accumulation in the chloroplast (Tognetti *et al.*, 2006). Using this experimental system Zurbirggen *et al*. (2009) demonstrated that chloroplasts are important initiators of signalling and programmed cell death (PCD) initiation in response to pathogens.

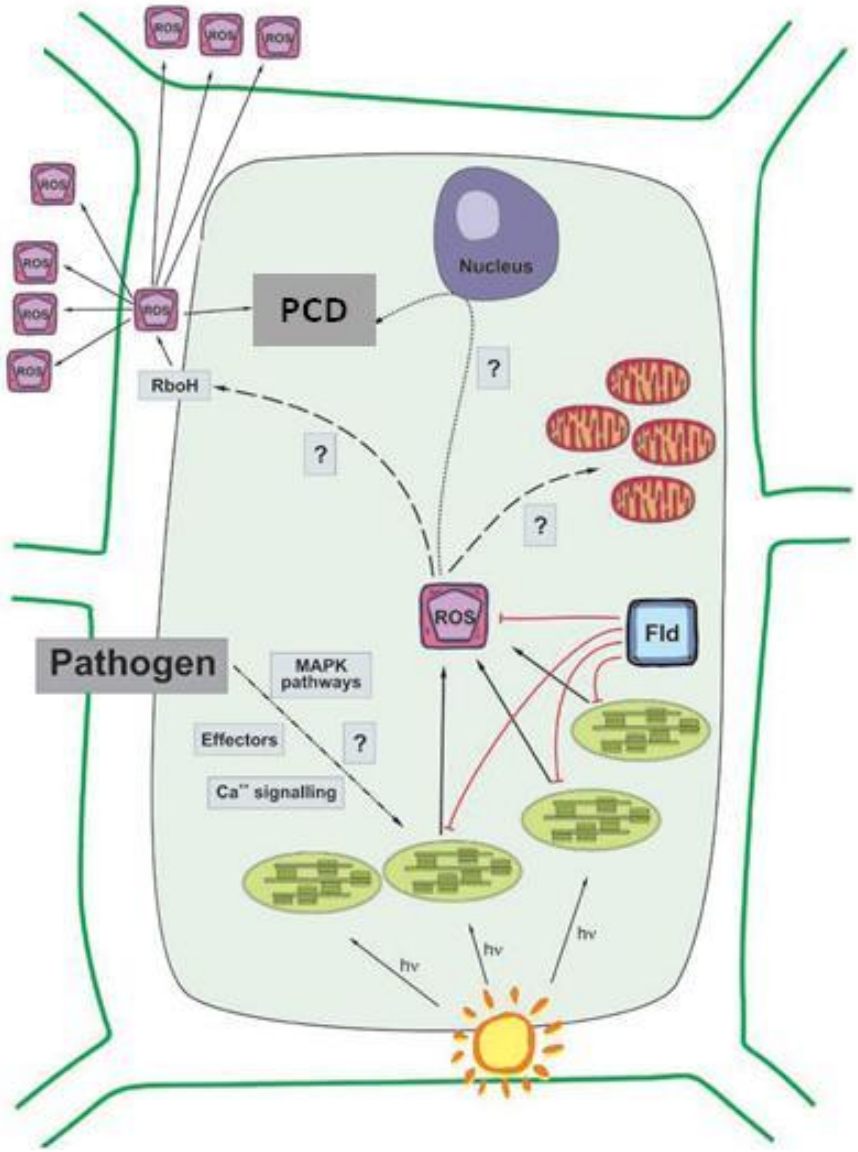

Figure 1.6 Schematic diagram of ROS signalling for the programmed cell death (PCD) in response to abiotic stress (high light) and pathogen interactions. ROS is generated in the chloroplast in response to abiotic stress exposure or pathogen challenge. Chloroplast-generated ROS then signal for further ROS production in the apoplast by activating NADPH oxidases, which are involved in propagation of the signal to adjacent cells. Retrograde signalling between the chloroplast and the nucleus and mitochondria also occurs by as yet unknowen means. Transgenic flavodoxin (Fld) expression in chloroplasts specifically blocks ROS generation in this organelle. Experiments using this system (Zurbriggen *et al.*, 2009) have elucidated that the chloroplast is a key initiator of abiotic and biotic signalling and PCD. Figure adapted from Zurbriggen *et al.* (2010).

1.4.4. **ROS leading to cell death**

Through ROS signalling, genes are activated to counteract oxidative damage. However, upon ROS reaching levels which exceed the cells antioxidant capacity, cell death can ensue. Thus, the cell has a cell death threshold below which signalling for acclimation is achieved and above which cell death is initiated, as illustrated in figure 1.7.

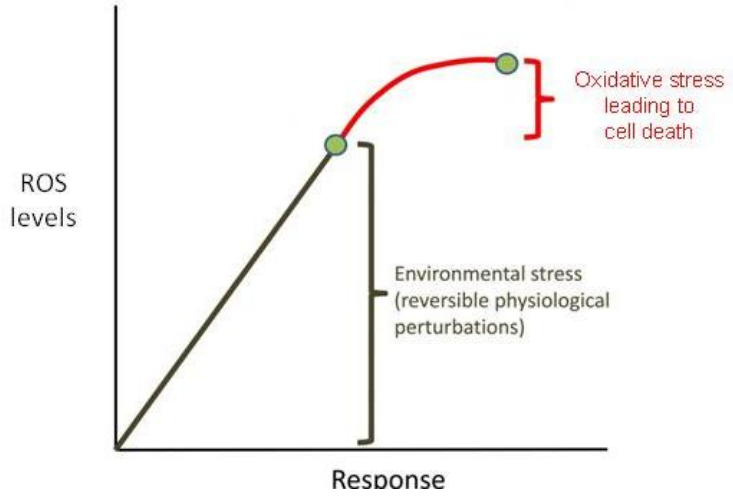

Figure 1.7. A model for the response of plant cells to increasing stress in the context of ROS levels. As stress increases so does the production of ROS which functions as a stress signal to initiate acclimatisation mechanisms. Cell death ensues once the ROS levels surpass a threshold. Figure adapted from Mullineaux and Baker (2010)

Plant cell death can take many forms. Sustained ROS production provokes the accumulation of phytotoxic levels of ROS which indiscriminately attack and damage cellular constituents resulting in membrane leakage and cell lysis. This type of uncontrolled cell death is referred to as necrosis (Van Breusegem & Dat, 2006). On the other hand, transient increases in ROS which exceed the cell death threshold can trigger signalling cascades to initiate programmed cell death (PCD). PCD is a genetically controlled process which displays biochemical and molecular hallmarks, such as DNA laddering, cytochrome c release, caspase involvement, ATP depletion, cytoplasmic swelling, and loss of membrane integrity (Pennell & Lamb, 1997).

Two major forms of PCD have been described in plants; apoptosis-like PCD and autophagic PCD (Reape *et al.*, 2008). Apoptosis-like PCD is a rapid form of cell death. The hypersensitive cell death is the best characterised form of apoptosis-like PCD in plants. The rapidity being essential to contain the invading pathogen. In contrast, autophagic PCD is a relatively slow form of cell death, for example cells undergo autophagic PCD during the senescence of leaves. Both pathways show similar morphological features and use a common set of cell death regulators (van Doorn & Woltering, 2005). A strong interplay exists between ROS and hormones to determine whether a cell undergoes apoptosis-like or autophagic PCD. Love *et al.* (2008) propose that SA, JA and ethylene dependent pathways may interact together with ROS signals to regulate which PCD response will be enacted. They suggest that SA promotes apoptosis-like PCD, as demonstrated with HR, while JA and ethylene promote autophagic PCD.

In mammalian cells both positive (proapoptotic) and negative (antiapoptotic) regulators of PCD have been identified (reviewed by; Chao & Korsmeyer, 1998). It is becoming increasingly evident that many of these regulators of cell death are functionally conserved across taxa. The transgenic expression of human, nematode and bacaulovirus antiapoptotic genes in tobacco conferred an increased resistance to pathogen-induced cell death (Mitsuhara *et al.*, 1999; Dickman *et al.*, 2001), suggesting a conserved role for these regulators in plant PCD. Human BAX inhibitor-1 (BI-1) was identified as a negative regulator of BAX induced cell death (Xu & Reed, 1998) and has since been identified in plants (reviewed by Huckelhoven, 2004). Overexpression of GFP-HvBI-1 in barley plants conferred increased resistance to cell death induced by the ectopic expression of mammalian proapoptotic *BAX* and increased resistance to pathogen-induced cell death (Babaeizad *et al.*, 2009). Whilst down regulation of tobacco *BI-1* accelerated abiotic induced cell death (Bolduc & Brisson, 2002). BI-1 has been demonstrated to be an important cellular protectant involved in the suppression of cell death resulting from mitochondrial dysfunction, accumulation of reactive oxygen species and elevated cytosolic Ca^{2+} levels (Huckelhoven, 2004).

Additional, plant-specific, negative regulators of cell death have been identified often associated with the spontaneous cell death phenotype evident in LoF mutants. For example the *mildew resistance locus o* (*mlo*) mutation in barley causes cell death lesions to form in fully expanded leaves (Schulze-Lefert & Vogel, 2000) and the *lesion simulating disease 1* (*lsd1*) mutants exhibit spontaneous cell death under certain, O_2^- eliciting, environmental conditions (Dietrich *et al.*, 1997). MLO dampens the ROS burst at points of attempted fungal penetration and suppresses a second oxidative burst, thus negatively controlling cell death. LSD1 is thought to regulate the timely expression of SOD and CAT and physically interacts with two positive regulators of cell death, bZIP10 and LOL1, suppressing their function (Kliebenstein *et al.*, 1999; Epple *et al.*, 2003; Kaminaka *et al.*, 2006). Both MLO and LSD1 have been implicated in biotic and abiotic stress responses (Piffanelli *et al.*, 2002; Mateo *et al.*, 2004).

1.4.5. **ROS mediated response to biotic stress**

The production of ROS is closely associated with plant defence response to pathogens. During pathogen attack ROS can have direct antimicrobial activity and also strengthen the cell wall by oxidative cross linking (Lamb & Dixon, 1997). ROS also act as a signal to activate gene expression, systemic responses and localised HR.

ROS can however have a dichotomous role in biotic stress response dependent on the lifestyle of the invading pathogen. Disease development upon challenge with a necrotrophic pathogen is generally promoted by the pathogen-induced oxidative burst (Gönner & Schlösser, 1993; Able, 2003). In fact, some necrotrophic pathogens have been observed to actively increase ROS levels,

either by producing ROS themselves or stimulating the host to do so, to promote disease development e.g. the necrotrophic pathogen, *Botrytis cinerea* (van der Vlugt-Bergmans *et al.*, 1997; Govrin & Levine, 2000; Kuzniak & Sklodowska, 2005). Suppression of ROS by infiltration of plants with antioxidant or diphenyleneiodonium (DPI; an inhibitor of NADPH oxidase) restricted disease development of *Botrytis cinerea* (Govrin & Levine, 2000). Similarly, *Sclerotinia sclerotiorum*, a broad host range necrotrophic pathogen, produces high amounts of oxalic acid (OA) which initiates PCD by inducing H_2O_2 production in the plant. Furthermore, mutants deficient in OA production are non-pathogenic suggesting that OA is an important pathogenicity factor for *S. sclerotiorum* (Kim *et al.*, 2008). Accordingly, resistant cultivars of barley challenged with two fungal necrotrophs, *Rhynchosporium secalis* or *Pyrenophora teres* were observed to suppress the oxidative burst specific to susceptible cultivars (Able, 2003).

In contrast, avirulent biotrophic pathogen challenge elicits the coordinated action of an increase in production of ROS and a down regulation of ROS scavenging mechanisms (Mittler *et al.*, 1998; Vanacker *et al.*, 1998; Mittler *et al.*, 1999). The resulting accumulated ROS results in localised hypersensitive cell death restricting pathogen access to water and nutrients, thus preventing disease development (reviewed by Mur *et al.*, 2008). As part of the hypersensitive response SA signalling is activated throughout the plant leading to the systemic activation of SA regulated defence responses, thus priming the plant for future (biotrophic) pathogen attack. This phenomenon is referred to as systemic acquired resistance (SAR; reviewed by Durrant & Dong, 2004).

1.4.6. **ROS mediated responses to abiotic stress**

Abiotic stresses such as drought, extremes in temperature, salinity, high light and UV-radiation result in a metabolic imbalance leading to an elevation of ROS production. ROS generated in this way is channelled into a signalling pathway alerting the plant to adversity. The stress signal serves to increase antioxidant mechanisms enabling the plant to restore its normal cellular redox balance and to induce defence mechanisms. For example, pathogenesis related (PR) proteins, glutathione S-transferase (GST) and phenylanine ammonia-lyase (PAL) are all up regulated upon exposure to abiotic stress (Levine *et al.*, 1994; Desikan *et al.*, 1998; Karpinski *et al.*, 1999). In addition, Lopez-Huertas *et al.* (2000) showed that the peroxisome biogenesis genes are induced in response to abiotic stress suggesting that peroxisome production is a key oxidative stress response. Acclimation tolerance to an abiotic stress is evident when a plant exposed to sub-lethal doses of one stress is protected from subsequent exposure to normally lethal doses of the same stress. Karpinski *et al.* (1999) has shown that one such oxidative protectant, *APX2*, is up regulated throughout the plant, even in tissues which were not exposed to the stress. This phenomenon is termed systemic acquired acclimation (SAA; Karpinski *et al.*, 1999). Active generation of ROS by NADPH oxidase has also been implicated in the response to drought and ozone through studies using the NADPH inhibitor

DPI (Pei *et al.*, 2000; Wohlgemuth *et al.*, 2002). This active generation of ROS may function in the propagation of the stress signal, routed via the plasma membrane, contributing to SAA (Mullineaux *et al.*, 2006).

1.4.7. **ROS mediated growth and development**

In addition to ROS' involvement in stress responses many studies have demonstrated an important role for ROS in the regulation of plant development (reviewed by Gapper & Dolan, 2006). For example, Foreman *et al.* (2003) demonstrate that plants defective in an Rboh protein (ROOT HAIR DEFECTIVE2 (RHD2)/AtrbohC) have roots 20% shorter than wild type and have decreased levels of ROS, suggesting that these plants are defective in cell expansion. Rodriguez *et al.* (2002) demonstrate that the zone of elongation in maize leaves is a site of ROS accumulation. Application of DPI reduces leaf expansion, again implicating NADPH oxidases as the generator of ROS which are required for cell expansion.

1.4.8. **DELLA and ROS**

Extensive work has been carried out on DELLAs role in development, as described above, however it is becoming increasingly evident that DELLA play an important role in abiotic and biotic processes and this may be partly achieved through the control of ROS.

Achard *et al.* (2006) demonstrated that salt treatment slows growth by a DELLA dependent mechanism associated with a reduction in levels of GA. This was supported by further experiments in which GFP-RGA accumulated in salt stressed tissue and LoF DELLA mutants exhibited reduced growth inhibition compared to wild type. Importantly, it was also demonstrated that stabilised DELLAs confer increased tolerance to salt stress. The *ga1-3* and *gai* plants, which have stabilised DELLA, had survival rates of 93.2 and 81.8% respectively in salt concentrations in which only 36.4% of wild type plants survived. Survival rate of the LoF DELLA mutants was approximately 5%. This study suggests that DELLA confers increased tolerance to abiotic stress. Subsequent work has demonstrated that GoF DELLA mutants in cereals (wheat and barley) also confer increased tolerance to salinity and heat shock (Boulton and Korolev, JIC, Norwich, UK, unpublished).

DELLA has also been associated with biotic stress responses in two back to back publications from Navarro *et al.* (2008) and Achard *et al.*(2008b). Navarro and colleagues (2008) demonstrate that DELLA proteins differentially affect responses to biotrophic and necrotrophic pathogens in Arabidopsis. The authors propose that DELLA may modulate the relative strengths of the SA and JA pathways. They observed quadruple-DELLA mutants (LoF of four of the five DELLA proteins) conferred enhanced resistance to virulent biotrophic *Pseudomonas syringae*, and that this was

associated with elevated SA. The quadruple DELLA mutants also showed attenuated induction of the JA marker, PDF1.2, which is consistent with an observed increased susceptibility to the necrotrophic pathogen *Alternaria brassicola.* The data provides an elegant explanation for why the aforementioned necrotrophic fungus, *Gibberella fujikuroi*, produces GA upon infection of rice plants, as GA degrades DELLA thus reducing JA signalling and potentiating SA signalling. Expanding on findings reported in 2006, Achard and colleagues (2008b) show a strong correlation between developmental effects of DELLA and the degree of salt-stress tolerance they confer. They demonstrate that following treatment with salt or challenge with the necrotrophic pathogen, *Botrytis cinerea*, quadruple DELLA mutants produce more ROS while DELLA stabilised mutants produce less ROS. They subsequently show that DELLAs reduce stress induced ROS accumulation by up regulation of the ROS scavenging system. Additionally, it is demonstrated that ROS dependent root hair growth (Foreman *et al.*, 2003) is repressed by DELLA. The authors conclude that DELLA mediated growth restraint, increased salt tolerance and increased resistance to necrotrophic pathogens is in part due to the modulation of ROS levels. Growth and stress responses are generally opposed. These studies suggest that DELLA may function as a key node of growth and stress response through its effect on ROS levels.

1.5. **Aims**

The semi-dwarfing alleles, *Rht-B1b* and *Rht-D1b*, are present in more than 70% of modern wheat cultivars (Evans, 1998). Although a lot of work has been carried out investigating developmental and pleiotropic effects of DELLA in the model dicot, Arabidopsis, very little work has been carried out in wheat since the cloning of the gene in 1999 (Peng *et al*., 1999). In contrast to Arabidopsis, monocot cereal species appear to contain a single DELLA encoding gene (Peng *et al.*, 1999; Chandler *et al.*, 2002), suggesting that Arabidopsis may not be a suitable model for GA signalling and DELLA related processes in wheat. The widespread use of these alleles neccesitates the understanding of their plietropic (potentially deleterious) effects on the plant. To this end, a central theme of this study will be the translation of findings from Arabidopsis to wheat, and monocotyledonous species as a whole.

In Chapter 2 the role transcriptional control of DELLA plays on growth control is investigated (component 1, Fig. 1.8). Using a characterised set of near isogenic lines differering at the *Rht* locus I shall investigate wheat *Rht* regulation at a transcriptional level under various environmental conditions and at various developmental stages assessing how the genotype affects the phenotype observed. The polyploidy nature of wheat means that there are multiple copies of a single DELLA encoding gene, differential regulation of the different copies may enable an appropriate growth response in wheat. A homoeologue specific qRTPCR assay was developed enabling the expression profiles of all three homoeologues to be measured.

In Chapter 3 the prospect of an additional DELLA encoding gene in the wheat genome is discussed (component 2, Fig. 1.8). A single DELLA encoding gene in wheat is thought to control GA-mediated growth fulfilled by five distinct genes in Arabidopsis. Rice also encodes a single DELLA protein (*Slr1*) however two additional genes, which share homology with *Slr1*, have been demonstrated to be involved in GA mediated growth and have been named DELLA-like genes. Evidence from a preceding study suggests that there may be an additional gene in the wheat genome which shares homology to *Rht*.A screen of the wheat Chinesse Spring BAC library was undertaken in an attempt to isolate this putative DELLA or DELLA-like gene from the wheat genome.

In Chapter 4 the plieotropic effect of DELLA on disease resistance is assessed in monocotyledonous crop species (component 3, Fig. 1.8). A plieotropic effect of DELLA has been reported in Arabidopsis in which DELLA GoF mutant plants are more susceptible to biotrophs whilst more resistant to necrotrophs. To investigate whether DELLA confers a similar pleiotropic effect in monocotyledonous crop species a series of defined wheat *Rht* NILs and barley GoF and LoF mutant DELLA lines were subjected to patho-tests with economically important cereal pathogens representing each of the trophic lifestyles (biotrophic, hemibiotrophic and necrotrophic).

In Chapter 5 DELLAs role in ROS homeostasis is assessed in respect to environment and how this affects the biotroph-necrotroph resistance trade-off observed in Chapter 4 (component 4, Fig. 1.8). Using barley GoF and LoF mutant DELLA lines treated with relative levels of light the effects of prior abiotic stress on ROS homeostasis and pathogen ressistance is assessed.

2. The regulation of *Rht* expression during development and in response to environmental stimuli and characterisation of mutant alleles.

2.1. Introduction

GA-mediated growth is controlled through the regulation of GA metabolism and signalling components some of which are responsive to endogenous and environmental cues leading to an appropriate growth response.

Arabidopsis contains five DELLA encoding genes, which form central components in GA signal transduction. The five proteins, GAI, RGA, RGL-1, -2 and -3 have distinct as well as overlapping roles during development. Studies in Arabidopsis demonstrate that DELLA protein abundance is predominantly regulated post-translationally, with protein degradation being determined by the level of bioactive GA and by post translational modifications.

GAI and *RGA* are highly expressed in most tissues whilst *RGL-1*,*-2* and *-3* are expressed in germinating seed, young seedlings and flowers suggesting developmental transcriptional control (Tyler *et al.*, 2004). Transcription of *RGL-2* has been shown to be altered during germination in response to environment with an 'optimal' environment causing a down regulation of *RGL-2* expression which is followed by rapid progression of germination (Lee *et al.*, 2002). Additionally, light and temperature have been shown to influence DELLA transcription suggesting environmental factors have a direct effect on DELLA transcript levels (Oh *et al.*, 2007; Achard *et al.*, 2008a).

The abundance of DELLA protein is inversely related to the levels of bioactive GA in wild type plants. Plant development and environmental stimuli affect the stability of DELLA through their influence on GA metabolism. For example, in Arabidopsis it has been shown that dark grown hypocotyls have increased GA levels (resulting from an increase in the GA biosynthetic enzymes, GA20ox1 and GA3ox1, and a decrease in the GA catabolising enzyme, GA2ox1) whilst light grown hypocotyls have relatively low GA levels (caused by low levels of GA20ox1 and GA3ox1 and a high GA2ox1 level). Therefore, DELLA is destabilised in dark grown hypocotyls leading to derepression of growth and the converse in light grown hypocotyls (Achard *et al.*, 2007). At low temperatures plant growth is restrained due to low GA levels (increased GA catabolising enzymes) causing an accumulation of DELLA (Achard *et al.*, 2008a). Similarly, salt treatment slows vegetative growth by decreasing GA levels through up regulation of a *GA2ox* gene (Magome *et al.*, 2008), resulting in enhanced DELLA protein stability. Accumulation (in wild type plants) or

stabilisation (for example in the GA insensitive mutant, gai) of DELLA was shown to lead to increased tolerance of salt and cold (Achard *et al.*, 2006).

Post-translational modifications of DELLA proteins also play a role in modulating DELLA mediated growth. The GA/GID1/DELLA complex interacts with the $SCF^{SLY1/GID2}$ complex resulting in the ubiquitination of the DELLA protein marking it for degradation by the 26S proteasome, the so-called relief of restraint model (Harberd, 2003). In contrast O-GlcNAcylation of DELLA proteins by SPINDLY (SPY), an O-linked N-acetylglucosamine transferase, is thought to activate the suppression function of DELLA proteins (Shimada *et al.*, 2006).

Many GA response pathway components, orthologous to those in Arabidopsis, have been either originally or subsequently identified in rice. For example the soluble GA receptor GID1 was first discovered in rice (Ueguchi-Tanaka *et al.*, 2005). Studies in rice revealed that the GA-GID1 complex interacts with the rice DELLA orthologue, SLR1, forming the first phase of the relief of restraint model.

Little is known about the control of DELLA mediated growth in wheat. In this chapter an *Rht* homoeologue specific qRT-PCR assay has been designed as a step towards understanding how DELLA mediated growth is regulated in wheat. The development of the assay has enabled the following areas to be explored.

Five distinct DELLA proteins coordinate growth in the diploid Arabidopsis genome at both a transcriptional and post-translational level with each protein providing distinct developmental and environmentally induced roles (Gallego-Bartolome *et al.*, 2010). I consider whether spatial, temporal and environmental induced transcriptional control of the three homoeologous DELLA encoding genes in the hexaploid wheat genome may be sufficient to fulfil the growth regulatory role that five distinct DELLA proteins contribute to in Arabidopsis.

Differential homoeologue contribution across tissues and in response to environment has been demonstrated in polyploids. Adams *et al.* (2003) demonstrated unequal genome contribution across organs in tetraploid cotton (*Gossypium*). Subsequently there have been investigations in wheat with Mochida *et al*. (2004) showing that 81% of the genes tested showed preferential expression from a particular genome in at least one tissue. This was done by identifying homoeologues within wheat ESTs by SNP analysis. Furthermore, Stamati *et al.* (2009) showed genome wide homoeologue contribution is altered by drought and temperature. More specifically Appleford *et al.* (2006) analysed the regulation of GA20ox and GA3ox, both of which are involved in the latter stages of GA metabolism in wheat. Northern analysis, using homoeologue-specific probes, showed that expression in the nodes, ears and germinating embryos is predominantly from the A and D

genomes. To date there are no reports of differential homoeologue contribution for *Rht* or other GA signalling components in wheat.

The *Rht* semi-dwarfing alleles, *Rht-B1b* and *Rht-D1b*, were cloned in 1999 (Peng *et al.*). The alleles were found to encode N-terminally truncated variants of DELLA proteins, due, in each case, to a nucleotide substitution in the DELLA region resulting in the introduction of a premature stop codon. The severe dwarfing alleles, *Rht-B1c* and *Rht-D1c*, were found to be allelic at these homoeoloci, but have not been sequenced. The similar mutations in *Rht-B1b* and *Rht-D1b* correlate with a similar degree of dwarfing, and I investigate if the comparable degree of dwarfing seen for the severe dwarfs, *Rht-B1c* and *Rht-D1c*, also is a result of related mutations.

The stems of semi-dwarf lines are shorter than *rht-tall* containing lines but the ears are the same size. The reason for this is not known but it is possible that *Rht-B1* and *Rht-D1* are not expressed in the ear. Likewise no *Rht-A1* mutants have been identified and it is possible that *Rht-A1* is not expressed in any tissues or that mutations in this gene are lethal due to *Rht-A1* involvement in a crucial developmental stage.

Unfavourable environmental conditions slow plant growth. As described above, in Arabidopsis adverse conditions have been demonstrated to result in a reduction of GA levels resulting in DELLA accumulation which restrains growth. In addition expression of DELLA encoding genes can be influenced in response to the environment. It is not known whether *Rht* is responsive to environmental factors at a transcriptional level or whether particular homoeologues predominate in response to particular environmental stimuli in wheat. *Rht* expression will be investigated in response to light and temperature. Arabidopsis mutant lines in which DELLA accumulates have been demonstrated to be more tolerant to salinity (Achard *et al.*, 2006) and extreme cold temperatures (Achard *et al.*, 2008a). It has since been demonstrated that wheat and barley GoF DELLA alleles also confer increased tolerance to salinity and extreme heat stress (Boulton, JIC, Norwich, UK, unpublished). To investigate whether stress causes differential affects on transcription of mutant and wild type plants, the effects of heat shock treatment, a short exposure stress, on *Rht* expression will be assessed.

2.2. **Material and Methods**

2.2.1. **Plant material**

Wheat cultivars Paragon, Mercia and Maris Huntsman were used throughout, as stated in particular experiments. Paragon contains the *rht-tall* allele and Mercia and Maris Huntsman cultivars are *rht-tall* containing lines for which a near isogenic *Rht* series has been developed (John Flintham, JIC,

Norwich, UK), enabling the effects of *Rht* mutant alleles to be assessed in isolation from background effects.

2.2.2. **ABD *Rht* Taqman® assay**

A Taqman® probe and flanking primers (Table 2.1, Fig. 2.1a) were designed to a region conserved in all three *Rht* homoeologues by the Sigma-Genosys probe design service. The homoeologues are insufficiently polymorphic to find suitable binding sites for both Taqman probe and flanking primers that are capable of distinguishing the homoeologues and, because of this SYBR assays were used for distinguishing the *Rht* homoeologues.

Table 2.1 ABD *Rht* Taqman® probe and primer sequences designed by the Sigma-Genosys probe design service.

ABD assay	**Sequence (5'-3')**
Forward	CTACGAGTCCTGCCCCTACC
Taqman probe	CGCSCACTTCACCGCCAACCAGG
Reverse	GACTTCGGCATCAAGCAGGG

S; an unnatural base pair, 2-amino-6-(2-thienyl)purine, which binds both Guanine and Cytosine.

2.2.3. ***Rht* Homoeologue specific SYBR assay**

Homoeologue specific primers (Table 2.2, Fig. 2.1b) were designed for SYBR qRT-PCR assays. Primers were designed in the 3' region of the gene where GC content is relatively low and polymorphisms between *Rht* homoeologues more frequent. The three homoeologous sequences (*Rht-A1*; Pearce (RRes, Harpenden, UK, unpublished), *Rht-B1*; Harberd (Oxford University, Oxford, UK, unpublished) and *Rht-D1*; NCBI AJ242531) were aligned and homoeologue specific SNPs identified. Appropriate SNPs were selected for primer binding sites to discriminate the three *Rht* genes. The ABD Taqman assay primers (Table 2.1.), designed in a region conserved across all group 4 *Rht* homoeologues and the barley orthologue, *Sln1* (Fig. 2.1a) can also be used in a ABD SYBR assay.

Table 2.2 *Rht* homoeologue specific primer sequences

Gene	**Forward**	**Reverse**	**Annealing temperature**
Rht-A1	CGGGTTCGAGACCGTG	CCGCCGGCGAATAGG	57˚C
Rht-B1	CACTACTACTCCACCATGTTCGATTCTCTG	GCGGCAGGAGCAGCAGCC	68.5˚C
Rht-D1	CCACGAGACGCTGGGC	CCTTCCTTCTCCTCCACCTTGTAG	64˚C

Amplification efficiency was calculated for each of the primer sets. A 10X dilution series of cDNA template (ranging from 1:10 to 1:10 000) was amplified in triplicate with each primer set. The cycle threshold (C_t) values for the dilution series were plotted and the slope calculated. The efficiency was calculated using the formula [10^1/(slope)]. The maximum efficiency equals 2, i.e. the concentration of cDNA doubles with each cycle, all primer sets had an efficiency of 1.9 or above.

Primer specificity and optimum PCR assay conditions were tested using nullisomic-tetrasomic or ditelosomic genomic DNA template. These are characterised deletion stocks of wheat cv. Chinese Spring developed by Sears (1954). To validate primer specificity a presence/absence screen was carried out by both meltcurve analysis and by separation of endpoint products through an electrophorisis gel. In each case Chinese Spring euploid DNA was used as a positive control template and a DNA template lacking the chromosome region on which the target was present was used as the negative template. *Rht-A1* and *Rht-D1* primer specificity was tested on nullisomic 4A and 4D template respectively. Material nullisomic for 4B is male sterile due to the presence of the male fertility gene, *ms1*, on the short arm of chromosome 4B. As an alternative, ditellosomic 4Bβ template DNA (DT4Bβ, i.e. lacks the short arm of chromosome 4B; Sears, 1954) was used. DT4Bβ lines can be maintained in a heterozygous state and after selfing progeny homozygous for 4Bβ was selected.

2.2.4. **RNA extraction**

Total RNA was isolated using Qiagen RNA easy spin columns from 100 mg of young leaf tissue ground in a pestle and mortar under liquid nitrogen. Removal of contaminating genomic DNA was carried out with the TURBO DNA-free kit (Ambion) using the rigorous treatment protocol described by the manufacturer. RNA concentration and purity was assessed using a Picodrop spectrophotometer (Picodrop Limited, Saffron Walden, UK). Samples which had an OD 260/280 ratio (a measure of purity) of less than 1.6 or more than 2.0 were processed using a phenol-chloroform clean-up.

2.2.5. **cDNA synthesis**

cDNA was synthesised from 1-5 µg of total RNA using the SuperScript III first strand synthesis reverse transcription kit (Invitrogen) following the manufacturer's instructions with the addition of random nonamers (50 µM, Invitrogen). RNA was digested with RNase-H (Invitrogen) from the RNA-DNA duplex to leave single stranded cDNA. cDNA was diluted 1:20 with nuclease-free water for qRT-PCR.

2.2.6. **qRT-PCR**

qRT-PCR reactions were performed using a DNA engine Opticon2 Continuous Fluorescence Detector (MJ Research Inc., Alameda, CA, USA). The cDNA was amplified using JumpStart™ Taq ReadyMix™ for Quantitative PCR or SYBR® Green JumpStart™ Taq ReadyMix™ (both Sigma-Aldrich) for the ABD Taqman assay and the SYBR homoeologue specific assays respectively. An initial activation step at 95°C for 4 min was followed by 40 cycles of 30 s at 95°C, 30 s at the appropriate annealing temperature (Table 2.2) and 30 s extension at 72°C. At least two technical replicates were analysed for each sample. Melt-curve analysis was performed at the end of each reaction to monitor primer–dimer formation and the amplification of gene-specific products.

Up to three reference genes (Table 2.3) were used for normalisation of the RT-PCR assay for each experiment. The software geNorm v3.5 (Vandesompele *et al.*, 2002) was then used to assess the stability of the expression of the reference genes under experimental conditions. The two genes with the most stable expression under the particular experimental condition were used to calculate a normalisation factor, the geometric mean of the two values, which was applied to the target gene expression values to normalise the data set.

Table 2.3. Primers designed for amplification of the genes used for normalisation of the RT-PCR assay.

Gene[a]	Forward	Reverse	Reference
Ubiquitin	CCTTCACTTGGTTCTCCGTCT	AACGACCAGGACGACAGACACA	(Van Riet *et al.*, 2006)
GAPDH	CCTTCCGTGTTCCCACTGTTG	ATGCCCTTGAGGTTTCCCTC	(McGrann *et al.*, 2009)
EF1α	TGGTGTCATCAAGCCTGGTATGGT	ACTCATGGTGCATCTCAACGGACT	(Coram *et al.*, 2008)

[a] GAPDH; Glyceraldehyde 3-phosphate dehydrogenase, EF1α; Elongation factor 1α

2.2.7. **Analysis of qRT-PCR data**

The average C_t value was calculated from duplicate readings for each sample (technical replication). Data were analysed using Opticon Monitor analysis software v2.02 (MJ Research Inc.). Target gene expression was calculated relative to the normalisation factor using the ΔC_t method (Pfaffl, 2001) and corrected for primer efficiencies ($\Delta\Delta C_t$). A minimum of three replicate samples were processed and analysed for each 'treatment' (biological replication). Data is presented as a mean of the three biological replicates and the error bars denote standard errors of the means. The generalised linear model (GLM) of regression analysis was carried out to determine statistical significance.

2.2.7.1. Absolute quantification of target gene expression

Absolute quantification might be necessary when comparing expression across different genes. Clones containing the open reading frame (ORF) of *Rht-A1*, *Rht-B1*, *Rht-D1* and the normalising gene, *ubiquitin*, were required. Bacterial clones containing *Rht-B1* and *Rht-D1 ORF* were provided by Alkaff (JIC, Norwich, UK) and Pearce (RRes, Harpenden, UK) respectively. *Rht-A1* and *Ubiquitin* containing clones were prepared in the present study. DNA was extracted from bacteria containing the clones using a miniprep kit (Qiagen) and product, containing the region of the gene of interest, was amplified by PCR and purified using a PCR purification kit (Qiagen).

The concentration of the purified product was quantified using a Picodrop spectrophotometer (Picodrop Limited, Saffron Walden, UK) and, together with the product length, was used to calculate the number of molecules using the formula below.

(Xg/µl mRNA/ [transcript length in nucleotides x 340]) x 6.022 x 10^{23} = Y molecules/µl

A dilution series of the quantified aliquot of each of the target genes was used as template for the respective primer sets. A standard curve for each of the primer sets was created by plotting molecules/µl against C_t value, from which absolute expression values were derived. Due to the ubiquitous nature of ubiquitin, expression levels are extremely high so target gene absolute quantification is presented as molecules/10,000 molecules of ubiquitin.

2.2.8. *Rht* expression analysis of wheat genomes with different ploidy level

The seed of CIMMYT synthetic line SHW 217, the donor parent lines; *Triticum durum* and *Aegilops tauschii*, and the conventional hexaploid *T. aestivum* cv. Paragon were surface sterilised using a weak bleach solution and stratified at 4˚C in the dark on wet 3MM grade 1 Whatman paper for two days. Germinated seeds were transferred to Petri dishes lined with wet 3MM grade 1 Whatman paper and placed in a controlled environment room (CER) at 22˚C with 16/8 h light/dark period. Shoot tissue was sampled after 5 days and RNA extracted and processed as described in section 3.2.4. Two biological replicates were sampled for each line, each consisting of at least five seedlings.

2.2.9. Growth stage-specific sampling of plant tissue

2.2.9.1. Germination stage

Seed of Paragon (*rht-tall*) were surface sterilised and stratified in the dark on wet 3MM grade 1 Whatman paper according to the treatments described below. Three biological replicates were collected for each treatment, and at least 20 excised embryos were collected for each biological replicate, depending on the developmental stage being assessed.

In experiment 1 seeds were incubated for 24 h at 4˚C or 24 h at 4˚C followed by 24 h at 22˚C. In experiment 2 seeds were incubated for either 24, 144 or 264 h at 4˚C, stratification of seed was commenced on successive days to enable simultaneous RNA extraction. RNA was extracted, and qRT-PCR assays carried out as described in sections 2.2.4 and 2.2.6.

2.2.9.2. Seedling stage

Seed of Mercia (*rht-tall)* were surface sterilised and stratified at 4˚C in the dark on wet 3MM grade 1 Whatman paper for 2 days. Germinated seeds were transferred to Petri dishes lined with wet 3MM grade 1 Whatman paper and placed in a CER (22˚C with 16/8 h light/dark period). The seedlings were grown in Petri dishes to facilitate root tissue harvesting and the filter paper was kept damp until shoot and root tissues were sampled separately after 4 days. Extraction of RNA from these tissues was done as described in section 2.2.4.

2.2.9.3. Mature plant stage

Seed of Paragon (*rht-tall*) were stratified at 4˚C in the dark on wet 3MM grade 1 Whatman paper for 2 days. Germinated seed were sown in P40 seed trays containing peat and sand mix. To ensure flowering and uniform development plants were exposed to a brief vernalisation period of 3 weeks at 5 ºC. Plants were transferred to a CER (18 ºC/15 ºC, 16/8 h light/dark period). At growth stage 20 (G.S; Zadoks *et al.*, 1974) plants were transplanted to 1 litre pots containing "cereal mix" compost and returned to the CER.

At G.S 50, at which the peduncle is extending, tissue was harvested for RNA extraction. three replicates were collected for each sample and 4 plants were sampled per replicate. The tissues sampled, illustrated in figure 2.2, are as follows; the ear (divided into developing grain and rachis), peduncle (P; divided into thirds; lower/middle/upper), peduncle node, P-1 internode (divided into thirds; lower/middle/upper) and the P-1 node. Additionally, ears were sampled at G.S 60-70 and G.S 70-80 and divided into developing grain and rachis. RNA was extracted from each of the tissue samples, and qRT-PCR assays carried out as described above. The experiment was repeated three times.

To quantify *Rht* expression in the stamen, pre-dehiscent anthers and filaments were collected from plants at G.S 60. Four replicates were collected with each replicate consisting of at least 40 individual florets. RNA was extracted, and qRT-PCR assays carried out as described in sections 2.2.4 and 2.2.6. The experiment was repeated.

2.2.10. **Molecular characterisation of *Rht* alleles**

Seed of Mercia (*rht-tall*, *Rht-B1b*, *Rht-D1b*, *Rht-B1c* and *Rht-D1c*) and Maris Huntsman (*rht-tall*, *Rht-B1b*, and *Rht-B1c*) NILs were surface sterilised and stratified at 4˚C in the dark on wet 3MM

grade 1 Whatman paper for 2 days. Seedlings were grown on Petri dishes in CER as described in section 2.2.9.2. Root and shoot tissue was collected for RNA extraction after 4 days, root (longest) and shoot lengths were measured immediately prior to sampling.

For molecular characterisation of the *Rht* dwarfing alleles, genomic DNA was extracted from leaves of the Mercia *Rht* NIL series plants using the CTAB DNA extraction method as described by Nicholson *et al.* (1996) for use as template in subsequent experiments.

Rht-B1 specific primers (Fwd-GGCAAGCAAAAGCTTGAGATA and Rev-GCCATGTCGGAC GCC) were designed to flank an apparent insert to the 5' end of the *Rht-B1c* ORF identified in preliminary work carried out by Bottley (JIC, Norwich, UK). *Rht-B1* specificity was assured by including the control template DNA from DT4Bβ plants in PCR experiments.

To analyse sequence upstream of the *Rht-D1* ORFs, *Rht-D1* specific primers were designed to amplify blocks of the promoter sequence of *Rht-D1a, Rht-D1b* and *Rht-D1c* (Fig. 2.3). Primers for PCR amplification were designed using the aligned promoter sequences of the *Rht* homoeologues from BAC sequence (sequence available from Wilhelm, NIAB, Cambridge, UK, unpublished data). Products were prepared for sequenceing using BigDye® (Applied Biosystems) and analysis of reaction products was performed by The Genome Analysis Centre, Norwich. Sequences were aligned using the BioEdit alignment tool (Tom Hall, Ibis Therapeutics, Carlsbad, CA, USA).

Genomic DNA of the Mercia *Rht-D1* allelic series (*Rht-D1a, -D1b, -D1c*) was used as template for homoeologue specific qPCR assays to assess copy number. The amplification signals of *Rht-B1* (used as an internal control) and *Rht-D1* (WT and mutant alleles) were normalised to ubiquitin using the $\Delta\Delta C_t$ method described previously.

The seed of wheat cultivars (Chinese Spring, Cadenza, JIC Synthetic, Mercia, Paragon, Kanred, Cappelle Desprez) identified as containing different *Rht-B1* promoter haplotypes (Table 2.4; Wilhelm, NIAB, Cambridge, UK, unpublished data) were surface sterilised and stratified at 4˚C in the dark on wet 3MM grade 1 Whatman paper for 2 days. Germinated seeds were transferred to Petri dishes lined with wet 3MM grade 1 Whatman paper and placed in a CER (22˚C with 16/8 h light/dark period). Shoot tissue was sampled after 5 days and RNA extracted and processed as described in section 2.2.4. Three biological replicates were sampled for each line, each consisting of at least 10 seedlings.

Table 2.4. Cultivars containing insertions in the promoter region of *Rht-B1* that were screened for aberrant *Rht-B1* expression.

Cultivar	Polymorphism[a]		
	16 bp insertion (-694)[b]	197 bp insertion (-592)[b]	160 bp insertion (-356)[b]
Chinese Spring	-	-	-
Cadenza	-	-	-
Mercia	-	-	+
Paragon	-	-	+
Kanred	-	+	-
Cappelle Desprez	-	+	-
JIC Synthetic	+	+	-

[a] + denotes presence of the insertion, [b] numbers in parentheses show co-ordinates relative to the Chinese Spring *Rht-B1* translation start site.

2.2.11. **Investigating the effect of the environment on *Rht* expression**

2.2.11.1. The effect of light

Seeds of Paragon (*rht-tall*) were surface sterilised and stratified in the dark at 5˚C on wet 3MM grade 1 Whatman paper. Germinated seed were transferred to Petri dishes lined with wet 3MM grade 1 Whatman paper and grown in a CER (16/8 h light/dark at 22˚C) under a propagator lid. Shoot samples were collected 88.5 h after seedlings were transferred to the CER and sampled every 2 hours thereafter over a 36 h period. Three biological replicates were sampled for each time point, and each biological replicate consisted of shoot tissue from at least 10 seedlings. RNA was extracted and processed as above. The full 36 h time course was not repeated however 'snapshot' experiments were carried out to confirm the findings.

Time-lapse photography was carried out to observe whether there is an effect of photoperiod on growth rate. Seed of Paragon (*rht-tall*) were prepared and grown as above and photographs were taken, *in situ*, every hour over a 96 hour period. The camera flash was fitted with a green filter to prevent the exposure of the plants to red and blue light during the dark period. Coleoptile growth was measured using ImageJ software (Abramoff *et al.*, 2004). The experiment was repeated several times.

2.2.11.2. The effect of ambient temperature

To determine whether ambient temperature affects *Rht* expression, seed of Mercia (*rht-tall*) were surface sterilised then stratified at 4˚C in the dark on wet 3MM grade 1 Whatman paper for 2 days. Germinated seeds were transferred to Petri dishes lined with wet 3MM grade 1 Whatman paper and placed in CERs at respective temperatures. All seedlings were grown under a foil covered propagator lid to eliminate the effect of different light intensities in the cabinets having an effect on *Rht* expression. Seedlings were grown at 25 ˚C or 10 ˚C and shoot tissue was harvested after 4 and 10 days respectively (equivalent thermal time period of 100 degree days) in order to collect tissue of a similar developmental stage. Three biological reps were collected for each treatment, from at least 10 seedlings per biological replicate. The experiment was repeated several times.

Seed of Mercia NILs (*rht-tall*, *Rht-B1b*, *Rht-D1b*, *Rht-B1c* and *Rht-D1c*) were grown at different temperatures to determine whether temperature affects growth differentially in wild type and mutant plants. Seeds were grown at 5°C, 15°C and 25°C for 20, 6.66 (160 h) and 4 days respectively (100 degree days) before measuring. The coleoptiles and longest root of at least 10 seedlings per replicate were measured, three biological replicates were taken for each temperature. The experiment was repeated several times.

2.2.11.3. The effect of heat shock

To examine whether heat shock affects *Rht* expression, seed of Mercia (*rht-tall*) were stratified at 4˚C in the dark on wet 3MM grade 1 Whatman paper for 2 days. Germinated seed were sown in P40 seed trays containing peat and sand mix. Seedlings were grown in a CER (Sanyo MLR-351H) at 20˚C for 2 weeks until they reached the 3 leaf emergence stage. Half of the plants (20 per genotype) were transferred to a second cabinet (Sanyo MLR-351H) preheated to 45˚C while the control plants remained at 20˚C. After 1h the treated plants were returned to the 20˚C cabinet. Samples were collected from the control and heat shocked plants immediately after treatment and then at 1, 2, 4, 6, 8, 24 and 48 h after treatment. The second leaf from 4 plants was sampled per replicate and three replicates were collected for each time point. RNA was extracted and processed for each sample as described above.

In two subsequent experiments, *Rht-B1c* and *Rht-D1c* Mercia NILs were treated alongside the *rht-tall* lines. However, in these experiments fewer sampling time points were taken due to plant growth space constraints. In addition to *Rht*, the expression of *BAX INHIBITOR-1* (*TaBI-1*) and the wheat orthologue of *RADICAL-INDUCED CELL DEATH 1*, *CLONE EIGHTY ONE* (*CEO*), both of which have a putative role in the negative regulation of cell death, were measured in this material using qRT-PCR and the primer sequences shown in Table 2.5.

Table 2.5. Primer sequences used for qRT-PCR analysis of expression of *BAX INHIBITOR-1* (*TaBI-1*) and *CLONE EIGHTY ONE* (*CEO*)

Gene	Forward	Reverse
BI-1	TACATGGTGTACGACACGCA	GTCCATGTCGCCGTGG
CEO	GCGTCTGTCTGTGAATCTGC	TGTTGATTGGACAAAAACCAA

2.3. **Results**

2.3.1. ***Rht* qRT-PCR assay development**

AqRT-PCR assay, designed to measure the expression of all three *Rht* homoeologues (ABD *Rht*) was initially developed due to the difficulties of designing qRT-PCR assays specific to each *Rht* homoeologue. The ABD assay was designed utilising Taqman technology which provides highly specific quantification of gene expression. Due to a high degree of sequence similarity between the three *Rht* homoeologues, coupled with their high GC content, it was not possible to design Taqman assays to differentiate the three homoeologues, thus SYBR assays were designed. The ABD assay was superseded once the homoeologue specific assays were designed and optimised because the aim of the project was to determine whether all *Rht* homoeologues were transcriptionally regulated similarly through development and responded similarly to environmental cues.

To establish *Rht-A1* and *Rht-D1* gene specific PCR amplification, a number of primer combinations were tested using an annealing temperature gradient and the nullisomic 4A and 4D DNA templates, respectively. In all cases, the Chinese Spring (C.S) euploid control was included to confirm the ability of the primers to amplify the homoeologous gene target. The optimum primers and conditions were selected by melt curve analysis. The *Rht-B1* assay required more extensive optimisation; 15 primer combinations (data not shown) were tested before absolute specificity was achieved and this assay required a very high annealing temperature.

Once optimised each of the *Rht* homoeologue specific quantitative PCR assays were validated for specificity using the respective nullisomic-tetrasomic or ditelosomic genomic DNA template. The data, presented as target product amplified relative to the ubiquitin normalising gene (Fig. 2.4a), shows absence of PCR product from respective aneuploid templates, and an increase in product (relative to the C.S template) when the homoeologue being amplified is present in a tetrasomic condition. The euploid genomic DNA template (C.S) used for validation provides a control in

which, assuming equal primer efficiency, *Rht* homoeologues should be present at a 1:1:1 (A:B:D) ratio. In figure 2.4a, *Rht*-A1 is over represented by approximately 2 fold in C.S and aneuploid templates.

In order to compare the contribution of each homoeologue to total *Rht* transcription, absolute quantification was determined. Absolute quantification calculates molecules of target gene per molecule of a normalising gene, in this case ubiquitin. Due to the high level of expression of ubiquitin, target molecules are calculated per 10,000 molecules of ubiquitin. Absolute quantification is based upon amplification of a dilution series of the target gene of known DNA concentration and conversion with an algorithm (section 2.2.7.1). The assay validation data is presented as absolute expression in figure 2.4b. The specificity of the amplification was confirmed by the absence of products from the respective nullisomic DNA templates. However, the homoeologue contribution is not as expected; instead of the 1:1:1 (A:B:D) homoeologue contribution expected from the euploid C.S template, a 1.6:6.5:1 ratio was observed. Normalisation of the data set, so that C.S had a 1:1:1 contribution from each homoeologue (Fig 2.4c) still did not provide the absolute quantification expected from genomic DNA (i.e. 0:1:2 for N4AT4D; 1:0:1 for DT4Bβ and 1:2:0 for N4DT4B). Data analysis of genomic DNA validation assays using absolute quantification has not fulfilled the absolute quantification of the *Rht* homoeologues sought for this study. The addition of a normalisation step will not provide accurate data when applied to expression data suggesting that absolute quantification is not a suitable method for presenting expression data. As such the following data is presented as relative quantification, with the *Rht-A1* over representation (Fig 2.4a) in mind. The use of relative expression means that expression data can only be compared within an experiment as the nature of relative expression means that C_t values are compared to one another within the experiment. Similar contribution of homoeologues can only be inferred by the expression of target homoeologue showing similar expression relative to the normalisation factor.

The endpoint products of the homoeologue specificity assay were separated by electrophoresis (Fig. 2.4d) confirming the absence of any product in assays in which the template is in a nullisomic condition. The absence of *Rht*-A1 product from chromosome 4A nullisomic template, but it's presence in all templates derived from plants containing the A genome, confirms, for the first time, that *Rht*-A1 is present on chromosome 4A. Melt curve analysis of optimum assay conditions are presented in figure 2.5.

2.3.2. **The effect of polyploidy on *Rht* homoeologue contribution**

The tetraploid, *T. durum* and diploid *A. tauschii* are the respective donors of the AABB and the DD genomes of *T. aestivum.* Synthetic lines are the products of crosses which recreate this

hybridisation event with the aim of recapturing genetic diversity that was lost through the genetic bottleneck of recent domestication and polyploidy speciation events. To investigate how the hybridisation event affects homoeologue contribution in the case of *Rht*, expression was measured in the donor parents, *T. durum* and *A. tauschii*, a synthetic line derived from a cross of the donor parents, and the *T. aestivum* cultivar Paragon (Fig. 2.6). As expected, *Rht-D1* is not expressed in *T. durum* and neither *Rht-A1* nor *Rht-B1* are expressed in *A. tauschii*. Interestingly the homoeologue contribution is similar in the newly hybridised line compared to the Paragon cultivar. Also for the first time it was demonstrated that *Rht-A1* is expressed, suggesting all three homoeologues are involved in controlling growth processes in *T. aestivum*.

2.3.3. **Investigating the expression of *Rht* during development**

2.3.3.1. Germination stage

Germination is a tightly controlled process in plant development in which the plant integrates exogenous and endogenous cues into an appropriate response. In order to investigate whether *Rht* transcriptional control contributes to this process expression was measured at various stages of germination in seed exposed to different environments.

In experiment 1 (Fig 2.7a) seed were incubated in the dark either at 4˚C for 24h or at 4˚C for 24 hours followed by 24h at 22˚C. Expression of all three *Rht* homoeologues was significantly (<0.001) lower in seeds that had been incubated at 22˚C for 24h than that observed in seed incubated at 4˚C for 24h, suggesting that transferring seed to a higher temperature could result in a decrease in *Rht* expression, although it cannot be ruled out that expression could decrease with increasing time at 4˚C.

In a second experiment *Rht* expression was measured in seed incubated at 4˚C for 24, 144 and 264 h. *Rht* expression increased with time (Fig. 2.7b). Expression of all three homoeologues was significantly greater at 144 h compared to 24 h (P=<0.01) and at 264 h compared to 144 h (P=<0.001). Once the seed, incubated for 264h was transferred to 22 ˚C germination progressed rapidly (visual observation, not quantified) likely coinciding with and resulting from a reduction in *Rht* expression (not measured in this study but based on observations in experiment 1). All *Rht* homoeologues are expressed in the germinating seed and all respond to a similar extent to developmental/environmental cues.

2.3.3.2. Seedling stage

For the rapid harvesting of both shoot and root material for RNA extraction, seedlings were grown in Petri dishes because root sampling is not feasible with soil grown plants. When seedlings were sampled four days after transfer to 22 ˚C, expression of all three *Rht* homoeologues, collectively

and independently (Fig. 2.8), is significantly (P=<0.01) less in the roots compared to the shoots. This result was evident throughout the study. *Rht*-A1 contribution was higher compared to that of *Rht-B1* and *Rht-D1*, but this is likely reflecting the overrepresentation of the *Rht*-A1 evident in the genomic DNA validation assay (Fig. 2.4a).

2.3.3.3. Mature plant stage

The expression of GA biosynthetic enzymes has been shown to be localised in particular tissues during stem elongation of wheat (Appleford *et al.*, 2006), furthermore differential homoeologue contribution of one of these genes, *GA20ox1*, has been reported. To investigate where *Rht* is expressed and whether certain homoeologues contribute to particular stages of development, material similar to that sampled by Appleford and colleagues was collected for this study across three independent experiments.

In a preliminary study in which biological replicates were pooled, accounting for the lack of error bars, *Rht* expression was measured in the peduncle and peduncle (P-) node (see Fig. 2.2) of wheat plants at three growth stages (Fig. 2.9a). At two of the three growth stages (G.S. 45 and G.S. 59), the *Rht* expression in the peduncle was higher in older tissue compared to younger tissue (the lower peduncle is younger than the upper peduncle) however *Rht* expression did not increase during GS progression. *Rht* expression in the P-node was at a similar level to that in the elongating peduncle. The lack of biological replication prevented statistical analysis of these data but the data were sufficient to inform the design of further studies.

In a second experiment, *Rht* expression was measured in peduncle, P-node, P-1 internode and P-1 node tissues (see Fig. 2.2) at a single growth stage (G.S 50; Fig. 2.9b). A general trend of increasing *Rht* expression with tissue maturity can be observed within the P-1 internode (where the lower internode tissue is younger than the upper internode), and comparing P-1 internode relative to the peduncle. *Rht* expression in the nodes is higher than in the young, actively elongating tissue (lower P-1 internode and peduncle).

To further investigate the effect of developmental stage on *Rht* expression, a more complete set of tissues were sampled with biological replication (Fig. 2.9c, and refer to Fig. 2.2 for details of the samples taken). In support of the previous findings *Rht* expression was greater in older, elongated tissue, relative to younger, actively elongating tissue. This was observed (1) within the peduncle; expression was higher in the older upper peduncle than in the younger lower peduncle, (2) between the older P-1 internode and the younger peduncle, and (3) between the rachis at post anthesis and the rachis at pre anthesis. *Rht* expression in the nodes was not as influenced by maturity of tissue (P-1 node older than P node). The increase in *Rht* expression in the rachis through development is marked whilst *Rht* expression in the grain remains the same through

development. No homoeologue in particular predominates *Rht* transcription in the ear. Indeed, the contribution from each of the homoeologues was similar in all tissues sampled.

Stamen development is an important stage for plant reproduction. In Arabidopsis, of the DELLA proteins, RGL1 and 2 predominantly regulate stamen development (Cheng *et al.*, 2004). *Rht* homoeologue contribution to stamen development at pre-anthesis (G.S 60, when pollen grains are developing and the filaments are beginning to extend) was assessed by qRT-PCR (Fig 2.10). To allow for any differences in developmental stage between florets at least 40 florets were sampled for each replicate. The data shows that expression of each *Rht* homoeologue was similar in these samples.

2.3.4. **The effect of *Rht* mutant alleles on *Rht* expression**

Peng *et al.* (1999) characterised the *Rht* semi-dwarf mutant alleles, *Rht-B1b* and *Rht-D1b*, in both cases they found that the dwarfism results from a nucleotide substitution which introduces a premature stop codon in the DELLA domain resulting in an N-terminally truncated product that has reduced GA sensitivity. The mutations causing the extreme dwarfing seen in *Rht-B1c* or *Rht-D1c* containing plants were unknown prior to this project.

The expression of *Rht* homoeologues in plants containing the wild type (*rht-tall*) or mutant alleles was investigated to determine whether incorporation of the mutant allele affected the relative contribution of the homoeologues. Homoeologue-specific *Rht* expression analysis in the Mercia *Rht* NILs revealed that *Rht-B1* expression was significantly reduced (P=<0.001) in plants containing the *Rht-B1c* allele and *Rht-D1* expression was significantly increased (P=<0.001) in plants carrying the *Rht-D1c* allele. The *Rht-A1* expression levels remained relatively unperturbed across the lines (Fig. 2.11). The reduction in *Rht-B1* expression in *Rht-B1c* containing lines was confirmed in Maris Huntsman NILs. A *Rht-D1c* containing NIL is not available in M. Huntsman. No significant differences in homoeologue-specific expression were observed between the semi-dwarf and *rht-tall* NILs.

2.3.4.1. Characterisation of the Rht-B1c allele

Using *Rht-B1* specific primers and DNA extracted from a Mercia *Rht-B1c* NIL plant, a product approximately 250 bp larger than that predicted was identified within the DELLA region of *Rht-B1c (*Fig. 2.12a). An insert was simultaneously identified in the DELLA region of *Rht-B1c* by Chandler *et al*. (CSIRO, Canberra, Australia; pers. comm.) concurrent with that seen in the present study. Subsequently, Pearce (RRes, Harpenden, UK) reported a 90 bp insertion in the DELLA region of cDNA derived from *Rht-B1c* (Fig. 2.12b). Alignment of the *Rht-B1c* insertion from

Chandler and Pearce revealed that the 90 bp insertion in the cDNA is a result of a splicing event removing part of the larger genomic DNA insertion (Pearce, RRes, Harpenden, UK, pers. comm.).

2.3.4.2. Characterisation of the Rht-D1c allele

No polymorphisms in the *Rht-D1c* ORF have been identified (Wilhelm, NIAB, Cambridge, UK, pers comm.). The increase in transcription of the *Rht D1* homoeologue in the presence of the *Rht-D1c* allele could be caused by changes in the promoter region 5' of the gene. For this reason, a 3695 bp region upstream of the ATG translation start site of *Rht* was sequenced from *Rht-D1a, -D1b and -D1c* genomic DNA. The only differences identified in the aligned promoter sequences of the *Rht-D1* allelic series (Appendix 7.1) was a variable cytosine repeat 1039 bp upstream of the ATG (Fig. 2.13). The number of cytosine residues did not correlate with a specific *Rht* allele (the Mercia *Rht-D1*a had three fewer cytosine residues compared to the Chinese Spring *Rht-D1*a and the Mercia *Rht-D1c* allele had seven fewer cytosine residues compared to the *Rht-D1c* progenitor, Ai-bian 1). This suggests that the cytosine repeat is not responsible for the severe dwarfing caused by *Rht-D1c*.

Amplification of *Rht-D1b* and *Rht-D1c* genomic DNA template with homoeologue specific primers using qRT-PCR revealed that the amplification signal for *Rht-D1* was approximately four times greater in *Rht-D1c* genomic DNA compared to *Rht-D1b*, whilst the *Rht-B1* amplification signal was similar for both templates (Fig. 2.14). *Rht-A1* was not included in this analysis because *Rht-B1* was considered a better reference for *Rht-D1* because as previously noted (Fig. 2.4a) *Rht*-A1 is overrepresented when using relative quantification. This suggests that the *Rht-D1c* allele is present as more than one copy in the genome.

Total *Rht* transcription and growth of the severe dwarf and wild type lines were next compared using the seedling bioassay to allow both shoots and roots to be sampled. ABD *Rht* expression of *rht-tall*, *Rht-B1c* and *Rht-D1c* lines reflects the alterations in expression resulting from their respective mutations (Fig. 2.15) i.e. ABD expression appears to be reduced in *Rht-B1c* shoots reflecting the reduction in *Rht-B1* expression in these lines, whilst overall expression is increased in roots and shoots, relative to *rht-tall*, in *Rht-D1c* lines reflecting the increase in *Rht-D1* in these plants. As previously observed in *rht-tall* plants (Fig 2.8) *Rht* expression is less in roots compared to shoots in *Rht-B1c* and *Rht-D1c* plants. Comparison of total shoot height and the length of the longest roots showed that *Rht-B1c* and *Rht-D1c* confer no difference in root length relative to *rht-tall*, whereas the shoot height of the plants containing the *Rht-B1c* and *Rht-D1c* alleles was approximately half that of plants containing the *rht-tall* allele. In light of the expression data this suggests that low levels of expression of the mutant protein in the roots may curtail the severe dwarf phenotype.

Large height differences are reported between wheat cultivars with *rht-tall* alleles and recent unpublished work by Wilhelm (NIAB, Cambridge, UK,) identified cultivars containing insertions upstream of the *Rht-B1* ORF (Table 2.4) .Representative cultivars were analysed to see whether the insertions result in altered *Rht-B1* expression. *Rht-D1* expression was also measured as a reference for *Rht-B1* expression as both have been shown to be represented similarly by relative quantification (Fig. 2.4a). In all but two cultivars the *Rht-B1* and *Rht-D1* contribution was similar (Fig. 2.16). *Rht-B1* expression was less than *Rht-D1* expression in Mercia and vice versa in Cappelle Desprez, however in neither case was the insertion likely to cause the disparity because other cultivars containing the same *Rht-B1* insert (Paragon and Kanred, respectively) showed similar contribution. Interestingly, Kanred, a particularly tall winter wheat cultivar showed reduced expression of both *Rht-B1* and *Rht-D1* relative to the other cultivars.

2.3.5. **Environmental effects on *Rht* expression**

2.3.5.1. The effect of light

A plant's growth is responsive to the environment, and, as demonstrated above during germination, transcriptional regulation of *Rht* likely contributes to this process. Light/dark cycling is a daily environmental variable which plants perceive and respond to. To determine whether *Rht* expression is affected by light, leaves of young plants were sampled throughout the photoperiod and expression analysed by qRT-PCR (Fig 2.17a). Using a 16/8 h light/dark cycle, expression of all three *Rht* homoeologues increased rapidly within 30 m of exposure to light. *Rht* expression peaked between 4h 30m and 6h 30m into the light period and was rapidly down regulated through the remaining light period and into the dark period, reaching its lowest point at the end of the dark period.

Homoeologue contribution seemed to alter within a 24h period, for example material sampled 8h 30m into the light period on day 4 (first data point, Fig. 2.17a) showed a similar contribution from each homoeologue whilst samples taken 24h later (8h 30m into light period of day 5) showed a greater contribution of *Rht-A1* followed by *Rht-D1* then *Rht-B1*, a trend seen in all four data points for which reciprocal data was available. To investigate further the effect of seedling age and light on *Rht* expression, another experiment was carried out in which samples were collected 1h 30m before and after the lights were switched on. Samples were taken on days 3-6 and day 10 after transfer of seeds to 22˚C and 16h photoperiod (Fig. 2.17b). The increase in *Rht* expression in response to light was not observed in this experiment, and in addition no significant alterations in *Rht* homoeologue contribution over time occurred.

To investigate whether growth of wheat seedlings occurs mainly in the light or dark and whether this could be correlated with the diurnal fluctuations in *Rht* transcription observed in the first

experiment, time lapse photography was used to measure growth in response to light/dark cycles during seedling development. The growth rate of wheat seedlings is independent of light (Fig. 2.18), evident from the linear growth seen over the first 4 days of growth. This experiment was repeated multiple times confirming the findings (data not shown).

2.3.5.2. The effect of ambient temperature

To investigate whether *Rht* transcription is affected by changes in ambient temperatures which could control the growth regulation of wheat seedlings, *Rht* expression was quantified in *rht-tall* plants grown in the dark at 10°C and 25˚C for an equivalent number of thermal days (Fig. 2.19). Expression of all three *Rht* homoeologues is lower in Mercia (*rht-tall)* NILs grown at 10˚C compared to plants grown at 25˚C. *Rht-A1* expression appears higher compared to *Rht-B1* and *Rht-D1* expression, but this is likely to be a result of the *Rht-A1* overrepresentation inherent in relative quantification of these homoeologues (Fig. 2.4a).

To investigate the effect of *Rht* mutant alleles on the growth response of plants at different temperatures, *rht-tall*, *Rht-B1b*, *Rht-D1b*, *Rht-B1c* and *Rht-D1c* Mercia NILs were grown at 5°C, 15°C and 25°C for 100 degree days and their root and shoot lengths measured at the seedling stage (Fig. 2.20). Growth of the shoot was reduced at lower temperatures in all Mercia *Rht* NILs tested apart from the *Rht-B1c* carrying line which remained a similar height, independent of temperature, when grown for an equivalent thermal day period. When shoot length reduction in plants grown at 5˚C compared to 25˚C was compared and expressed as % length reduction, it was clear that growth was allele-dependent with *rht-tall* NILs showing the greatest reduction (approx 45%) at 5˚C, *Rht-B1c* the least reduction (approx 5%) and *Rht-B1b*, *-D1b* and *-D1c* all showing a similar intermediate (approx 25-30%) reduction in height. The expected height ratio observed in plants grown at 25˚C (*rht-tall>Rht-B1b=Rht-D1b>Rht-B1c>Rht-D1c*) is therefore altered in plants grown at 5˚C (*Rht-B1b=Rht-D1b>rht-tall>Rht-B1c>Rht-D1c*) resulting in a situation where *Rht* semi-dwarf allele carrying NILs are taller than the *rht-tall* NILs.

Root growth was reduced with decreasing temperatures in all lines. When root lengths at 5˚C were compared with those at 25˚C, the roots of plants containing the semi-dwarfing alleles were slightly less responsive to the reduction in temperature than those of plants containing the *rht-tall* and severe dwarfing alleles (Fig 2.20d). As reported previously in this chapter (Fig. 2.15), the root lengths of *Rht-B1c* and *Rht-D1c* carrying plants were not shortened compared to the *rht-tall* carrying lines at ambient temperature and this remained true at 5˚C and 15˚C. In contrast, the roots of *Rht-B1b* and *Rht-D1b* carrying plants are longer than the *rht-tall* carrying plants at all three temperatures.

2.3.5.3. Heat shock

It has been demonstrated that *Rht* mutant allele containing lines, particularly *Rht-B1c* and *Rht-D1c*, have increased tolerance to heat shock (45˚C for 1h) compared to *rht-tall* containing lines (Korolev, JIC). To investigate whether *Rht* expression may be affected by heat stress and whether this response is altered in *Rht* mutant allele containing plants, *Rht* expression was measured in *rht-tall* and severe-dwarf lines in response to heat treatment.

Rht expression was initially analysed over a time course in both treated and control (not heat stressed) *rht-tall* plants and the data expressed as treated minus untreated (Fig. 2.21). A general trend was observed in all three *Rht* homoeologues in which expression was rapidly (within 1 h) down regulated after treatment followed by a gradual increase in expression relative to control plants reaching a peak somewhere between 24 and 48h after heat treatment. By 48h expression returned to that of the control samples.

Experiments comparing *Rht-B1c* to *rht-tall* and *Rht-D1c* to *rht-tall* were carried out separately due to sample number limitations (Fig 2.22). *Rht* expression perturbations in severe dwarf plants were noticeable compared to respective *rht-tall* lines. In all lines *Rht* expression was reduced 1 m after treatment removal. In the case of the *Rht-B1c* allele carrying lines, *Rht-B1* transcription was reduced but not by as much as the other homoeologues, whilst *Rht-D1c* allele carrying lines showed a greater decrease in *Rht-D1* transcription in response to heat treatment relative to the other homoeologues and compared to *rht-tall* lines tested in the same experiment.

A potential mechanism for tolerance to a short sharp shock experienced by the plants is a reduction of cell death. The Arabidopsis *AtRCD1* protein homologue in wheat, *CEO*, and *BI-1* are both thought to negatively regulate oxidative stress responses leading to cell death (Overmyer *et al.*, 2000; Huckelhoven, 2004). To see whether the increased survival of plants conferred by the severe dwarfing alleles was associated with an increase in negative regulation of cell death, the expression of *CEO* and *BI-1* was measured in the *rht-tall* and severe dwarf, treated and control material (Fig 2.23). There was no clear effect of heat stress treatment on *CEO* transcription in response to treatment or *Rht* allele. However, *BI-1* is rapidly (1m after treatment) up regulated in response to treatment. *BI-1* expression returns to that of untreated controls within 24 hours after treatment. *BI-1* expression is greater in treated *Rht-B1c* plants compared to treated *rht-tall* plants 1m and 1h after treatment, while there is no statistical difference in *BI-1* expression between *rht-tall* and *Rht-D1c*.

2.4. **Discussion**

A homoeologue specific assay has been developed enabling for the first time the quantification of relative homoeologue contribution to the *Rht* transcriptome in wheat. The development of an assay to measure *Rht* expression is a step towards a greater understanding of DELLA mediated growth in

wheat and to determining whether different homoeologues may play different roles in development. However, it must be borne in mind that expression of mRNA does not necessarily correlate with protein levels, and DELLA protein stability is influenced by GA metabolism and other GA signalling components. During the course of this project neither suitable wheat RHT antibodies nor RHT-GFP lines were available to monitor RHT protein levels in conjunction with *Rht* expression. However the design and implementation of the homoeologue specific assay has enabled the confirmation of *Rht-A1* expression, the identification of *Rht* homoeologue expression perturbations in *Rht* mutant alleles and observation of how the *Rht* homoeologues may contribute to growth control during development and in response to environment, which would not have been possible using the ABD *Rht* assay.

2.4.1. **The effect of polyploidy on *Rht* homoeologue contribution**

The process of polyploidisation has the potential to change plant physiology due to increased gene dosage. Tolerance strategies to polyploidisation events include sequence elimination, methylation alterations and gene silencing. In the present study I investigated whether the homoeologue contribution of *Rht*, an important dose dependent growth regulatory gene, was altered as a result of polyploidisation. By recreating polyploidisation events with synthetic lines, the effects of ploypoloidisation on the contribution of homoeologues can be assessed against known pedigree. *Rht* homoeologue contribution in young shoots was equivalent in a 10 year old synthetic hexaploid compared to an approximately 10,000 year old conventional hexaploid suggesting that no *Rht* transcriptional silencing or altered methylation has occurred to compensate the increased gene dosage of *Rht*. Adams *et al.* (2004) showed that silencing of certain gene homoeologues is differentially affected in different organs in newly synthesized cotton tetraploids, suggesting that subfuntionalisation of homoeologues by tissue specific transcriptional regulation can arise. It may be that *Rht* homoeologue contribution to the *Rht* transcriptome is altered in organs other than young seedlings in newly synthesised wheat lines relative to conventional hexaploid cultivars.

2.4.2. **The effect of development on *Rht* expression**

The five DELLA proteins of Arabidopsis control growth in distinct organs and developmental stages, for example, GAI and RGA are the main regulators of cell expansion in vegetative tissues (Dill & Sun, 2001), RGL2 primarily controls germination (Lee *et al.*, 2002) and in combination with RGL1 and RGA also controls flower development (Cheng *et al.*, 2004). Tyler *et al* (2004) demonstrated that RGA and GAI are mainly regulated by ubiquitin mediated proteolysis whilst RGL1, 2, and 3 are regulated at the transcript level in addition to posttranslational regulation. The level of transcriptional control is evident in a study by Gallego-Bartolome *et al.* (2010) who showed that *RGL2* expressed under the control of the RGA promoter complements the loss of RGA

function, and showed that RGA and RGL2 have an equivalent capacity to interact with downstream basic Helix-Loop-Helix (bHLH) transcription factors, suggesting that at the protein level RGA and RGL2 function is equivalent. The study concludes from expression analysis that Arabidopsis DELLA subfunctionalisation, at least in part, has arisen from differences in transcriptional regulation. In contrast to Arabidopsis, wheat is thought to contain only one DELLA encoding gene, and thus there is a possibility that the three copies of *Rht* are differentially regulated at a transcriptional level to fulfil the growth regulatory role that requires five DELLA proteins in Arabidopsis. However data obtained in this study suggest that gene expression of all three homoeologues is regulated similarly in all of the organs tested.

2.4.2.1. Germination stage

RGL2 plays the most important role, of the DELLA proteins, in the control of germination in Arabidopsis (Lee *et al*., 2002). *RGL2* expression increases during imbibition at 4˚C and rapidly decreases after transfer of seed to higher temperatures (23˚C), in response to increases in GA, which coincides with the completion of germination. The present study demonstrates that, similar to *RGL2*, *Rht* expression increases with time at 4˚C and rapidly decreases when seed is transferred to higher temperatures.

The data in the present study shows that all three homoeologues respond similarly in the germinating seed suggesting that they may function collectively in repressing germination. Tyler *et al* (2004) showed that RGL2, RGA, GAI and RGL3 transcripts all accumulate in imbibed seeds and that RGA protein is relatively abundant in comparison to RGL2. The authors suggest that RGL2 may have a higher specific activity than RGA proteins in repressing germination. It therefore remains a possibility that a particular *Rht* homoeologue has a greater role in germination repression at the protein level. This would be determined by the sequence at the C-terminal functional domain necessary for interactions with downstream GA-mediated components.

Gubler *et al*. (2002) showed that down regulation of DELLA encoding genes is not essential for the progression of germination in barley. In contrast to the findings of Tyler *et al.* (2004) in Arabidopsis and the findings of this study in wheat, it was suggested that the function of SLN1 in aleurone cells of barley is regulated solely at a post-translational level. A similar finding was reported also for tomato and soybean (Bassel *et al.*, 2004). A more detailed timescale of *Rht* expression analysis related to the progression of germination will enable a better understanding of the extent to which transcriptional regulation of *Rht* controls germination progression in wheat. Clearly it will also be important to analyse the RHT levels should appropriate antibodies become available. However, it is unlikely that the contribution of each RHT protein could be assessed because the high level of sequence conservation will prevent homoeologue-specific antibody production.

DELLA encoding genes in apple (*Maloideae*) are regulated transcriptionally, with high levels of mRNA in summer arrested shoot tips and in autumn vegetative buds (Foster *et al.*, 2007). Controlling growth in response to environmental stimuli is crucial to the plant, and the transcriptional regulation of DELLA, as demonstrated in the present study and the studies mentioned above, may provide another level of growth control, additional to the control of GA metabolism with resulting effects on DELLA stability. For example, when transcription is reduced, residual DELLA protein can be rapidly degraded, enabling rapid response to optimal environmental conditions. In contrast, enhanced transcription can lead to rapid accumulation of protein with a concomitant growth repression.

The transcription of *Rht* in germinating plants containing mutant alleles was not studied but extrapolating from the transcriptional and sequence analysis of plants containing the severe dwarfing alleles in this chapter it could be postulated that *Rht-B1c* probably results in increased protein stability, and therefore would be less responsive to the environment induced transcriptional changes during germination and in response to other transcriptionally regulated processes. Pre-harvest sprouting is the premature germination of wheat seeds while still on the head in the field and is strongly associated with environmental conditions. *Rht-B1c* containing lines are highly tolerant to pre-harvest sprouting (Flintham & Gale, 1982) possibly reflecting *Rht-B1c* having a reduced responsiveness to environmental conditions at a transcriptional level.

2.4.2.2. Mature plant stage

Rht transcript levels are different in different organs suggesting *Rht* may be under spatial and temporal transcriptional control, furthermore relative levels in different organs were different across experiments. The differences between experiments likely reflects slightly different stages of development in the organs because younger actively elongating tissue, such as the lower peduncle and the rachis at G.S 50 (Fig. 2.9 c), has reduced *Rht* expression compared to older elongated tissue, such as the upper P-1 internode or the rachis at G.S 70-80. This would suggest a low level of *Rht* expression in younger tissue enables cell elongation and *Rht* expression gradually increases as the cells mature and growth slows. Interestingly, increases in transcription do not occur with maturity in non-elongating tissue, for example *Rht* expression is equivalent in older P-1 node relative to the P-node (Fig. 2.9 b & c), similarly *Rht* expression in the developing grain is equivalent at G.S 50 compared to G.S 70-80 (Fig. 2.9 c).

In rice, studies using plants transformed with a SLR1 promoter:GUS construct have shown, contrary to the findings in the present study, that high expression of *SLR1* was correlated with actively developing and/or elongating cells, for example strong GUS activity was observed in the divisional zone immediately above the node and extended into the elongation zone but little or no activity was seen in the elongated zone (upper internode; (Kaneko *et al.*, 2003)). Similarly, in

barley *SLN1* mRNA is preferentially expressed in elongating regions of the leaf of barley and this correlates with the level of SLN1 protein (Chandler *et al*., 2002). In wheat, Pearce (RRes, Harpenden, UK, unpublished data) shows that *Rht* expression is greater in younger elongating tissues using the assay developed in the present study and this was corroborated using a wheat line transformed with an*Rht*-A1 promoter:GUS construct which revealed that *Rht*-A1 expression is greatest in the lower part of the peduncle. Each of these studies contradicts the findings of the present study, but differences are likely explained by the developmental stage at which samples were taken for expression analysis highlighting the labile nature of *Rht* transcription through development.

In addition to DELLA expression data, studies have been carried out to analyse the role of gene expression in control of GA metabolism in cereals. Studies in rice demonstrated that the expression of GA metabolism enzymes are coincident with those involved in GA signalling (Kaneko *et al*., 2003). The transcription of the GA biosynthetic enzymes, GA20ox and GA3ox, has also been analysed in the elongating stem and ear of wheat plants (Appleford *et al*., 2006). The data show that *GA20ox1* and *GA3ox2* expression was greatest in the node subtending the peduncle. In the P-1 internode *GA3ox2* expression was higher in the lower, actively elongating tissue compared to the upper elongated tissue. Interestingly, using homoeologue-specific probes, the authors noted that homoeologue contribution to the expression of *GA20ox1* is unequal, they showed that the A and D genomes contribute equally whereas expression from the B genome was not detected in the peduncle node or ear.

The data in the present study suggest that all three homoeologues are expressed in the rachis of the ear to relatively high levels compared to the internode or peduncle tissue, and expression increases with maturity. GUS localisation in the rachis of a GUS:*Rht-A1* line (Pearce, RRes, Harpenden, UK, unpublished data) confirms increased expression in the rachis compared to the grain. Assuming similar contribution of the *Rht* homoeologues, as the qRT-PCR data suggest, *Rht-B1* and *Rht-D1* will also be expected to be expressed to high level in this tissue. It is a surprise therefore that the dwarf phenotype is not observed in the ears of semi-dwarf and severe-dwarf allele containing lines whilst it is clearly evident in the stem. It is possible that the level of *Rht* is at saturation point in the rachis meaning an increase in RHT protein stability, as is thought to be the case in the semi-dwarf and severe-dwarf alleles, will not have an equivalent effect in the growth of the rachis as is observed in the stem.

In this study *Rht* expression was shown to be lower in the root compared to the shoot and probably the lack of a reduction in the root length in the semi-dwarf or severe dwarf lines reflects low levels of RHT protein in the roots. In the current study root growth was increased in semi-dwarf allele containing plants relative to wild type although in contrast no significant differences in root length

were found between semi-dwarfing lines and the control lines in a study by Wojciechowski *et al* (2009). However the author found that differences in root length between the severe dwarfs and the control lines depended on the experimental methodology. For example, the roots of severe dwarf plants were 40% longer than control (*rht-tall*) plants when grown in gel chambers whilst in soil media their root length reduced by 24-33% relative to control (*rht-tall*) plants. Kaneko *et al*. (2003) showed that the expression of *Slr1*, *GA3ox* and *GA20ox* was less in the roots compared to the shoots of rice. Taken together, these findings may indicate that other, GA independent, processes play more of a predominant role in regulating root growth.

2.4.3. **Molecular characterisation of the severe dwarf alleles**

Although the phenotypes of *Rht-B1c* and *Rht-D1c* are similarly severe, the mutations differ and result in altered *Rht* expression. Transcription of *Rht-B1* is reduced in *Rht-B1c* lines resulting in an overall decrease in *Rht* transcript relative to that seen in *rht-tall* lines, but paradoxically *Rht-B1c* plants are approximately half the height of *rht-tall* lines. Sequence analysis of the *Rht-B1c* allele lead to the identification of an insertion in the DELLA region of the gene. This finding was corroborated by Chandler *et al*. (CSIRO, Canberra, Australia, pers. comm.) who sequenced the insert. Subsequently, Pearce *et al*. (RRes, Harpenden, UK, unpublished data) identified a 90 bp insertion in *Rht-B1c* cDNA. This 90 bp insertion lies within the region that was identified by PCR amplification in the present study and aligns within the sequence of Chandler *et al*. Analysis of the genomic DNA sequence with splice site programmes suggests that splicing accounts for the presence of only a 90 nucleotide insertion in the DELLA region of the mRNA/cDNA sequence (Pearce, RRes, Harpenden, UK, pers. comm.)

The DELLA region is crucial for the interaction of the DELLA proteins with GID1 (Ueguchi-Tanaka *et al.*, 2007). To test whether The GID1-DELLA interaction was affected by the 30 amino acid insertion in the RHT-B1 protein, Pearce carried out a yeast-2-hybrid screen with RHT and RHT-B1c proteins and the GID1 protein in the presence of bioactive GA. The 30 a.a. insertion in the DELLA region abolished the GID1-RHT-B1c interaction (Pearce, RRes, Harpenden, UK, unpublished data). The GID1-RHT interaction in wild type plants results in SLY1/GID2 recruitment which leads to protein degradation. If this does not occur in *Rht-B1c* lines, RHT-B1c protein is likely to be stabilised resulting in the continued repression of growth in the presence of GA and presumably causing negative feedback on further expression observed in this study. Interestingly, this negative feedback seems to be B genome specific, presenting the possibility that the *Rht* homoeologues have the capacity to be independently regulated at the transcriptional level.

Transcription of *Rht-D1* is elevated in *Rht-D1c* lines resulting in an overall increase in *Rht* transcription in these lines relative to *rht-tall*. The nucleotide substitution in *Rht-D1b*, resulting in a premature stop codon in the DELLA region (Peng *et al.*, 1999) is also present in the *Rht-D1c* mutant as identified by Pestsova *et al*.(2008) using perfect primers designed to the nucleotide substitution responsible for the mutation (Ellis *et al.*, 2002) and this has subsequently been independently confirmed by sequencing (Wilhelm, NIAB, Cambridge, UK, pers. comm.). The *Rht-D1c* ORF contains no additional mutations (Wilhelm, NIAB, Cambridge, UK, pers. comm.). In order to explain the increased dwarfing, of *Rht-D1c* relative to *Rht-D1b* an additional mutation must be present.

A region encompassing approximately 4 kb upstream of the *Rht-D1* allelic series was sequenced, and only one polymorphic region was identified. The polymorphism, a variable cytosine repeat, was not consistent when sequence upstream of an additional *Rht-D1c* allele (provided by Jia, CAAS, Beijing, China) was analysed and therefore was not considered causative of the increases in expression observed. The region is highly GC rich and a span of 13-20 cytosine residues lead up to the polymorphic site. Repeat elements and GC richness can result in the introduction of PCR or sequencing errors therefore it cannot be discounted that the polymorphism is a result of errors in sequencing or PCR amplification.

Amplification of *Rht-D1b* and *Rht-D1c* genomic DNA template using the *Rht-D1* qPCR assay resulted in *Rht-D1c* template producing an signal significantly greater than *rht-tall* whilst *Rht-B1* amplification signal, used as an internal control, remained similar, suggesting *Rht-D1c* is a copy number variant of *Rht-D1b*. This result could explain the increase in *Rht-D1* expression observed by qRT-PCR. The resulting increase in RHT-D1b protein is likely to cause the increased GA insensitivity observed. Copy number variants for *Ppd-B1* have been identified in wheat resulting in early flowering, and cultivars containing different numbers of tandem repeats have been identified (David Laurie, JIC, pers. comm.). It would be interesting to screen cultivars using the *Rht* homoeologue specific primers to find further *Rht* homoeologue copy number variants.

McVittie *et al*. (1978) mapped *Rht-B1b* and *Rht-B1c* 13 map units, and *Rht-D1b* 15 map units, from the centromere on the short arms of chromosomes 4B and 4D, respectively using telocentric mapping. *Rht-B1b* and *Rht-B1c* had previously been shown to be allelic (Gale & Marshall, 1976). Subsequently, Izumi *et al*. (1981) reported an additional GA insensitive dwarfing gene on chromosome 4D in a Chinese variety, Ai-bian 1. Telocentric analysis of this allele, now known as *Rht-D1c*, suggested that it was located on the short arm of chromosome 4D more than 50 map units from the centromere (Izumi *et al.*, 1983). Other studies also mapped *Rht-D1c* more distal from the centromere than *Rht-D1b* (Borner *et al.*, 1997; Cao *et al.*, 2009). However test crosses have shown that *Rht-D1b* and *Rht-D1c* are allelic (Borner & Mettin, 1988). The discrepancy in the mapping of

Rht-D1b and *Rht-D1c* to the same loci on the short arm of chromosome 4D may be due to the increase in copy number of *Rht-D1* in *Rht-D1c* lines observed in this study. The sequence from an *Rht-D1c* containing BAC isolated from a Ai-bian 1*Chinese Spring BAC library has been made available by Jia (CAAS, Beijing, China). Analysis of the sequence reveals a single copy of *Rht-D1* at the distal end of the available 200 kb of sequence. Isolation and analysis of the adjacent BAC(s) may provide sequence evidence of duplication of *Rht-D1c* in Ai-bian1 and may reveal the extent of sequence duplication either side of the *Rht-D1* gene. Additionally, similar to the approach used in the present study with homoeologue specific SYBR assays, the application of the ABD Taqman assay to compare relative amplification signal from *Rht-D1c* template compared to *Rht-D1b* control gDNA template may confirm the gene copy number differential.

Identification of cultivars with insertions *in the Rht-B1* promoter and subsequent expression analysis of these cultivars demonstrate that these insertions do not affect transcription under the conditions tested. Neither transcription start sites of *Rht* homoeologues nor motifs in the promoter regions essential for transcriptional regulation of *Rht* have yet been identified. Upstream sequence of each homoeologue is available and preliminary sequence alignment with equivalent regions in Arabidopsis, rice and Brachypodium has shown high conservation for approximately 2 kb upstream of the *DELLA* (Boulton, JIC, Norwich, UK, pers. comm.). Gallego-Bartolome *et al*. (2010) analysed the promoter regions of the Arabidopsis DELLA encoding genes but were unable to identify specific sequences within the promoter to explain the divergence of DELLA transcription observed by Tyler *et al*., 2004. However, using microarray data available through Genevestigator, Zimmermann *et al*. (2004) showed clear tissue- and developmental stage specific expression. No such tissue- or stage specific expression was observed between the wheat homoeologues in this present study suggesting that promoter regions conferring such specificity may be conserved.

In this study, Kanred, a particularly tall winter wheat cultivar, showed reduced expression of both *Rht-B1* and *Rht-D1* relative to the other cultivars which correlated with increased height. This suggests that transcriptional regulation of *Rht* expression could be an inheritable, tractable trait for controlling plant height. To test this further a correlation analysis could be undertaken; a representative sample of worldwide wheat diversity is available in a collection of 372 wheat cultivars known as the INRA core collection (Balfourier *et al.*, 2007) and further screening of this collection for promoter differences (currently being undertaken by Wilhelm, NIAB, Cambridge, UK) in combination with quantification of *Rht* expression of promising candidates may identify novel sources of dwarfing for use in the field.

2.4.4. **Characterising the *Rht-A1* locus**

Prior to this study the location, sequence and expression level of *Rht-A1* was unknown. Based on *Rht-A1* sequence obtained from Pearce (RRes, Harpenden, UK) homoeologue specific primers were designed. The absence of *Rht-A1* product in the nullisomic 4A template shown in the present study, for the first time, provides evidence that the gene resides on chromosome 4A. The exact location of *Rht-A1* on chromosome 4A is not known, but it is likely to be '4AL' due to the translocation event, pericentric inversion (including centromere) and paracentric inversion (not including centromere) events that have occurred during the evolution of chromosome 4A (Devos *et al.*, 1995). The *Rht-A1* specific primers could be used to confirm the location by a presence/absence screen with DT4AL and DT4AS aneuploid templates. Similarly, the physical map position of *Rht-A1* could be determined using chromosome 4A deletion bin stocks (Endo & Gill, 1996). Further mapping studies are being carried out by Wilhelm (NIAB, Cambridge, UK).

Assuming equivalent protein activity of *Rht-B1* and *Rht-D1* in the stems, the equal transcript contribution of *Rht-B1* and *Rht-D1* to the transcriptome is substantiated by the equivalent degree of dwarfism conferred by *Rht-B1b* and *Rht-D1b*, which both carry very similar mutations. Relative quantification used in this study caused an overrepresentation of *Rht*-A1, evident from analyses using the genomic DNA template. Thus, the relative level of *Rht-A1* compared to *Rht-B1* and *Rht-D1* expression cannot be inferred in the present study. However, the finding that *Rht*-A1 is expressed challenges the idea that the lack of *Rht-A1* mutants is due to *Rht-A1* not contributing to the transcriptome of this gene.

The involvement of *Rht-A1* in developmental stages was considered as another possible reason to explain the absence of *Rht-A1* mutants. Action of RHT-A1 may be required at a certain stage so that a mutation disrupting normal function may be lethal. Germination and stamen development were identified as important stages which in Arabidopsis have been shown to be controlled chiefly by the transcriptional regulation of a single DELLA gene. In Arabidopsis germination is controlled chiefly by the transcriptional regulation of *RGL2* (Lee *et al.*, 2002). In the present study it was demonstrated that all three homoeologues are transcriptionally regulated to the same extent during germination in wheat. However the specific activities of the RHT proteins are not known, so one may have higher specificity than the others in the control of germination. Similarly, RGL2 chiefly controls stamen development in Arabidopsis (Cheng *et al.*, 2004) whilst in wheat all three *Rht* homoeologues contributed.

2.4.5. **Environmental effects on *Rht* expression**

2.4.5.1. The effect of light

Plants grow in constantly fluctuating environments. The availability of light oscillates diurnally and through the seasons and plants rely on light for energy and to regulate their development. An appropriate growth response to light is therefore essential. Work in Arabidopsis has shown that the main components regulating growth in response to light are light activated phytochrome B (PhyB), growth promoting phytochrome interacting factor 4 (PIF4) and growth repressing DELLA proteins. Continuous light represses hypocotyl growth due to the photo-activation of PhyB causing its migration into the nucleus and targeting of PIF4 for proteasome degradation. Dark grown hypocotyls become etiolated because PIF4 is able to accumulate and directly activate the expression of genes involved in cell elongation. In the light DELLA proteins accumulate (due to a decrease in GA) and sequester PIF4 in an inactive DELLA-PIF4 complex. In the dark, DELLA is degraded (due to an increase in GA) enabling the accumulation of free (growth promoting) PIF4. As a consequence, Arabidopsis hypocotyls exhibit rhythmic elongation with the window of maximum growth at the end of the dark period (Nozue *et al.*, 2007). The peak of growth at the end of the night coincides with PIF4 accumulation and DELLA destabilisation. Interestingly hypocotyls of quadruple DELLA mutant plants (lacking four of the five DELLA genes) exhibit arrhythmic elongation, with growth occurring in the light, in which PIF4 is broken down, suggesting PIF4 independent hypocotyl growth also occurs (Veronica Arana, IBMCP, Valencia, Spain; pers. comm.).

In the present study, *Rht* expression showed diurnal regulation, peaking at the start of the light period. In contrast, Achard *et al.* (2007) found that *AtRGA* expression remained constant when light or dark grown plants were transferred to dark or light respectively over 24 hours, although transcript levels of GA metabolism genes were influenced markedly. The *GA2ox1* and *GA2ox2* (GA catabolising genes) transcripts rapidly decreased in the dark and rapidly increased in light, both within 1 hour of transfer, conversely *GA5* (a GA biosynthesis gene) rapidly decreased in the light and increased in the dark resulting in alterations in DELLA stability. Overall the data from studies in Arabidopsis suggest that DELLA protein stability, rather than transcription, is responsible for the rhythmic elongation observed and that this is influenced by alterations in GA levels which are regulated at the transcriptional level. In wheat, unlike Arabidopsis, rhythmic elongation was not observed during seedling growth therefore the oscillations of *Rht* transcript observed in seedling tissue may be buffered by alterations in RHT protein levels as observed in Arabidopsis resulting in linear growth.

Plant growth is slowed by unfavourable environmental conditions such as reduced temperature, partly as a result of a reduction of physiological and metabolic processes (Stitt & Hurry, 2002) but also, distinctly as part of a tolerance response that is mediated by DELLA (Achard *et al.*, 2008a).

Achard *et al.* (2008a) show that reduced growth rate in response to low temperature (4˚C) in Arabidopsis, at least in part, is due to the action of the cold inducible factor CBF1 which increases *GA2ox* expression resulting in a reduction in GA levels and an accumulation of DELLA protein. The authors therefore suggest that growth rate reduction is due to a reduction of DELLA degradation through the action of CBF1, however, interestingly the authors note that CBF1-ox lines (which over express CBF1) specifically enhance *RGL3* expression.

In the present study it was observed that the expression of all three *Rht* homoeologues was lower at 10°C than 25°C, suggesting that reduction of growth in response to lower temperatures in wheat is likely to be due to a stabilisation of RHT, as observed in the Arabidopsis orthologues. In accordance with the stabilisation of RHT at lower temperatures Reid *et al.* (1974) found that GA content of a winter wheat cultivar (cv. Kharkov) was much lower at 2˚C compared to 20˚C.
From growth measurements in this study it has been observed that lower temperature reduces coleoptile length differentially in seedlings containing different *Rht* mutant alleles. Through work carried out in this and other studies it has been shown that *Rht-B1c* is thought to encode a highly stabilised RHT-B1 protein, RHT-B1 and –D1 are partially stabilised in *Rht-B1b* and –D1b plants respectively, whilst *Rht-D1c* plants encode a partially stabilised RHT-D1 protein that is highly expressed.

In terms of coleoptile growth wild type plants are the most sensitive to decreasing temperature. The next most sensitive lines were those encoding partially stabilised RHT (*Rht-B1b*, *Rht-D1b* and *Rht-D1c*) that all showed a similar reduction in growth in response to decreasing temperature, whilst the coleoptile growth of *Rht-B1c* line was barely affected; possibly suggesting the latter is temperature insensitive. Addisu *et al.* (2009) also showed that wheat seedlings containing *Rht-B1c* are less affected by low temperature compared to those with the *Rht-D1c* allele. If RHT protein is stabilised in response to decreasing temperature, as observed for the Arabidopsis orthologues, then it is possible that *Rht-B1c* lines may be resistant to the growth rate reduction because RHT-B1c is not affected by increasing stability due to the protein being inherently stable.

My work has shown that shoot growth of Mercia *Rht-B1b* and *Rht-D1b* lines is greater than wild type lines at lower temperatures (5˚C). Bush and Evans (1988) observed that leaf areas are smaller in semi-dwarf than in *rht-tall* NILs at warm temperatures (24/19 ˚C), but larger at cool temperatures (15/10˚C), for example the leaf area of *Rht-B1b* + *Rht-D1b* plants at anthesis was 41% greater than that of the near isogenic *rht-tall* line (cv. Yaqui 50). The observation that growth of plants containing semi-dwarf alleles is greater than that of the *rht-tall* containing plants at lower

temperatures could be due to higher levels of endogenous GA in DELLA GoF lines (Peng and Harberd, 1993) resulting in increased rate of DELLA protein turnover at lower temperatures compared to wild type.

As observed previously the effects of dwarfing alleles is not reflected in root length. In contrast to observations of shoot growth all lines show a similar level of growth reduction in response to a reduction in temperature. The roots of severe dwarf lines are a similar length, whilst the roots of semi-dwarf lines are longer compared to wild type lines at all temperatures tested. It has previously been considered that the reduction in the effect of dwarfing alleles in root tissue is due to reduced expression of *Rht* in the roots and is also likely to reflect the reduced role of GA-mediated growth in the roots which involves other regulatory pathways, namely auxin. The increase of semi-dwarf lines compared to wild type lines may be a result of increased GA levels in these lines as described above which suggests GA regulated processes do contribute, albeit at a reduced capacity relative to shoot growth.

2.4.6. **The effect of heat shock on *Rht* expression**

DELLA stabilisation has been shown to increase tolerance to both salinity and cold stress in Arabidopsis (Achard *et al.*, 2006; Achard *et al.*, 2008a). Translational research lead by Boulton (JIC, Norwich, UK) has demonstrated that *Rht* GoF mutants in wheat also exhibit increased tolerance to salinity, and furthermore has demonstrated their increased tolerance to heat shock (unpublished). In the present study *Rht* expression was found to be reduced within one hour of heat treatment although transcription rapidly returned to levels in untreated plants once treatment ceased. Similarly, analysis of microarray data available through Genevestigator (Zimmermann *et al*. 2004) showed down regulation of all five DELLA encoding genes in response to heat shock, suggesting a commonality of response in cereals and Arabidopsis. However, in Arabidopsis GAI and RGL2 were more highly down-regulated than the other three *DELLA*s whereas in the present study no one *Rht* homoeologue in particular was more transcriptionally responsive to heat treatment in wild type lines.

The severe dwarf lines were heat treated alongside wild type lines in two separate experiments. *Rht* transcript reduction in response to treatment is evident in both mutant lines. In *Rht-B1c*, *Rht-B1* transcript is reduced relative to *Rht*-A1 and *Rht-D1* in untreated controls and heat treatment further reduces *Rht-B1* expression but not by as much as the other homoeologues. Whilst in *Rht-D1c* plants, the elevated *Rht-D1* expression is evident, at least in comparison to *Rht-B1* which I have found to be the better marker for relative quantification of expression, in all untreated controls, but after treatment *Rht-D1* contributes similarly to other homoeologues.

DELLA stabilisation as a result of salinity and cold stress have been shown to be a result of decreasing GA levels. The reduction of *Rht* expression in response to heat stress observed in this study would be consistent with treatment causing a stabilisation of DELLA protein, perhaps via a reduction in GA levels, resulting in the negative feedback observed. The severe dwarfs constitutively have higher levels of DELLA and therefore the plants are effectively primed for the onset of stress. It is thought that DELLAs promote survival of adversity in part by reducing the levels of reactive oxygen species (ROS; Achard *et al.*, 2008b). *BI-1* and *CEO* are both involved in the negative regulation of ROS induced cell death. Transcript accumulation of *BI-1* and *CEO* was measured in the heat treated material to see whether tolerance conferred by DELLA was a result of either gene being highly expressed. *CEO* expression did not increase in response to treatment relative to untreated controls however expression was not constant, even in untreated controls, possibly suggestive that the gene was responding to other challenges during the experiment. *BI-1* on the other hand was clearly responsive to heat treatment in all lines, with expression rapidly increasing (within 1 minute) but having returned to untreated levels within 24h. . Expression was increased more in heat treated *Rht-B1c* lines relative to *rht-tall* lines but no differences were observed between *Rht-D1c* and *rht-tall* lines. These latter data suggest that *Rht* may not directly determine the heat stress-dependent increase in expression of *BI-1*.

2.4.7. ***Rht* expression is only part of the story**

In the present study a homoeologue specific *Rht* assay has been developed enabling the quantification of transcription of a central growth regulatory gene in a spatial-temporal context and in response to environment. As described previously DELLA-mediated growth is regulated by additional means. The development of monitoring systems for additional aspects of DELLA-mediated growth i.e. GA biosynthesis and DELLA protein accumulation will allow a more complete picture of the regulation of DELLA mediated growth in wheat. The *Rht* expression assay provides a first step to understanding this regulatory pathway however the mRNA levels measured in this work are not necessarily correlative to RHT protein accumulation; additionally protein stability is influenced by GA levels and post translational modifications. Also the feedback and feed forward control between GA metabolism and signalling pathways makes dissection difficult in the complex genome of wheat. These factors necessitate the development of tools to measure all of the components of GA metabolism and signalling to gain greater understanding of GA mediated growth in wheat. A. Phillips *et al*. (RRes, Harpenden, UK) have been developing GUS reporter lines and homoeologue-specific qRT-PCR assays for each of the GA biosynthesis genes and Boulton *et al*. (JIC, Norwich, UK) have been generating RHT-B1-GFP fusion constructs for wheat transformation as part of a collaborative project. Additionally both groups have been trying to produce antibodies specific for RHT. However these monitoring systems were still in development

as this project finished, highlighting the difficulties of working with this pathway. For example, qRT-PCR assay development for the GA biosynthetic genes has been hindered by the low levels of expression (A. Phillips, pers. comm.), whilst difficulties with cloning the RHT-B1: GFP constructs arose due to the high level of secondary structure of the protein (Nadia Al-Kaff, pers. comm.)

2.4.8. **Conclusions**

The subfunctionalisation of *Rht*homoeologues by transcriptional regulation is not evident in the present study. All three homoeologues are expressed in all of the tissues, developmental stages and environmental conditions tested. The homoeologues are therefore unlikely to be regulated for specific roles as hypothesised in order to fulfil the function of the five DELLA proteins in Arabidopsis. Specific down regulation of *Rht-B1* transcription in *Rht-B1c* mutant allele lines, speculated to be a result of RHT-B1 negative feedback, would suggest independent homoeologue transcriptional regulation is possible. As *Rht* is expressed in all tissues tested, this suggests the RHT-1 protein is present so is likely to regulate GA responses in these tissues. It is not known whether subfunctionalisation of RHT-1 protein homoeologues has occurred i.e. altered affinity to bind downstream components such as basic helix loop helix containing transcription factors or directly to promoters. However, the protein sequence of the homoeologues is very similar and so this is unlikely. The data presented suggest that one DELLA gene is capable of controlling all of the GA mediated growth processes required in wheat, a theory supported by the presence of a single functional DELLA encoding gene in tomato (Bassel *et al*. 2004; Gallego-Bartolome *et al*., 2010) and most cereal and grass species (Ikeda *et al.*, 2001; Chandler *et al.*, 2002; Vogel *et al.*, 2010). However the discovery of DELLA like genes in the rice genome (Itoh *et al.*, 2005) may suggest additional proteins are required for fine control of GA mediated growth which are yet to be identified in wheat, a hypothesis discussed further in Chapter 3.

DELLA stability as influenced by GA metabolism and post translational modification is likely to play a significant role in GA mediated growth processes in wheat however growth regulation conferred by *Rht* at a transcriptional level has been demonstrated to be important in certain aspects of growth in the present study. For example the progression of germination coincided with a reduction in *Rht* transcription in response to seeds being transferred to a favourable environment. Thus transcriptional regulation of *Rht* may provide an additional node of growth control when a rapid response to environmental conditions is required.

The development of a homoeologue-specific assay has revealed that differential *Rht* homoeologue expression is conferred by the previously uncharacterised severe dwarf alleles, *Rht-B1c* and *Rht-D1c.* The molecular basis of the mutations of *Rht-B1c* and *Rht-D1c* have been presented. As a

consequence of characterisation *Rht-B1c* and *Rht-D1c* can be used as models for the effects of increased protein stability and increased transcription of *Rht* respectively.

3. Exploration for an additional *Rht* member; Are there additional copies of *Rht* in wheat?

3.1. Introduction

DELLA encoding genes have been well characterised using the model plant, *Arabidopsis thaliana* because of their integral role in plant development but also in crops because of the agronomic potential of height reduction in a variety of crop species.

The Arabidopsis genome contains a family of five DELLA encoding genes, *GAI, RGA, RGL-1, -2, -3,* (Peng *et al.*, 1997; Silverstone *et al.*, 1998; Wen & Chang, 2002) which perform distinct but also overlapping roles in plant development. For example, GAI and RGA are the main regulators of cell expansion in vegetative tissues. This was demonstrated by the restoration of a near normal growth phenotype in *ga1-3* (GA deficient) plants lacking both GAI and RGA (Dill & Sun, 2001; King *et al.*, 2001). In contrast, RGL-2 is the main regulator of germination because knockout mutations of RGL-2 in *ga1-3* restore germination while all other DELLA hnockout mutations do not (Lee *et al.*, 2002). In contrast to Arabidopsis, most cereal species contain only a single DELLA gene. Rice and barley encode SLR1 and SLN1, respectively, which are the orthologues of GAI (Ikeda *et al.*, 2001; Chandler *et al.*, 2002). In wheat, the group 4 *Rht* homoeologous genes are the only known DELLA encoding genes in the genome. How is it that a single gene in wheat can control developmental processes that, in Arabidopsis, require five distinct DELLA encoding genes? Spatial and temporal transcriptional regulation of the three copies of the single DELLA encoding gene is a possibility, as discussed in chapter 2. Another possibility, considered in this chapter, is the presence of additional DELLA encoding genes in the wheat genome.

A candidate for an additional DELLA encoding gene was suggested following SSCP analysis of DNA from group 4 nullisomic-tetrasomic lines using *Rht* specific primers (personal communication, Bottley, JIC, Norwich, UK). In this gel each of the group 4 *Rht* products disappeared in the respective nullisomic-tetrasomic DNA templates, but in all templates one product remained (Fig. 3.1a). Subsequently, Bottley (JIC, Norwich, UK) located the product to the short arm of chromosome 7D (Fig. 3.1b) and sequence analysis of the 381bp product showed it to be similar to the group 4 *Rht* genes (Fig. 3.2).

Dwarfing genes have been pivotal for increasing yield in wheat. The 1960's brought massive yield increases thanks in part to the reduction in stem height conferred by the allelic variants of the group 4 *Rht* genes; *Rht-B1b* and *Rht-D1b*. *Rht-B1b* or *Rht-D1b* are present in the majority of commercial cultivars (Worland *et al.*, 1998). However the semi-dwarfing (GoF) alleles have been reported to carry negative pleiotropic and linkage effects. For example, their seedling vigour and coleoptile length is reduced compared to wild type lines meaning, *Rht-B1b* and *Rht-D1b* containing cultivars

have to be sown in shallow drills. Also, the *Rht-B1b* allele confers increased susceptibility to *Mycosphaerella graminicola* (Baltazar *et al*., 1990), whilst the *Rht-D1b* allele appears to be linked to a nearby gene thought to be a *Fusarium graminearum* susceptibility factor (Srinivasachary *et al*., 2008) carried as a linkage block into modern day *Rht-D1b* cultivars which has, to my knowledge, yet to be broken. In contrast, GoF *Rht* alleles confer increased resistance to the necrotrophic pathogens *Oculimacula acuformis* and *O. yallundae*, and increase type 2 resistance (resistance to spread) to *Fusarium graminearum* (as discussed in chapter 4) as well as tolerance to abiotic stresses (Boulton, JIC, Norwich, UK, unpublished data).

Rht 8 is an alternative GA sensitive dwarfing gene present on chromosome 2DS (Korzun *et al*., 1998). The *Rht* 8 allele is used as a source of dwarfing in drier environments where seed are sown deeper to facilitate access of the germinating seed to limited water. The Group 4 *Rht* semi-dwarfing genes are not suitable for this agronomic practice as the coleoptile length is reduced. The coleoptile length of the *Rht* 8 containing cultivars is equivalent to the wild type; dwarfism is only evident later in plant development, during culm extension.

Discovery of novel loci controlling height will allow breeders to tailor the selection of alleles to those which may be better suited to particular environments and disease pressures, furthermore the characterisation of additional DELLA encoding genes could clarify whether a single DELLA encoding gene regulates all developmental processes in wheat. In this chapter I have attempted to isolate the novel candidate height gene.

3.2. **Materials and Methods**

3.2.1. **Phylogenetic analysis**

Sequences of GRAS gene family members from a variety of plant species were retrieved from the literature and by using Basic Local Alignment Search Tool (BLAST; (Altschul *et al.*, 1997)) searches of the NCBI database with *AtGAI* nucleotide sequence. The C terminal region of the collected sequences which corresponds to the known sequence of the 7D candidate gene were aligned using the ClustalW program followed by manual alignment using BioEdit (Tom Hall, Ibis Therapeutics, Carlsbad, CA, USA). The alignments were analysed using the neighbour joining method and performed by Phylogenetic Analysis Using Parsimony (PAUP) software (Swofford, 2000). Bootstrap values were calculated to statistically support the tree. The tree was rooted using the C-terminal region of a member of the signal transducers and activators of transcription (STAT) family of proteins (STAT2P562), which are structurally and functionally similar to the GRAS proteins (Richards *et al.*, 2000).

3.2.2. **Bacterial Artificial Chromosome (BAC) library**

The *Triticum aestivum* cv. Chinese Spring BAC library was constructed in a collaborative project between BBSRC (UK) and INRA (France) (Allouis *et al.*, 2003). The complete library consists of 1,200,000 clones with an average insert size of 130 Kb which provides 9.3X coverage of the hexaploid wheat genome. The library has been subdivided into two components, which are referred to as the UK and French libraries. The French component was screened in this study; it provides approximately 3X coverage on 26 high density colony filters.

3.2.3. **Probe Preparation**

Using the primers listed in figure 3.3 two products were amplified from Chinese Spring gDNA template to be used to probe the BAC library. Rht-16_F and Rht-11_R amplify a 195 bp product which spans a conserved region of the DELLA motif of the chromosome 4 *Rht* homoeologues, refered to as Generic *Rht*. MRH F2 and MRH R2 (7D candidate gene specific primers) amplify a 133 bp product refered to as *Rht-7D*. The PCR conditions were as follows: initial denaturation at 95 ºC for 5 minutes, 30 cycles of 94 ºC for 30 s, 62 ºC for 30 s and 72 ºC for 30 s, then a final incubation of 72 ºC for 10 minutes.

Products were separated on a 1% agarose gel stained with ethidium bromide to ensure the amplicons were of the correct size. To ensure the correct product had been amplified, probes were purified using the Qiagen PCR purification kit as described in the manufacturer's instructions.

Products were prepared for sequenceing using BigDye® (Applied Biosystems) and analysis of reaction products was performed by The Genome Analysis Centre, Norwich.

Probes were radiolabelled with the Rediprime ll DNA labelling system (GE Healthcare Life Sciences). 20 ng of the probe DNA (section 3.2.3) was diluted in water to a final volume of 45 µl and denatured at 95 °C for 5 minutes. The denatured probe was added to the Rediprime reaction tube (containing dATP, dGTP, dTTP, Klenow DNA polymerase and random hexanucleotide primers) and 3 µl of P^{32}-dCTP was added and incubated for 2 hours at 37 °C. Finally, 2.5 µl of 4M NaOH was added to denature the DNA and stop the reaction.

3.2.4. **Hybridisation screening of the BAC library**

Filters containing the French portion of the Chinese Spring BAC library were pre-hybridised in Church and Gilbert (1984) hybridisation buffer (15 ml 1M Na_2HPO_4/1M NaH_2PO_4 solution, 60 µl of 0.5M EDTA pH 8, 10.5 ml of 20% SDS in a total volume of 30 ml) at 65 °C for 2 hours with agitation.

The labelled probes (a pool of probes containing the generic *Rht* and *Rht-7D* probes with additional probes unrelated to this project) were added to the pre-hybridisation buffer and the filters incubated at 65 °C overnight with agitation.

The filters were transferred to clean containers and washed once in wash l buffer (2x SSC, 0.5% SDS in a total volume of 2L) then washed one to three times in wash ll buffer (0.5x SSC, 0.5% SDS in a total volume of 2L) depending on the intensity of radioactivity remaining on the filters. Washing steps were carried out at 65 °C for 15 minutes under agitation, wash buffers were preheated to 65°C.

The filters were wrapped in Saran wrap™ and exposed to phosphorimager screens overnight. The exposed films were scanned using a phosphorimager (Typhoon 8600, Amersham Biosciences).

3.2.5. **Preparation and screening of the BAC sub-library**

To prepare a sub-library of BAC clones identified as containing *Rht* sequence by hybridisation, selected clones were picked from glycerol stocks and transferred to 96-well plates. Each well contained 200 µl of Lauria Broth (LB) medium with chloramphenicol (CAM; 12.5 µg/ml). The bacteria were incubated overnight at 37 °C with shaking. The bacterial cultures were transferred onto Hybond-N+ membrane (Amersham Biosciences) using a 96-pronged hedgehog device, placed on LB agar and incubated overnight at 37 °C. The membrane was processed through the following steps: Denaturation solution (0.5 M NaOH, 1.5 mM NaCl) for 7 minutes; Neutralisation solution (1.5 mM NaCl, 0.5 M Tris, pH 7.6) for 7 minutes; air drying on Whatman paper for 1 hour; 0.4 M

NaOH for 20 minutes; 2 x SSC for 7 minutes and air dried overnight. Dry filters were placed between two sheets of Whatman paper and baked at 80°C for 1 hour. 2 sets of replica filters were prepared for this project.

The filters were pre-hybridised, probed, washed and developed as described in section 3.2.5. except that the *Rht-7D* and Generic *Rht* probes were used separately and no additional probes were included.

3.2.6. **Preparation of the sub-clone library**

The TOPO® shotgun sub-cloning kit (Invitrogen) was used to construct two sub-clone libraries from two clones selected in section 3.2.4. Unless stated otherwise, the reagents and disposables used in this section were provided in the kit

Plasmid DNA was extracted from the selected BAC clones using the Plasmid Midi kit (Qiagen) according to the manufacturer's instructions.

Plasmid DNA (6.5 µg) was added to 750 µl of shearing buffer and transferred to a nebulizer kept on ice. DNA was fragmented by passing compressed air through the nebulizer unit for 20 s maintaining pressure at 10-30 psi. The sheared DNA was cleaned by isopropanol precipitation and resuspended to a concentration of 100-200 ng/µl in sterile distilled water.

Fragmented DNA (~3 µg in 35 µl deionised water) was incubated at room temperature for 30 minutes after adding the following solutions: 5 µl 10 x blunting buffer, 1 µl BSA, 5 µl dNTP mix, 2 µl T4 DNA polymerase and 2 µl Klenow DNA polymerase. Incubation at 75°C for 20 m was used to inactivate the enzymes.

To the blunt-end repaired DNA fragments, 35 µl sterile water, 10 µl 10X dephosphorylation buffer and 5 µl calf intestinal phosphatase was added before incubating at 37 °C for 60 minutes. The DNA was cleaned with a phenol: chloroform extraction followed by an ethanol precipitation step and the pellet was resuspended to a concentration of 50-100 ng/µl in sterile distilled water.

Blunt-end DNA (~100 ng) was ligated into pCR®4Blunt-TOPO® vector by incubating for 5 minutes at room temperature.

Electrocompetent cells (20 µl; One shot® Top10, Invitrogen) were transformed with ligated plasmid (6 µl) by electroporation (electrical pulse of 400 V and a capacitance of 370 µF). The cells were quickly transferred into 1 ml of SOC medium and incubated at 37 °C for 1 hour under agitation. 100 µl of culture was spread onto X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside) selective LB agar plates and incubated at 37 °C overnight. A total of 480 white (insert-containing) clones were selected for each of the two original BAC clones for subsequent screening.

3.2.7. **Screening of the sub-clone library**

3.2.7.1. PCR amplification with 7D-specific primers

The 480 clones selected from the BAC E11 sub-library was screened by colony PCR. Bacterial colonies were transferred to a 96 well plate, containing 50 µl of sterile distilled water, with a toothpick. 1 µl of the resuspended culture was used for PCR amplification with 50 ng each of primers MRH F2 and MRH R2 (Fig. 3.3c.), 1 mM dNTPs, 2 µl of 5X Go Green Flexi Buffer (Promega), 0.6 µl 100% glycerol, and 0.1 µl of lab-produced Taq (unknown concentration) made up to 10 µl with sterile distilled water. Amplified products were separated on a 1% agarose gel stained with ethidium bromide and clones were selected if a product of the size expected following amplification of *Rht* 7D was present. Clones from which the product was obtained were restriction digested, sequenced and analysed as described below.

3.2.7.2. Colony Hybridisation

The subclone libraries of both BAC clones were screened by Southern hybridisation. Filters were prepared as described in section 3.2.5 and screened using the *Rht-7D* and Generic *Rht* probes (section 3.2.3) as described in section 3.2.6.

3.2.7.3. Characterisation of inserts in selected sub-clones

DNA was isolated from each of the clones that hybridised to the probes using a miniprep kit (Qiagen). Plasmid insert size was estimated by either PCR amplification of the insert or restriction digest of the miniprep DNA. The inserts were amplified using M13 primer binding sites which flank the insertion site or digested using *Eco*R1 sites of which flank the insertion. Amplification or digestion products were separated on a 1% agarose electrophoresis gel containing ethidium bromide. Insert size was estimated relative to a marker ladder. Products were transferred to a nylon membrane for Southern hybridisation as described by Sambrook (2001). The positive sub-clones were prepared for sequencing directly from the plasmid DNA using BigDye® (Applied Biosystems) and analysis of reaction products was performed by The Genome Analysis Centre, Norwich. Sequences were assembled in Vector NTI contig express (Invitrogen) and were used to interrogate the NCBI database using the BLAST tool. Two clones were selected for sequence analysis of the entire insert. This was obtained by "primer walking" - using successive sequencing reactions, each time basing primer design on newly acquired sequence.

3.3. **Results**

3.3.1. **Phylogenetic analysis places *Rht-7D* in cereal DELLA clade.**

Phylogenetic analysis can be used to test for relatedness within a genome (paralogs) in addition to looking at evolutionary relatedness and was performed to assess the relatedness of the 7D candidate gene with the group 4 *Rht* homoeologues. GRAS related genes from a variety of plant species were included in the analysis and a strict consensus tree was constructed from 585 trees. A total of 212 characters were analysed, of which 182 were informative. The resulting dendrogram is shown in Fig. 3.4. DELLA encoding genes grouped in a clade distinct from the other GRAS containing genes with a highly significant bootstrap value of 88. A cereal DELLA sub-clade was recognised within the DELLA group, with a highly significant bootstrap value of 100. The candidate DELLA on chromosome 7D, *Rht-7D*, groups within the wheat/barley DELLA sub-cluster with a reduced but still significant bootstrap value of 69 (significance = ≥50). The rice slender-like genes (*OsSLRL1* and *2 (Itoh et al., 2005)*) form a clade, close to, but distinct from the DELLA clade.

3.3.2. **BAC library screening**

The screening of the BAC library with the *Rht-7D* probe (MRHF2/R2 amplicon) revealed no positive hybridisation signals, in contrast, a hybridisation signal was obtained for 73 clones with the generic *Rht* probe (Rht16F/11R amplicon). Of these 73 clones, 11 produced a strong hybridisation signal, and 62 produced a weaker signal (data not shown). Clones producing the strong signal (11) and 25 clones which produced a weaker signal were selected for further analysis.

The DNA of the 36 candidate DELLA clones were PCR-screened using Rht-7D specific primers (MRHF2/R2; Fig. 3.3), to test whether any contained the 7D candidate sequence, on the assumption that a DELLA encoding gene would contain a DELLA motif and so would be represented in the positive clones selected with the DELLA probes. Separation of the products on an electrophoresis gel (Fig. 3.5a) revealed that two of the selected BAC clones (E11 and M13) contained the predicted 133 bp product. Both BAC clones had produced a weak signal when hybridised with the generic *Rht* probe. Blotting of the electrophoresis gel and subsequent hybridisation with the *Rht-7D* probe resulted in strong hybridisation of both of these products (Fig. 3.5b). In addition, several other clones produced a hybridisation signal, with bands of the expected size. However, the non-template controls (ntc) also produced a hybridisation signal. This reflects the sensitivity of the technique, in which the probe is binding trace amounts of the products of endpoint PCR. Due to concerns of gDNA contamination, the PCR screening was repeated with the E11 and M13 BAC clone templates confirming amplification of the 133 bp product (data not shown). To allow further characterisation of these clones, sheared DNA of the two BAC clones

was sub cloned and the clones obtained shown to contain inserts of 1 to 5 kb (compared to the 45 to 350 kb insert size (Allouis *et al.*, 2003) of the original Chinese Spring BAC library clones).

3.3.3. **Sub-clone library screening**

Two methods were adopted for screening the sub-clone libraries, colony PCR (for BAC sub-library E11) and colony hybridisation. Following colony PCR using Rht-7D specific primers (MRHF2/R2; Fig. 3.3) a 133 bp product was amplified from 19 clones. No hybridisation was seen for any of the 960 clones probed using the Rht-7D probe, whereas the generic *Rht* probe hybridised to 8 clones. None of the positive clones were identified by both techniques.

Using the M13 primers, approximately 1 kb of sequence from left and right plasmid borders was obtained for all 19 sub-clones but BLAST annotation (Table 3.1, sequence data in Appendix 7.2) showed that 30% of the positive sub clones selected by the generic *Rht* probe contained *E. coli* sequence. The remaining clone inserts were all identified as *T. aestivum* sequence. The most frequently retrieved annotation for both E11 and M13 derived sub clone inserts was 3B specific BAC library contigs (37% of the clones). Of the remaining clones nothing suggested a chromosome 7 location or a DELLA-like sequence based BLAST annotation alone.

Two sub-clones, E11-171 and M13-48, were selected as the best candidates for sequencing the entire insert because of their strong hybridisation signal with the generic *Rht* probe in the initial screen of the sub clone library (Fig. 3.6a & b), the relatively large size of the inserts (approx. 5.5 and 4 kb respectively Fig. 3.6c), and the hybridisation of a digested fragment to the generic *Rht* probe (Fig. 3.6d). Additionally, BLAST annotation of the initial sequence of both sub clones suggested homology to '3B specific BAC library contigs' which, on the basis that many clones had homology to this sequence may have been linked to the target sequence (Rht-7D). Forward and reverse reads of the E11-171 clone overlapped after four rounds of 'primer walking', however sequence from the forward and reverse reads of M13-48 did not, despite the fact that the total retrieved sequence (5497 bp) was more than the size of the insert (4000 bp) estimated by restriction enzyme digestion,suggesting the two insert sequences derived from the M13-48 sub-clone are in fact from two separate sub clones.

BLAST annotations suggest that BACs E11 and M13 both contain sequence originated from chromosome 3BS. Comparison of the sequences of the E11_171 insert and the Rht-7D probe using the NCBI BLAST 2 sequences alignment tool (Tatusova & Madden, 1999) to identify regions of homology showed short regions (10-30 bp) of high homology which were GC rich (70-80%). The generic *Rht* probe has 73% GC content and this could have resulted in non-specific hybridisation to E11_171 and perhaps to the other selected sub clones, and by inference, the BAC clones E11 and M13.

3.4. **Discussion**

The constitutive GA response phenotypes of *slr1* and *sln1* (LoF) mutants of rice and barley (Ikeda *et al.*, 2001; Chandler *et al.*, 2002) suggest that they encode single DELLA proteins. Further analysis of the assembled rice genome sequence identified two additional genes, *SLR1-like 1* and *-2* (*SLRL1* and *-2*), similar to *Slr1* but lacking the N-terminal, GA responsive DELLA domain (Itoh *et al.*, 2005). The authors demonstrate that the genes encode products that are non-responsive to GA and that they repress growth, and suggest that the SLRL genes may function as a fine control mechanism of growth in rice. Itoh *et al*. (2005) also identified a DELLA-like gene in maize suggesting a single DELLA encoding gene alone is not sufficient to control GA-regulated growth processes in rice and maize. Database searches of the *Brachypodium distachyon* genome (http://www.modelcrop.org) show that a single DELLA encoding gene is, located on a region of chromosome Bd1 collinear with the short arm of the group 4 chromosomes of wheat. No DELLA-like genes have been reported in barley, although the complete genome sequence is not yet available. Experimental work carried out in this chapter failed to identify an additional *Rht* gene from the wheat genome. However, evidence suggests an *Rht* gene may be present on chromosome 7DS, with possible homoeo-loci on other group 7 chromosomes.

Many studies have located height QTLs on the group 7 chromosomes of wheat, for example, Röder *et al*. (2008) identified a grain weight QTL designated *gw1*, also associated with plant height, located on chromosome 7DS using introgression lines of synthetic wheat W-7984 in the genetic background of Prinz. Lines carrying the introgressed segment of Prinz chromosome arm 7DS had reduced height, and reduced grain weight, although the authors did not conclude whether the association between these traits were a result of pleiotropy or linkage. Huang *et al*. (2004) also identified a QTL for plant height in the same region in a population derived from crossing cultivar 'Flair' with XX86. Additionally, QTL for lodging resistance, for which short stature is a key determinant, were located on 7D (arm not specified) along with the group 4 *Rht* loci (Verma *et al.*, 2005). Cadalen *et al*. (1998) identified a marker associated with plant height that has been mapped to 7DS, although this locus was not consistently present across multiple experiments, the plant height effect may have been hidden by other height reducing loci of greater effect in this population. These mapping studies have provided evidence of potential height reducing loci present on the short arm of chromosome 7D however the gene(s) responsible have yet to be isolated.

GA sensitivity tests on the Prinz-W-7984 introgressed lines (Röder *et al*., 2008) show that lines containing the Prinz 7DS introgressed segment are GA insensitive, but due to the presumed presence of *Rht-D1b* in Prinz, no conclusions can be drawn as to whether the 'Prinz' *Rht* gene on 7DS is GA sensitive or insensitive. Ent-kaurenoic acid oxidase (KAO) encoding genes have been located on the short arms of chromosomes 7A and 7D (Spielmeyer *et al.*, 2004) which represent

possible GA sensitive height reducing loci on the group 7 chromosomes. Khlestkina *et al*. (2010) used the Prinz x W-7984 population mentioned above to partially clone and map a homoeoloci set of KAO encoding genes to 7AS, 4AL and 7DS , corresponding to the 7BS/4AL translocation region. However KAO3, which mapped to 7DS, was 50 cM distal to the 'Prinz' *Rht* gene identified by Röder *et al*. (2008).

The GA sensitivity tests on the Prinz-W-7984 introgressed lines were inconclusive due to the presence of *Rht-D1b* in the Prinz background. By crossing the Prinz 7DS introgressed segment into a background containing wild type *Rht-1* alleles GA sensitivity could be assessed in the absence of GA insensitive alleles and if a GA insensitive phenotype resulted then the 7D candidate gene (Rht-7D) sought in this study, which shares similarities with the group 4 *Rht* (DELLA) encoding genes, would be a good candidate.

As part of the PhD project of Wilhelm (NIAB, Cambridge, UK), 73 clones were isolated from the CS BAC library by Southern hybridisation with a DELLA motif spanning probe (generic *Rht*) in order to isolate *Rht-A1*, *B1* and *D1* containing BACs. Of the 73 clones, 11 produced a strong hybridisation signal, but when they were screened by PCR using a generic set of *Rht* primers only two contained the group 4 *Rht* target gene. Both of these clones were derived from the D genome (Febrer *et al.*, 2009). The remaining clones were screened for presence of the candidate gene, Rht-7D, in the present study. The lack of specificity of the DELLA spanning probe coupled with the limitations of hybridisation as a technique are evident from the low number of *Rht-1* containing BACs recovered and the high number of false positives selected in the study by Wilhelm (NIAB, Cambridge, UK). Non-specificity of the probe was also evident from the high number of false sub-clone positives retrieved in the present study. This is likely to be due to the high GC content (>70%) of the short probe which will hybridize strongly to regions of the target with high GC content.

In contrast, screening of the same CS BAC library by PCR (described by; Febrer *et al.*, 2009) using DELLA specific primer sets Wilhelm identified 13 group 4 *Rht* containing clones. This screening method may offer a more practical approach to isolate the 7D candidate gene. Alternativly, using the sequence data available for the 7D candidate gene, sequence specific primers could be designed to undertake Rapid Amplification of cDNA Ends (RACE) enabling the whole gene to be sequenced. If isolated, Prinz will be a useful cultivar in which to identify a potential allelic variant.

The rice expressed sequence tag (EST), D39460, encodes a potential polypeptide containing a sequence nearly identical to the 17 amino acids near the amino terminus of GAI, that when knocked out results in GA insensitivity in Arabidopsis(Peng *et al*., 1997). Peng *et al*. (1999) isolated clone C15 from wheat complementary DNA using EST D39460 and hybridised the probe to *Dra*1-digested DNA from the nullisomic-tetrasomic series and the euploid control (Chinese

Spring) in order to locate DELLA encoding genes in the wheat genome. Digestion products were absent in each of the corresponding group 4 nullisomic lines therefore locating the *Rht* genes to the group 4 chromosomes. The remaining product bands not assigned to the group 4 chromosomes did not disappear in any of the remaining nullisomic lines, including group 7 (pers. comm. P. Nicholson) suggesting that if an *Rht* gene is present on chromosome 7DS it is unlikely to encode the 17 amino acids essential for GA sensitivity. In this case the 7D candidate gene could encode a DELLA –like protein orthologous to SLRL1 and 2 in rice.

The complete assembled sequences of *B. distachyon* and *Sorghum bicolour* coupled with the collinearity that exists between grass species (Moore *et al.*, 1995) provide a useful resource for wheat genome analysis. The *SLRL1* and *SLRL2* genes, identified in the rice genome database are located on the long arm of chromosome 1 and 5 respectively (Itoh *et al.*, 2005). These genes are likely to be duplicates, arising from the reported duplication event of rice chromosome 1 and 5 (Kurata *et al.*, 1994). Analysis of the *B. distachyon* and *S. bicolour* databases with the *SLRL2* sequence revealed multiple hits. Additional to the 'true' DELLA genes, on chromosome 1 and 8 of *B. distachyon* and *S. bicolour* respectively, sequence possibly representing DELLA like genes was also retrieved. Two genes in particular, g45120 and g029470, present on *B. distachyon* chromosome 2 and *S. bicolour* chromosome 3 respectively, showed a high level of similarity across most of the *SLRL2* gene. Further analysis of the collinearity between these gene locations shows rice chromosome 1, on which *Slrl1* resides, is collinear to both chromosome 2 in *Brachypodium* and chromosome 3 in *Sorghum* (Vogel *et al.*, 2010). The main regions of collinearity to wheat are on chromosomes 1 and 3. Collinear regions of wheat chromosome 7DS, on which the Rht-7D candidate gene sought in this study have been located, are chromosomes 1 and 3 in *Brachypodium* and 6 in rice. Bioinformatic analysis would therefore suggest that the Rht-7D candidate is unlikely to encode a DELLA-like protein.

Analysis of the Arabidopsis genome revealed no homologous sequences to SLRL (that lack the DELLA domain) genes in rice. Rice and maize encode DELLA-like genes (Itoh *et al.*, 2005) and from the database analysis in this present study there is evidence that *Brachypodium* and *Sorghum* also encode DELLA-like genes, therefore it may be that DELLA like genes exist in monocots, which generally encode a single 'true' DELLA, but not in dicots which generally encode multiple DELLAs. Whether wheat encodes DELLA-like genes is yet to be established but on the basis of wheats shared collinearity with other grass species and the bioinformatics data presented above it is likely, but unlikely to be the Rht-7D candidate gene investigated in this chapter, which would explain why rice SLRL genes were distinct from the Rht-7D candidate gene in the phylogenetic analysis.The complete assembly of the wheat genome sequence approaching this will serve to facilitate the identification of any additional DELLA (and DELLA-like) encoding genes present in the wheat genome.

The objective of this chapter was to isolate an additional *Rht* family member thought to reside on chromosome 7DS, based on initial evidence from Bottley. Ultimately this objective failed. Present evidence suggest that cereal genomes are likely to contain only a single DELLA encoding gene, however using fully sequenced cereal genomes Itoh *et al*. (2005) provide evidence that certain cereal species (rice and maize) encode additional DELLA-like proteins and database interrogation of *B. distachyon* and *S. bicolour* genome sequence in this present study also imply the presence of DELLA-like genes in these genomes. The wheat genome is likely to contain DELLA-like genes, on the basis of collinearity with these fully sequenced grass species, but these are unlikely to represent the Rht-7D candidate gene sought in the present study. Many plant height QTL have been reported on the short arms of group 7 chromosomes, and although many genes affect plant height the location is coincident with the Rht-7D candidate gene. Further work, as suggested above, is required to identify whether the candidate gene on 7D encodes an additional functional DELLA protein in the wheat genome. If identified it could provide useful, novel alleles for plant height reduction in commercial wheat cultivars.

4. The 'Green Revolution' dwarfing genes play a role in disease resistance in *Triticum aestivum* and *Hordeum vulgare*

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4.1. Introduction

The plant growth hormone gibberellic acid (GA) has a central role in the complex network of hormones responsible for the regulation of plant growth and development (Santner & Estelle, 2009). DELLA proteins are nuclear localised repressors of growth that are core components of the GA signal transduction pathway (Peng *et al.*, 1997). In the presence of GA the soluble GA receptor, GIBBERELLIN INSENSITIVE DWARF1, GID1 (Ueguchi-Tanaka *et al.*, 2005) interacts with DELLA resulting in a conformational change of the DELLA protein. This enables DELLA to interact with the F-box protein SLY1/GID2, leading to the polyubiquitylation, and subsequent degradation of DELLA protein by the 26S proteasome (Ueguchi-Tanaka *et al.*, 2007). The *Arabidopsis thaliana* genome contains five DELLA genes that encode distinct proteins (GAI, RGA, RGL1, RGL2 and RGL3; (Silverstone *et al.*, 1998; Peng *et al.*, 1999; Wen & Chang, 2002). Recent studies in Arabidopsis have implicated DELLA proteins in resistance to biotic stress (Navarro *et al.*, 2008), suggesting that in addition to their role in plant development, DELLA encoding genes have a role in disease resistance.

Depending on their mode of infection, plant pathogens can be broadly classified into three trophic lifestyles; biotrophs, necrotrophs and hemibiotrophs. Biotrophs derive nutrients from living cells whilst necrotrophs kill host cells in order to derive energy (Lewis, 1973). A Hemibiotrophic pathogen requires an initial biotrophic phase before switching to necrotrophy to complete its life cycle (Perfect & Green, 2001).

A plant subject to pathogen attack is required to respond appropriately. An interplay between the phytohormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) activates distinct defence pathways, depending on the lifestyle of the invading pathogen (Glazebrook, 2005). The antagonistic cross-talk between the SA and JA/ET signalling pathways enhance biotroph and necrotroph resistance respectively. Navarro *et al*. (2008) suggested that DELLA proteins differentially affect responses to biotrophic and necrotrophic pathogens through their influence on the SA-JA balance. Accumulation of DELLA results in potentiated JA signalling, and consequently a dampening of SA signalling. Accordingly, resistance to necrotrophs in Arabidopsis is enhanced, and resistance to biotrophs reduced, in DELLA accumulating mutants.

In contrast to Arabidopsis, monocot cereal species appear to contain a single DELLA encoding gene (Peng *et al.*, 1999; Chandler *et al.*, 2002). The *Reduced height* (*Rht*) gene of *Triticum*

aestivum (bread wheat) and the *Slender 1* (*Sln1*) gene of *Hordeum vulgare* (barley) are both orthologous to *GAI*. Mutations disrupting the conserved DELLA domain, essential for GID1 interaction, reduces the susceptibility of DELLA to GA-induced degradation (Peng *et al.*, 1997; Chandler *et al.*, 2002). Increased DELLA stability results in a dwarf phenotype, a trait which has been exploited by wheat breeders through introduction of the semi-dwarfing genes, *Rht-B1b* and *Rht-D1b*, into modern wheat cultivars, which underpinned the so-called 'Green Revolution' in wheat (Hedden, 2003).

Reduced height has been associated with increased susceptibility to splash dispersed pathogens (Vanbeuningen & Kohli, 1990; Eriksen *et al.*, 2003; Gervais *et al.*, 2003; Draeger *et al.*, 2007; Klahr *et al.*, 2007) which has been thought to be due to the reduced distances between consecutive leaves facilitating progress of the pathogen up the stem (the "ladder effect" (Bahat *et al.*, 1980)) and alterations in canopy structure providing a micro-climate more favourable for pathogen establishment (Scott *et al.*, 1982; Scott *et al.*, 1985). However, not all plant height QTL are coincident with those for pathogen susceptibility, as has been demonstrated with fusarium head blight (FHB, Draeger *et al*., 2007) suggesting resistance is not an effect of height *per se* but rather of linkage or pleiotrophy. The *Rht* loci on chromosome 4B and 4D which encode DELLA proteins are coincident with FHB resistance loci suggesting that they may have a pleiotropic effect on susceptibility to FHB (Srinivasachary *et al.*, 2009).

In this chapter, I report the effect of DELLA on responses to pathogens with different trophic lifestyles in cereals. I assessed the relative resistance of both wheat and barley lines differing in DELLA status against several cereal fungal pathogens representing each of the three classes of pathogen lifestyle and collate wth data from experiments in the *Fussarium* pathosystem carried out by Gosman (JIC, Norwich, UK). The effect of DELLA gain of function (GoF) and loss of function (LoF) mutants was studied using barley and the effect of GoF mutants of differing severity in a polyploid genome was investigated using wheat.

4.2. **Materials and Methods**

4.2.1. **Plant material**

Near-isogenic lines (NILs) of wheat (*Triticum aestivum*) varieties Mercia and Maris Huntsman differing in the alleles at the *Reduced height* (*Rht*) loci on chromosome 4B and 4D were kindly supplied by Dr. J. Flintham of the John Innes Centre, Norwich, England. The semi-dwarf GoF alleles *Rht-B1b* and *-D1b* (formerly *Rht1* and *2*) and the severe dwarf alleles *Rht-B1c* and *-D1c* (formerly *Rht3* and *10*) are dominant gibberellin (GA) insensitive alleles, thought to accumulate DELLA to higher levels than wild type. The wild type (*rht-tall*) parental lines carry GA sensitive alleles at all three homoeologous loci (Flintham *et al.*, 1997). Barley (*Hordeum vulgare*) variety

Himalaya, the dwarf (GoF) mutant (*Sln1d*) and the constitutive growth (LoF) mutant (M770) were kindly supplied by Dr. P. Chandler of CSIRO, Canberra, Australia. M640 carries a dominant GA insensitive allele (*Sln1d*) at the *Slender 1* (*Sln1*) locus that is orthologous to *Rht* of wheat (Chandler *et al.*, 2002). *sln1c* arises from an early termination codon at the *Sln1* locus, resulting in a truncated protein lacking the COOH-terminal 17 amino acid residues. This loss of function allele has been designated *sln1c.* In the homozygous state, the loss of functional DELLA results in male sterility and therefore homozygous *sln1c*/*sln1c* plants have to be selected in a 1:3 ratio from a heterozygous parent.

Table 4.1. A list of the genotypes tested in each of the pathosystems investigated in this study

				Pathosystem				
Species	**Locus**	**Allele**	**Phenotype**	*Blumeria graminis*	*Ramularia collo-cygni*	*Oculimacula spp.*	*Fusarium graminearum*	*(Deoxynivalenol)*
Triticum aestivum	*Rht-B1*	*rht-tall*	Wildtype	+		+	+	+
		Rht-B1b	semi-dwarf				+	+
		Rht-B1c	severe-dwarf	+		+	+	+
	Rht-D1	*rht-tall*	Wildtype	+		+		
		Rht-D1b	semi-dwarf					
		Rht-D1c	severe-dwarf	+		+		
Hordeum vulgare	*Sln1*	WT	Wildtype	+	+	+	+	
		Sln1d	dwarf (GoF)	+	+	+	+	
		sln1c	slender (LoF)	+	+	+		

+ denotes that the line was tested in respective pathosystem.

4.2.2. **Influence of DELLA alleles on resistance of wheat and barley to *B. graminis***

Near isogenic lines of wheat cvs. Mercia (*rht-tall, Rht-B1c* and *Rht-D1c*), Maris Huntsman (*rht-tall,Rht-B1c* and the double mutant *Rht-B1c+D1b*) and April Bearded (*rht-tall* and the double mutant *Rht-B1c+D1b*), and barley cv. Himalaya *Sln1a*, *Sln1d* and *sln1c* plants were grown to growth stage 12 (G.S; Zadoks *et al.*, 1974, i.e. second seedling leaf unfurled) in controlled environment cabinets under 16h photoperiod and 17^{o}C/12^{o}C temperature regime. Detached sections (2.5 cm) of leaf 2 were placed in agar boxes and inoculated with the spores of virulent *Blumeria graminis* isolates (either f. sp. *tritici* (*Bgt*) or f. sp. *hordei* (*Bgh*)) in aluminium settling towers as

described by Brown & Wolfe (1990). Experiments were conducted in a randomised block design of 3 replicates with 4 detached leaves of each line per replicate. Each experiment was repeated.

Bgh inoculated barley leaves were collected 48 and 60 hours post inoculation (hpi) for microscopic analysis. Leaf tissue was cleared and fungal structures were scored for papillae defence and host cell death response, at each time point as described by Boyd *et al.* (1994). Fungal structures and plant cellular autofluorescence were observed using a Nikon Microphot-SA with Nomarski (DIC) and fluorescence filter; FITC (450-490 nm > 520LP). Macroscopic symptoms were assessed 8 days post inoculation (dpi) by quantifying colonies/cm^2 of leaf area.

4.2.3. **Influence of DELLA alleles on resistance of barley to *R. collo-cygni***

Barley cv. Himalaya *Sln1a* and *Sln1d* were grown and inoculated with *R. collo-cygni* according to the methods reported previously (Makepeace *et al.*, 2008). This experiment was conducted in a randomised block design with 3 blocks each containing 5 plants per line. Symptoms were assessed 15 dpi as percentage of leaf area covered with lesions. This experiment was replicated three times with the addition of *sln1c* in the third replicate.

4.2.4. **Influence of DELLA alleles on resistance of wheat and barley to *O. acuformis* and *O. yallundae***

Wheat cv. Mercia *rht-tall, Rht-B1c* and *Rht-D1c* NILs, and barley cv. Himalaya *Sln1a and Sln1d* lines were grown and inoculated with either *O. acuformis* or *O. yallundae* as described by Chapman *et al*. (2008). Plants were harvested 6-8 weeks after inoculation and scored for fungal penetration of leaf sheaths according to the scale devised by Scott (1971). The experiments were conducted in a randomised block design with five blocks, each containing ten plants per line of which five were inoculated with *O. acuformis* and five were inoculated with *O. yallundae.* The experiment was replicated.

Himalaya *Sln1a, Sln1d* and *sln1c* lines were inoculated with *O. acuformis*. Due to the elongated nature of the *sln1c* mutant line, the method described by Chapman *et al*. (2008) was modified slightly by using longer tubes to contain the inoculum. This experiment was arranged in a randomised block design with five blocks as described above. The experiment was carried out only once because of limited stocks of *sln1c* seed.

4.2.5. **Influence of DELLA alleles on Type 1 resistance of wheat and barley heads to *F. graminearum***

Mercia NILs (*rht-tall, Rht-B1b* and *Rht-B1c*) were phenotyped for resistance to initial infection (Type 1 resistance as defined by Schroeder & Christensen (1963)). Lines were inoculated by

spraying until run-off with a conidial suspension of *F. graminearum* (1 x 10^5 conidia mL^{-1}) at GS 65 (Zadoks *et al.*, 1974) as described previously (Gosman *et al.*, 2005). The experiment was conducted in a randomised complete block design consisting of four blocks within which were seven plants of each line. Disease severity was visually assessed as percentage of spikelets infected at 14 dpi. The data from 4 independent experiments were combined prior to analysis.

4.2.6. **Influence of DELLA alleles on Type 2 resistance of wheat and barley heads to *F. graminearum***

Mercia and Maris Huntsman NILs (*rht-tall*, *Rht-B1b* and *Rht-B1c*) were phenotyped for resistance to spread within the spike (Type 2 resistance as defined by Schroeder & Christensen (1963)) in two experiments in an unheated polytunnel. Experiments were arranged in a randomised complete block design with four replicate blocks with seven plants per line in each.

Inoculation and disease assessment were as described by Gosman *et al.* (2007). Lines were inoculated at GS 65 (Zadoks *et al.*, 1974) by point inoculation with 50 µl of conidial suspension (1 x 10^6 mL^{-1}) of a deoxynivalenol (DON) producing isolate of *F. graminearum* (UK1), injected into a single floret within the central portion of each spike. High humidity was maintained for 72 hpi by misting. Disease severity was measured as the number of diseased spikelets 14 dpi.

4.2.7. **Influence of DELLA alleles on foliar disease resistance of wheat and barley to *F. graminearum***

Plants of wheat cv. Maris Huntsman (*rht-tall* and *Rht-B1c* NILs) and barley cv. Himalaya (*Sln1a* and *Sln1d*) were grown to GS 12 in controlled environment cabinets under 16h/8h, 15°C/12°C, light/dark with 70% relative humidity. Sections (5 cm) of leaf two were inoculated with conidia of *F. graminearum* (5 µl of 1x10^6 conidia mL^{-1}) as described by Chen *et al.* (2009). Leaves were returned to the growth cabinet and lesion areas were measured after 6 days using ImageJ (Abramoff *et al.*, 2004)

4.2.8. **Influence of DELLA on resistance of wheat heads to deoxynivalenol**

Maris Huntsman (*rht-tall, Rht-B1b* and *Rht-B1c)* NILs were tested for resistance to DON in an unheated polytunnel. DON was kindly supplied by Dr. M. Lemmens (IFA-Tulln, Austria). At GS 65, two spikes per plant were inoculated with DON according to the method of Lemmens *et al.* (2005) with the following modification; DON solution was applied to a single clipped spikelet on each wheat head instead of two. Pots containing individual plants were arranged in a randomised

complete block design of four replicates of seven plants per line. Following treatment, the number of damaged spikelets per head was assessed 14 dpi.

4.2.9. **Influence of DELLA on deoxynivalenol-induced expression of negative cell death regulators**

Maris Huntsman (*Rht-B1a, Rht-B1b* and *Rht-B1c*) NILs were incubated in darkness at 20°C for five days on moist filter paper. Roots were submerged in water or Deoxynivalenol solution (DON) (14 ppm) and incubated for a further eight hours. Total RNA was isolated using Qiagen RNA easy spin columns from 100mg of leaf tissue, ground in a pestle and mortar under liquid nitrogen. DNase treatment was carried out using the Turbo DNA-free kit (Ambion) and cDNA was synthesised from 5 µg of RNA using SuperScript III (Invitrogen) following the manufacturers instructions with the addition of random nonamers (50 µM, Invitrogen). RNA was digested with RNase-H (Invitrogen) from the RNA-DNA duplex to leave single stranded cDNA. cDNA was diluted 1:20 for qRT-PCR. qRT-PCR reactions were carried out using a DNA engine Opticon2 Continuous Fluorescence Detector (MJ Research Inc., Alameda, CA, USA). Amplification was carried out using SYBR Green Jumpstart Taq ready mix with gene specific primers (Table 4.2). An initial activation step at 95°C for 4 min was followed by 40 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C. Target gene expression was calculated relative to the expression of the reference gene, 18S (Table 4.2) using the $\Delta\Delta C_t$ method (Pfaffl, 2001). cDNA was diluted 1:100 for quantification of the expression of 18S rRNA.

Table 4.2. Table of primer sequences.

Gene name	Forward primer	Reverse primer
BI-1	TACATGGTGTACGACACGCA	GTCCATGTCGCCGTGG
CEO	GCGTCTGTCTGTGAATCTGC	TGTTGATTGGACAAAAACCAA
18S rRNA	AGTAAGCGCGAGTCATCAGCT	CATTCAATCGGTAGGAGCGAC

BI-1(*BAX INHIBITOR-1*) and *CEO* (*CLONE EIGHTY ONE*, the wheat homologue of Arabidopsis *RADICAL-INDUCED CELL DEATH 1*).

4.2.10. **Statistical analysis**

All statistical analyses were performed using GenStat for Windows 12th edition (Payne *et al.*, 2009). Analysis of variance (ANOVA) was carried out using the generalised linear model (GLM) of regression analysis to estimate variance attributable to experiment, replicate and genotype.

4.3. **Results**

4.3.1. **Interaction between DELLA and *B. graminis***

B. graminis f. sp. *tritici* (*Bgt*) and f. sp. *hordei* (*Bgh*) are obligate biotrophs which infect wheat and barley, respectively, causing powdery mildew. The DELLA GoF barley line, *Sln1d*, showed a significant ($P<0.001$) increase in susceptibility to *Bgh* compared to the Himalaya wild type (Fig. 4.1). The LoF DELLA mutant line, *sln1c*, was not significantly more resistant compared to the Himalaya wild type, which expressed a high level of resistance relative to the check cultivar Golden Promise. However, the *sln1c* line exhibited a spreading hypersensitive cell death phenotype (Fig. 4.1b), which is absent in the Himalaya wild type line. The wild type and severe dwarf (*Rht-B1c* or *Rht-D1c*) NILs of Mercia and Maris Huntsman were equally infected by *B. graminis* (Fig 4.2). However, lines containing both semi-dwarf and severe dwarfing alleles (*Rht-B1c* and *Rht-D1b*) were more susceptible (assessed as colonies cm^{-2}) than the respective wild type plants (Fig 4.3).

Cytological analysis of the barley lines showed that a significantly ($P<0.001$) higher proportion of spores infected plant host epidermal cells in *Sln1d* (57%) compared to *Sln1a* Himalaya (18%), and none of the spores infected the cells of *sln1c* leaves. In the *sln1c* line a high proportion (73%) of the unsuccessful interactions were due to host cell death. In *Sln1d* lines, by contrast, a significantly ($P<0.001$) lower proportion (15%) of unsuccessful interactions were due to cell death restriction, with the majority correlated with papillae formation (77%; Fig 4.4) and the remaining spores not being advanced enough to elicit either response. Interestingly, while restriction of attempted penetration was associated with enhanced cell death, the proportion of cells in which the fungus was restricted by papillae formation alone was independent of the DELLA status of the line. The fungal colonisation process was more rapid in the *Sln1d* interaction than in the wild type, with all spores which had successfully infected epidermal cells reaching the hyphal stage 60 hpi whilst only 17% of the successful spores on wild type leaves were at the hyphal stage and most (83%) were only at the balloon/digitate haustorium stage.

4.3.2. **Interaction between DELLA and *R. collo-cygni***

The responses of barley lines, differing in DELLA status, to the hemibiotroph, *Ramularia collo-cygni* (*Rcc*) were assessed. *Rcc* is a barley infecting pathogen which exhibits a long biotrophic, endophytic phase before switching to a necrotrophic lifestyle late in infection (Stabentheiner *et al.*, 2009). The DELLA accumulating (GoF) *Sln1d* containing barley line was significantly ($P<0.001$) more susceptible (assessed as % diseased leaf area) to *R. collo-cygni* than the wild type line. In the third experiment the LoF DELLA line (*sln1c*) was included and found to be significantly ($P=0.03$) more resistant than the wild type (Fig 4.5).

4.3.3. **Interaction between DELLA and *Oculimacula* spp.**

O. acuformis and *O. yallundae* infect the stem base of cereal hosts and present contrasting pathogenic lifestyles. The former is considered a necrotroph whilst the latter exhibits a short biotrophic phase of establishment before switching to necrotrophic nutrition (Blein *et al.*, 2009). Wheat GoF mutant lines showed significantly greater resistance to both *O. acuformis* and *O. yallundae* ($P<0.01$ and <0.05 respectively) compared to *rht-tall* lines (Fig. 4.6a). Although still significant, the disease resistance conferred by DELLA stabilisation was less for *O. yallundae* than for *O. acuformis*. Barley GoF mutants also exhibited significantly ($P<0.001$) increased resistance to both forms of the disease relative to wild type plants, in contrast, the LoF DELLA mutant line *sln1c*, exhibited greater susceptibility to *O. acuformis* (Fig 4.6b).

4.3.4. **Interaction between DELLA and FHB caused by *F. graminearum***

F. graminearum is one of the predominant causative agents of Fusarium head blight (FHB). Schroeder & Christensen (1963) describe 2 main components of resistance to FHB: resistance to initial infection (Type 1) and resistance to spread within the head (Type 2). These components can be broadly dissected using different inoculation techniques (Miedaner *et al.*, 2003), namely spray or point inoculation.

Spray inoculation of wheat heads assesses resistance to initial infection (type 1 resistance) during which the fungus is thought to exhibit a short biotrophic phase (Brown *et al.*, 2010). Experiments carried out by Gosman (JIC, Norwich, UK) show that following spray inoculation the Mercia *rht-tall* line showed the greatest resistance to initial infection with only 19% of spikelets infected (Fig. 4.7). The semi-dwarf *Rht-B1b* line showed significantly greater ($P<0.001$) susceptibility to initial infection (24%) and greater still in the severe dwarf *Rht-B1c* NIL (36%, $P<0.001$) compared to wild type.

Point inoculation of individual spikelets, which bypasses the plant defence against initial infection, is used by Gosman (JIC, Norwich, UK) to assess resistance to disease spread (type 2 resistance), during which the fungus is in the necrotrophic phase of infection(Boddu *et al.*, 2006). The wild type line of Maris Huntsman was highly susceptible to spread of *F. graminearum* with an average of 10.0 spikelets showing disease 14 dpi (Fig.4.8a & b). *Rht-B1b* NILs showed significantly less ($P<0.001$) symptom spread than the wild type line with an average of 8.9 spikelets showing disease at this stage. The reduction in symptoms was even greater for the *Rht-B1c* NILs with only 5.2 spikelets exhibiting disease ($P<0.001$). A similar trend was observed with Mercia wild type, *Rht-B1b* and *Rht-B1c* NILs (data not shown).

4.3.5. **Interaction between DELLA and DON induced lesion development**

F. graminearum produces the trichothecene mycotoxin deoxynivalenol, not essential for initial infection (Proctor *et al.*, 1995) but necessary for disease spread in wheat heads (Bai *et al.*, 2002). To determine whether DELLA accumulating lines were more resistant to DON Gosman (JIC, Norwich, UK) assessed bleaching symptoms caused by this mycotoxin. In the wild type Maris Huntsman line, bleaching symptoms spread an average of 4.3 spikelets from the point of inoculation at 14 dpi (Fig.4.8a & b), whereas in the *Rht-B1b* line, spikelets adjacent to the inoculated spikelet developed symptoms but the rate of symptom spread was significantly less ($P<0.005$) than in the wild type line, with an average of 3.1 spikelets exhibiting symptoms at 14 dpi. Most strikingly, following injection of the *Rht-B1c* line, symptoms were restricted to the inoculated spikelet (Fig. 4.8 a/b). Overall, these results closely resembled those appearing following point inoculation with a DON-producing fungus.

4.3.6. **Influence of DELLA on lesion development induced by *F. graminearum* on leaves of wheat and barley**

Wound inoculation of leaves with *F. graminearum* was used by Gosman (JIC, Norwich, UK) to further assess relative resistance to disease spread (type 2 resistance). At 6 dpi the zone of cell death about the inoculation point was significantly greater ($P=0.006$) in Maris Huntsman wild type than the *Rht-B1c* NIL (Fig. 4.9). Similarly, the zone was significantly greater ($P<0.001$) in Himalaya wild type than the *Sln1d* line (Fig. 4.9).Cell death was not observed beyond the point of inoculation in wheat or barley following wounding alone (data not shown).

4.3.7. **Influence of DELLA on deoxynivalenol-induced expression of negative cell death regulators.**

Deoxynivalenol is a virulence factor, which has been demonstrated to induce H_2O_2 production and promote host cell death (Desmond *et al.*, 2008). To test what the mechanism of DELLA conferred type 2 resistance to deoxynivalenol and deoxynivalenol producing isolates may involve, the expression of two candidate negative regulators of cell death, *BI-1* and *CEO*, was quantified in the *Rht-B1* allelic series treated with water (control) or DON solution (Fig 4.10). The expression of both *BI-1* and *CEO* is less in *Rht* GoF lines relative to wild type in control samples suggesting endogenously reduced levels. Expression of both *BI-1* and *CEO* increases in *Rht* GoF lines in response to DON treatment but expression of *BI-1* remains the same and *CEO* is reduced, in wild type lines in response to DON treatment compared to water control.

4.4. **Discussion**

The reduced GA sensitivity of the DELLA *Rht-B1b* and *Rht-D1b* semi-dwarfing alleles in wheat were central to enhanced crop yields achieved as part of the Green Revolution (Hedden, 2003). These alleles are believed to encode stabilised versions of DELLA proteins that resist degradation in the presence of GA (Peng *et al.*, 1999). Previous studies have demonstrated that Arabidopsis DELLA proteins are involved in the response to pathogens. Using DELLA mutants in Arabidopsis Navarro *et al*. (2008) showed that DELLAs promote susceptibility to biotrophs and resistance to necrotrophs. In the current study I investigated the role of DELLA proteins in response to pathogens in two important monocotyledonous crop species. *H. vulgare* (barley) is a diploid species which contains only a single DELLA-encoding gene, *and T. aestivum* (bread wheat) is a polyploid species originating from the hybridisation of three diploid progenitors and as such contains three DELLA-encoding genes. Multiple *Rht* alleles with differing severity of dwarfing are available in NILs of wheat (Flintham *et al.*, 1997). I have demonstrated a trade-off effect on resistance to a range of necrotrophic and biotrophic fungal pathogens that are responsible for economically important diseases of cereals. Barley has enabled us to observe GoF and LoF mutations in *Sln1* without the gene redundancy present in Arabidopsis and wheat. Additionally, I assessed the effect of polyploidy, observing how disease resistance is influenced by DELLA alleles conferring semi-dwarf and severe dwarf phenotypes, functioning in the presence of background wild type homoeologous DELLA-encoding genes.

Biotrophic pathogens derive their nutrients from living host cells. All obligate biotrophic pathogens possess specialist feeding structures, known as haustoria, which penetrate the cell wall. Recognition of the invading pathogen results in an increase in ROS production leading to the hypersensitive response (HR), a type of programmed cell death which deprives biotrophic pathogens of their food source. Studies in Arabidopsis have implicated DELLA proteins in processes leading to cell death. Achard *et al*. (2008) demonstrated that DELLAs delay reactive oxygen species (ROS) -induced cell death and Navarro *et al*. (2008) reported that DELLAs suppress accumulation of salicylic acid (SA) and cell death in response to infection by *Pseudomonas syringae* pv. *tomato*. Microscopic analysis of the host defence response in the barley-*B.graminis* (biotroph) interaction in this study showed that the GoF line is associated with a reduced frequency of hypersensitive cell death reactions, resulting in a higher number of successful haustorial establishment events, whilst the the cells of the DELLA LoF line appear to be hypersensitive to ROS-induced cell death resulting in complete resistance (Fig. 4.4). DELLA accumulation delays ROS-induced cell death which in turn reduces the effectiveness of the HR therefore increasing susceptibility both to an obligate biotroph, *B. graminis* (Figs. 4.1-4.3) and to a hemibiotroph with a long biotrophic or endophytic phase, *Rcc* (Fig. 4.5).

Stabilisation of the single DELLA protein (GoF) in the dwarf barley line *Sln1d* was sufficient to confer a striking increase, relative to wild type, in susceptibility to *B. graminis* whilst the *sln1c* (LoF) line expressed increased cell death, macroscopically visible as necrotic lesions (Figs. 4.1 and 4.4). The height reduction associated with the *Sln-1d* (GoF) allele in barley is similar to that associated with *Rht-B1c* and *-D1c* alleles in wheat (ca. 50% of respective wild type). In contrast to the significantly enhanced susceptibility to *B. graminis* conferred by barley *Sln1d* lines, wheat lines carrying single mutant alleles, *Rht-B1c* or *Rht-D1c*, exhibited no similar increase to *B. graminis* susceptibility, however the combination of dwarf and semi-dwarf mutant alleles (*Rht-B1c* + *Rht-D1b*) did result in a significant increase in susceptibility relative to wild type. Wild type homoeologous copies of the *Rht* genes might therefore be sufficient to buffer the negative effect of a single mutation on susceptibility to *B. graminis*. It is conceivable that DELLA accumulation must pass a threshold in order to significantly delay cell death induced by *B. graminis* and that this threshold is exceeded in dwarf + semi-dwarf NILs carrying mutations in two of their three *Rht* (DELLA) homoeologues but not in lines carrying a mutation in a single homoeologue. Further investigation with other biotrophic pathogens is necessary to determine whether this phenomenon is specific to *B. graminis* or common to all biotrophs.

Hemibiotrophic pathogens exhibit an initial biotrophic phase before switching to a necrotophic lifestyle. The barley pathogen, *R. collo-cygni*, is a hemibiotroph with a prolonged biotrophic phase in which the fungus grows intercellularly, colonising the spaces between mesophyll cells (Sutton & Waller, 1988; Stabentheiner *et al.*, 2009). The metabolic changes that underlie the switch from biotrophic to necrotrophic growth, required for the pathogen to complete its lifecycle, are not yet fully understood but it is thought to be triggered by the vegetative to reproductive transition of the host (Salamati & Reitan, 2006). One component contributing to the necrotrophic phase is thought to be the biosynthesis and secretion of rubellin, a host non-specific toxin which triggers light dependent production of ROS (Heiser *et al.*, 2003) leading to necrosis of the leaf, thereby enabling the fungus to complete its lifecycle. The barley lines carrying the GoF or LoF DELLA mutations showed differential responses to *R. collo-cygni* with the GoF mutant being significantly more susceptible, and the LoF significantly more resistant, than wild type. These results suggest that DELLA-mediated prevention of cell death benefits the initial biotrophic phase of *R. collo-cygni* infection and support the current view that *R. collo-cygni* has a long biotrophic phase.

Necrotrophic pathogens derive their nutrients from dead host cells. These pathogens have evolved a number of strategies to kill host cells including the secretion of toxins, cell-wall degrading enzymes and eliciting ROS production. If DELLA prevents ROS induced cell death then it would be predicted that plants with increased DELLA accumulation would be more resistant to necrotrophs. *Oculimacula yallundae* and *O. acuformis* are considered to be typical necrotrophic pathogens that cause eyespot disease on the stem base of cereals and considered the most serious disease of winter

wheat in the UK. GoF mutations of DELLA in both wheat and barley resulted in significantly increased resistance to both *Oculimacula* species as might be anticipated given their necrotrophic lifestyles. Supportive of DELLA increasing resistance to necrotrophs, the barley LoF mutant exhibits enhanced susceptibility to eyespot caused by *O. acuformis.*

Interestingly, the effect on the GoF mutation in wheat was greater for *O. acuformis* than for *O. yallundae*. The two *Oculimacula* species used in this study have different infection strategies, in that *O. yallundae* penetrates the coleoptile in a more ordered, intramural manner whilst *O. acuformis* exhibits a more invasive growth habit (Daniels *et al.,* 1991). Coleoptile penetration by *O. yallundae* has been demonstrated to be asymptomatic, with no sign of host cell death (Blein *et al.*, 2009). Once at the first leaf sheath the pathogen initiates production of an infection plaque hyphal growth penetrates the first, then successive leaf sheaths facilitated by the secretion of cell-wall degrading enzymes (Mbwaga *et al.*, 1997). These observations suggest that *O. yallundae* may be considered to be a hemibiotroph with a very short initial phase of biotrophic growth before switching to a necrotrophic phase once the pathogen reaches the first leaf sheath (Blein *et al.*, 2009). In contrast *O. acuformis* exhibits the traits of a necrotroph. I speculate that the more pronounced DELLA-associated increase in resistance for *O. acuformis* than for *O. yallundae* reflects the different growth habits of the two species. It is anticipated that the GoF mutants will be more susceptible to the initial biotrophic establishment phase of *O. yallundae*, which partially counters the enhanced resistance, relative to the wild type, when the fungus switches to necrotrophic nutrition.

F. graminearum was originally considered to be entirely necrotrophic. However, evidence is accumulating to indicate that this may not be the case for some of the diseases caused by this fungus. Analysis of the interaction at the cellular level shows that *F. graminearum* exhibits extracellular growth during the early stages of infection (Pritsch *et al.*, 2000) and sub-cuticular growth reminiscent of *O. yallundae* (Rittenour and Harris, 2010). It appears that *F. graminearum* requires a transient biotrophic phase of establishment before switching to necrotrophic nutrition during infection of wheat leading to FHB (Goswami and Kistler 2004). This view is supported by studies on the interaction of FHB with the SA and JA signalling pathways. SA content and expression of the SA-inducible *PR1* gene in *Arabidopisis* inoculated with *F. graminearum* showed that SA signalling is activated in the early stages of infection (Makandar *et al.,* 2010). Furthermore, over-expression of a gene that regulates SA signalling (*NPR1*), in wheat and Arabidopsis, increases resistance to *F. graminearum* (Makandar *et al.*, 2006). Methyl jasmonate (MJ), however, has dichotomous effects on the susceptibility of Arabidopsis to *F. graminearum*. Application of MJ during the early stages of infection enhanced disease severity, presumably due to JA attenuating SA signalling, whist, when applied at later stages of infection, MJ reduced disease severity

(Makandar *et al*., 2010). Thus DELLA would be predicted to have contrasting effects depending on whether resistance is assessed during the biotrophic or necrotrophic phase.

Short wheat varieties tend to be more susceptible to FHB than tall ones and it has been demonstrated that semi-dwarf lines carrying the *Rht-B1b* allele are more susceptible to initial infection than those carrying the wild type allele (Srinivasachary *et al.*, 2009). In this study I report work under taken by Gosman (JIC, Norwich, UK) that demonstrates both *Rht-B1b* and *Rht-B1c* NILs exhibit increased susceptibility relative to wild type, and in addition it was observed that this effect is associated with the severity of the DELLA effect on plant height. In contrast, following point inoculation *Rht-B1b* and *Rht-B1c* NILs exhibited increased resistance to disease spread relative to wild type and this also correlates with the DELLA effect on plant height. Dwarf lines of both wheat and barley also showed enhanced resistance to cell death following wound-inoculation of leaves with *F. graminearum.* Overall these results indicate that DELLA accumulating lines are more susceptible to the initial establishment phase whilst being more resistant to the later colonisation phase. I propose that this differential reflects the different trophic modes of growth employed by *F. graminearum* and represents a case for the biotroph/necrotroph trade-off within a single pathogen.

F. graminearum produces the trichothecene mycotoxin deoxynivalenol (DON), that functions as a virulence factor, not essential for initial infection (Proctor *et al.*, 1995) but necessary and sufficient for disease spread in wheat heads (Bai *et al.*, 2002). Accordingly up regulation of trichothecene biosynthesis pathway components and subsequent DON accumulation have been observed 48 hpi (Boddu *et al.*, 2006), and this is thought to signal the switch from biotrophic to necrotrophic growth. DON induces H_2O_2 production in the host, promoting cell death (Desmond *et al.*, 2008). In turn, *in vitro* experiments have demonstrated that H_2O_2 induces DON production in the fungus (Ponts *et al.*, 2006) leading to a cycle that ultimately favours the necrotrophic fungus. Plant lines with enhanced capabilities of alleviating oxidative stress would be anticipated to exhibit increased resistance to *F. graminearum* and DON. This view is supported by a number of studies. For example, increases in superoxide dismutase (SOD) and catalase (CAT) activity positively correlated with FHB resistance in a set of wheat varieties inoculated with DON producing isolates (Chen *et al.*, 1997). Similarly, the Arabidopsis *radical-induced cell death 1* (*AtRCD1*) protein homologue in wheat, *Clone eighty one* (*CEO*), thought to negatively regulate oxidative stress responses (Overmyer *et al.*, 2000) accumulated in higher amounts in DON tolerant lines compared to susceptible lines (Walter *et al.*, 2008).

In the present study it was shown that GoF DELLA lines were more resistant to colonisation by *F. graminearum* and DON-induced cell death. I postulate that this is due, at least in part, to a reduced propensity to initiate or undergo cell death. DELLA proteins have been shown to up-regulate genes

involved in the ROS scavenging system such as *CSD1/2* which encode SOD in response to stress (Achard *et al.*, 2008a). I observed that expression of *CEO* and the wheat homologue of *Bax Inhibitor-1* (*TaBI-1*), a second gene with a putative role in the regulation of cell death (Huckelhoven, 2004), is greater in wheat DELLA GoF lines than in wild type in response to DON (Fig. 4.10). Interestingly, a trade-off in resistance to pathogens with opposing lifestyles has been observed in *HvBI-1* over-expression lines which exhibit enhanced susceptibility to *Blumeria* and enhanced resistance to *Fusarium* associated with a decreased propensity to cell death (Babaeizad *et al.*, 2009).

Susceptibility to biotrophs is often accompanied by resistance to necrotrophs, and vice versa, as has been demonstrated with *HvBI-1* over-expression lines and in lines lacking MLO function, which exhibit increased resistance to *Blumeria* whilst enhancing susceptibility to necrotrophic pathogens (Jarosch *et al.*, 1999; Kumar *et al.*, 2001). In this present study I show that DELLA influence a biotroph-necrotoph resistance trade-off and propose that DELLA are involved in regulating downstream cell death processes, highlighting DELLAs significance in disease responses.

Our data demonstrate that DELLA plays a central and dichotomous role in resistance to necrotrophic and biotrophic pathogens in cereal monocot species in a manner similar to that observed in Arabidopsis (Navarro *et al.* 2008). Single DELLA GoF mutants of both barley (diploid) and wheat (hexaploid) exhibited enhanced resistance to necrotrophic pathogens (*F. graminearum,* and *Oculimacula* species). In contrast, however, significantly enhanced susceptibility to a biotrophic pathogen (*Blumeria f. sp*.) was only observed in wheat when two of the three DELLA homoeologues were mutated. These findings indicate that the semi-dwarfing alleles deployed in present day wheat cultivars provide increased tolerance to necrotrophic pathogens, and, due to the polyploid nature of wheat confer only a negligible effect on susceptibility to *Blumeria* (and possibly additional obligate biotrophs). A better understanding of the role of DELLA in biotic stress of cereals will inform breeding strategies for improving broad spectrum disease resistance.

5. Reactive Oxygen Species and DELLA; how the DELLA conferred resistance trade off is affected by the environment

5.1. Introduction

Plants generate reactive oxygen species (ROS) as a by-product of normal metabolism via photosynthetic and respiratory electron transport (PET and RET; Asada, 1999; Dat *et al.*, 2000). Under adverse abiotic conditions (non-living environmental factors) ROS production is increased. For example, prolonged exposure to high light causes elevated ROS levels due to the energy absorbed being in excess of that which can be transduced by photosystem II (PSII). This phenomenon is known as excess excitation energy (EEE; Karpinski *et al*., 1999). EEE can be strongly enhanced and even initiated under otherwise benign light levels by additional abiotic stresses such as drought, ozone, UV or extremes of temperature (Wise & Naylor, 1987; Munne-Bosch *et al.*, 2001; Rossetti & Bonatti, 2001; Hideg *et al.*, 2002). ROS accumulation as a result of EEE can result in cellular oxidative damage. To protect themselves plants induce antioxidant defences in order to control the increased levels of ROS locally and in addition, initiate signalling that results in systemic acquired acclimation (SAA) which leads to the stimulation of the antioxidant system throughout the plant protecting unexposed parts of the plant to future exposure to stress (Karpinski *et al*., 1999).

ROS can also be actively produced by the plant, for example induction of ROS production plays a central role in the plants response to pathogen infection (biotic stress). Membrane bound NADPH oxidases significantly contribute to the "oxidative burst" (HammondKosack & Jones, 1996), a rapid increase in ROS production at the site of attempted invasion. Intracellular sources of pathogen induced ROS have also been reported to contribute to the oxidative burst including the chloroplast, mitochondria, and peroxisomes (Kariola *et al*., 2005; Kuzniak & Sklodowska, 2005; Amirsadeghi *et al.*, 2007). Recent evidence suggests that chloroplasts are important signal initiators which subsequently activate NADPH oxidase (Liu *et al*., 2007). Increased production of ROS in combination with nitric oxide (NO), salicylic acid (SA), and calcium fluxes leads to localised hypersensitive cell death (Mur *et al*., 2008), defence gene induction and systemic acquired resistance (SAR) preparing systemic tissue for a faster defence response to future pathogen attack.

The ROS production induced by a potential pathogen has a dichotomous role in the disease resistance outcome that is dependent on the pathogen's nutritional lifestyle: biotrophic or necrotrophic. Following the perception of a pathogen threat a ROS burst occurs in both compatible and incompatible biotrophic interactions. A second larger burst occurs only in incompatible reactions resulting in hypersensitive cell death and resistance (Figure 5.1a; Grant & Loake, 2000).

The first burst is initiated through the recognition of pathogen associated molecular patterns (PAMPs) and can bring about PAMP triggered immunity (PTI). Successful pathogens subsequently deliver effectors to either interfere with PTI or enable pathogen nutrition and dispersal leading to effector triggered susceptibility (ETS) in the case of virulent isolates. However, if the plant posses an associated receptor which recognises an effector a second ROS burst is initiated of greater amplitude leading to a hypersensitive cell death, as is the case of avirulent isolates. This model is referred to as the zig-zag-zig model as proposed by Jones & Dangl (2006). Conversely, hypersensitive cell death resulting from ROS accumulation may be beneficial to necrotrophic pathogens (Govrin & Levine, 2000). Accordingly, Able (2003) showed that resistant barley cultivars suppress a second burst of O_2^- with increased SOD activity in response to challenge with the necrotrophic fungus *Rhynchosporium secalis*, whilst elevated levels of O_2^- in susceptible cultivars lead to disease (Fig. 5.1b.). In agreement with this, lines with elevated antioxidant activity have been shown to be more tolerant to necrotrophs (Chen *et al.,* 1997). This resistance trade-off has also been identified in DELLA accumulating Arabidopsis lines which were demonstrated to have enhance ROS scavenging systems (Achard *et al* 2008) and subsequently in GoF DELLA barley lines (chapter 4).

When plants are challenged with a compatible isolate of a biotrophic pathogen effectors delivered by the pathogen will reduce an oxidative burst resulting in establishment. DELLA GoF mutants exhibit an increase in susceptibility suggesting a reduction in ROS levels, conferred by an increase in DELLA, is resulting in a further reduction in the effectiveness of HR. In contrast, LoF lines are hyper responsive to the ROS accumulation caused by pathogen attack due to the absence of functional DELLA leading to a higher propensity to cell death and resistance to pathogen establishment. Conversely necrotrophic pathogens produce virulence factors to intensify the ROS burst leading to virulence in wild type lines which is increased in LoF lines, leading to an increase in susceptibility, whilst dampened in GoF lines leading to a containment of pathogen spread. No DELLA dependent resistant differential is observed upon challenge with incompatible biotrophic isolates (Navarro *et al*., 2008), likely because the host response is so strong that the DELLA affect is inconsequential. In addition to DELLAs influence on ROS accumulation, it has also been demonstrated that DELLA alter the relative strength of salicylic acid and jasmonic acid (JA) signaling pathways, which greatly contribute to biotroph and necrotroph resistance respectively. The effect of DELLA on SA and JA signalling was shown to influence the resistance trade-off observed in Arabidopsis (Navarro *et al*., 2008) and as such likely to contribute to the trade-off observed in chapter 4.

The influence of EEE and SAA on ROS homeostasis in the plant is likely to result in an altered outcome (resistance or susceptibility) of a subsequent pathogen attack. This has been shown in several studies, for example Bechtold *et al.* (2005) show that Arabidopsis plants grown in high

light at 10˚C show increased resistance to a virulent strain of *Pseudomonas syringae pv tomato* DC3000 compared to plants grown at 22˚C in low light. Conversely, Keon *et al.* (2007) demonstrated that increased resistance is conferred to wheat plants grown in the shade (low light) after inoculation with *Mycosphaerella graminicola* compared to plants grown in medium light. Symptoms were assessed 13 days after inoculation, by which point this hemibiotroph had switched to a necrotrophic mode of nutrition. Interestingly, Zeier *et al.* (2004) found no significant difference in disease susceptibility between Arabidopsis plants exposed to high light (500 µmol m^{-2} sec^{-1}) or medium light (70 µmol m^{-2} sec^{-1}) when inoculated with an avirulent strain of *Pseudomonas syringae pv. maculicola*, likely because the oxidative burst associated with incompatible interactions are so great that the altered ROS homeostasis resulting from the preconditioning of the plants is of no consequence.

From these studies it appears that a preceding abiotic stress, which stimulates the antioxidative system of a plant, will influence a subsequent biotic stress. The outcome with respect to resistance will be dependent on the nutritional preference of the pathogen (biotrophic/hemibiotrophic or necrotrophic). In this chapter, as a continuation of my previous findings, the influence of DELLA on ROS accumulation will be investigated. The environment greatly influences plant ROS homeostasis. I will also assess how the environment (light) influences disease outcome dependent on host DELLA status (wild type and GoF) and pathogen lifestyle (biotrophic/hemibiotrophic or necrotrophic). Preliminary work will be presented on how DELLA may influence ROS metabolism in response to both pathogen and environmentally induced ROS processes.

5.2. **Material and Methods**

5.2.1. **Plant material and growth conditions**

Throughout this chapter Barley cv. Himalaya *Sln1a*, *Sln1d* (GoF, dwarf) and *sln1c* (LoF, elongated) lines were used as test cultivars. Barley cvs. Kirsty and Golden Promise were used for *Ramularia* and *Blumeria* pathosystems respectively as check cultivars.

Plants were grown at three different light intensities; 10, 130 and 740 µmol $m^{-2}s^{-1}$, with all other environmental variables remaining constant (15˚C, 70% humidity, 16/8 h light/dark). Seed were pre-germinated and sown in P40 trays containing peat and sand. To ensure all plants were at a similar developmental stage at the time of inoculation plants grown in low light (LL; 10 µmol $m^{-2}s^{-1}$) were sown first, plants grown at medium light (ML; 130 µmol $m^{-2}s^{-1}$) were sown 2 days later and plants grown at high light (HL; 740 µmol $m^{-2}s^{-1}$), 2 days after that.

5.2.2. **ROS inducers**

The ROS inducers paraquat (methyl viologen), alloxan and menadione were used in this study. When applied to the plant paraquat and menadione induce superoxide production in the chloroplast and mitochondria respectively and alloxan induces hydrogen peroxide production in the mitochondria.

A range of concentrations of each chemical were applied to Himalaya *Sln1a* leaves. The optimum concentration, i.e. the concentration at which cell death lesions were visible at 2-4 days after treatment, were as follows; Paraquat, 25 µM; Alloxan, 500 mM and Menadione, 10 mM. Detached leaves (5 cm) were suspended across agar bridges as described above, 2 µl of treatment supplemented with 0.01% Tween 20 to facilitate drop adhesion was placed onto the leaf surface with a pipette. Leaves were incubated at 15˚C in constant light, necessary to maximise ROS production. Subsequent assays included *Sln1d* and *sln1c* lines. Assays were repeated many times with similar results.

5.2.3. **ROS staining**

Nitroblue tetrazolium (NBT) is reduced to a blue NBT formazan precipitate upon its reduction by O_2^-. NBT (Sigma) was dissolved at a concentration of 300µM in a 10mM potassium phosphate buffer (45.4 ml of K_2HPO_4 (1M) and 4.6 ml of KH_2PO_4 (1M) adjusted to pH 7.8 and diluted 1:10 with water). Detached leaves were submerged in staining solution contained within a Petri dish and vacuum infiltrated three times for 3 min. Chlorophyll was removed from the detached leaves using a clearing/fixing solution (1:3, chloroform:ethanol).

2', 7' Dichlorfluorescein-diacetate (DCFH-DA) emits green fluorescence when oxidised by H_2O_2. DCFH-DA (Sigma) was dissolved at a concentration of 60 mM in DMSO and diluted 1:100 with a buffer (KCl (1mM), $MgCl_2$ (1mM), $CaC1_2$ (1mM), 2-morpholinoethanesulfonic acid monohydrate (5mM) and adjusted to pH 6.1 with NaOH) for a 60µM working solution. Detached leaves were submerged in DCFH-DA solution contained within a Petri dish and vacuum infiltrated three times for 3 min. Stained leaves were mounted on microscope slides and observed under a fluorescence microscope (Nikon Eclipse 800, 2x magnification lens fitted with a FITC filter). Due to the light sensitivity of DCFH-DA all steps were carried out under aluminium foil.

In both cases the mechanical damage at the cut ends of the detached leaf provide a useful control for staining.

5.2.4. **Oxidative burst assay**

Oxidative burst assays were carried out as described by Felix *et al.* (1999). Briefly, leaf disks were cut from leaves using a cork borer and placed in individual wells of a 96 well white microtitre plate each containing 200 µl of water and incubated overnight under aluminium foil. 20 µl of Luminol dissolved to a concentration of 100nM in DMSO and 20 µl of peroxidise dissolved in water (10 mg/ml) were combined with 10 ml of water. Luminol solution was supplemented with either an elicitor (flg22 to a final concentration of 100 nM or chitin to a final concentration of 4 g/L) or water (control). The water was removed from the wells of the prepared leaf disks and replaced with 100 µl of PAMP solution. Fluorescence was measured using a Varioskan flash reader (Thermo Scientific).

5.2.5. **qRT-PCR**

The expression of two superoxide dismutase genes (*Cu/ZnSOD* and *MnSOD*) and a catalase gene (*CAT-1*) were measured. Cu/ZnSOD and MnSOD are localised to the chloroplast and the mitochondria respectively so provide useful markers for the antioxidant activity in these organelles (Foyer & Noctor, 2003). RNA was extracted and processed as previously described. Primers (Table 5.1) were designed from alignments of barley EST sequence to annotated Arabidopsis sequence.

Table 5.1. Table of primers for monitoring expression of antioxidant genes.

Gene	Forward	Reverse
Cu/Zn SOD	TCGGTGACACGACTAATGGA	TGTCTCTGCCACACCTTCAG
Mn SOD	CAACAAGGCGCTCGAG	ATTTGATGGCGCTCTGG
CAT-1	CCCGTCTGGAACAACAAC	CCCCGTGCATGAACAAC

5.2.6. **Pathosystems**

5.2.6.1. Barley – *B. graminis*

Golden Promise (*Sln1a*) and Himalaya lines (*Sln1a*, *Sln1d* and *sln1c*) grown at three light intensities, as described above, were tested for relative resistance to *B. graminis f. sp hordei*. Leaf one of plants of each line/treatment was isolated when the HL grown plants were 10 days old. Sections (2 cm) of detached leaves were inoculated as described previously. Leaves were incubated under LL after inoculation and scored by counting colonies per cm^2 and assessing infection type 8 dpi.

Subsequently the experiment was carried out on whole plants. The first leaves of the seedlings were fastened onto Perspex settling tables with string. Plants were inoculated in a large settling tower

with an incorporated rotating table to ensure homogeneous inoculum load. Seedlings were incubated under LL after inoculation and scored as above. Microscope slides and check cultivar (Golden Promise) seedlings were positioned around the treated lines to confirm homogeneity of inoculation. Spore numbers were counted on the slides and subsequently colonies were counted on check cultivars 8 dpi.

In a separate experiment a cross-over design was used. Using the detached leaf assay described above, lines grown in either HL or LL before treatment were incubated in either LL or HL for 24 hours after treatment before being returned to LL for the remaining 7 days, then scored as above.

Barley DELLA differential lines were treated with an inappropriate *forma specialis*, *B. graminis f. sp. tritici* isolate, using the detached leaf assay previously described. Leaves of wheat cv. Cerco were included as susceptible controls.

5.2.6.2. Barley – *R. collo-cygni*

Kirsty (*Sln1a*) and Himalaya lines (*Sln1a* and *Sln1d*) grown at three light intensities, as described above, were tested for relative resistance to *R. collo-cygni.* Whole plants were inoculated when HL grown plants were 10 days old. Inoculum was prepared and applied as described by Makepeace *et al.*(2008). The experiment was repeated twice.

5.2.6.3. Barley – *F. graminearum*

Himalaya lines (*Sln1a* and *Sln1d*) grown at three light intensities, as described above, were tested for relative resistance to *F. gaminearum.* The first and second leaves of each line/treatment were collected when the HL grown plants were 10 days old. Sections (5 cm) of the detached leaves were suspended over agar bridges and wound inoculated as described previously. A highly virulent DON producing *F. graminearum* isolate, S1 was used due to the inherently high level of resistance of barley. Leaves were incubated in low light at 20˚C and high humidity was maintained by spraying the lid of the boxes containing the leaf segments with water. Lesion areas were measured after 4 days.

5.3. Results

5.3.1. Influence of DELLA on ROS induced cell death

In chapter 4 I demonstrated that wheat and barley lines in which the DELLA protein is thought to accumulate showed a general increase in resistance to necrotrophs and an increase in susceptibility to biotrophs, and the converse was shown in barley DELLA loss of function lines. In order to test whether DELLA influences ROS induced cell death a range of pharmacological ROS inducers

were applied and resulting cell death was measured. Barley lines were used because the presence of a single DELLA encoding gene means that both GoF and LoF lines are available.

Cell death resulting from paraquat treatment (Fig 5.2 a & b), which induces superoxide production in the chloroplast, was significantly ($P<0.01$) reduced in *Sln1d* lines compared to *Sln1a* containing lines. Conversely *sln1c* lines exhibited extensive cell death lesions around the treatment site which was significantly ($P<0.001$) greater than *Sln1a* containing lines. No cell death was observed in control (water supplemented with 0.01% Tween) leaves (data not shown).

Alloxan treatment, which induces hydrogen peroxide production in the mitochondria, resulted in extensive cell death in all three lines tested however no significant difference in cell death lesion size was observed (Fig 5.2 c & d).

To test whether the origin or type of ROS elicited by paraquat and alloxan resulted in the DELLA dependent differential observed the same lines were treated with menadione, which induces superoxide production in the mitochondria. At the concentration used, menadione treatment caused relatively little cell death, however cell death spread was significantly ($P<0.01$) reduced in *Sln1d* lines and significantly ($P<0.001$) increased in *sln1c* lines relative to *Sln1a* (Fig 5.2 e & f).

5.3.2. **Influence of DELLA on ROS accumulation**

The superoxide anion, actively produced by NADPH oxidase and by organelles in response to pathogen attack, has been associated with the elicitation of defence mechanisms including the HR. Treatment of barley *Sln1a*, *Sln1d* and *sln1c* lines with paraquat and menadione, which both induce superoxide production, show a DELLA dependent propensity to cell death. The accumulation of the superoxide anion was therefore measured in paraquat treated barley lines allelic at the *Sln1* locus.

Two hours after paraquat treatment of *Sln1a* lines blue formazan precipitate was observed in most of the cells throughout the detached leaf suggesting a systemic accumulation of O_2^- (Fig. 5.3a). Control samples and samples stained 30m, 1h and 4h after paraquat treatment only showed formazan precipitation at the cut ends providing a good control for the staining procedure in these leaves. Interestingly, the amount of blue precipitate was greatest distal from the treatment site whilst reduced in the area surrounding the site (end nearest diagonal cut end). Apart from staining at the cut ends no blue precipitate was observed in the detached leaves of *Sln1d* lines at any of the time points measured in this experiment. In contrast, a high level of O_2^- accumulation was observed in *sln1c* untreated control leaves, evident from the deep blue precipitation of formazan. As a result of the control showing high levels of O_2^- accumulation it is not evident whether blue formazan precipitation reporting O_2^- accumulation at subsequent time points after treatment is a result of

paraquat treatment or because of endogenously high levels of O_2^-. Interestingly, a reduction in blue precipitate is evident in the cells underlying and surrounding the treated area at 1 and 2 h after treatment (arrows in

5.3a). After 4 h O_2^- accumulation appeared to be reduced systemically in *sln1c* relative to the control sample.

The dismutation product of O_2^-, H_2O_2, was measured in *Sln1a* lines and detected 4 h after treatment with paraquat (data not shown) using DCFH-DA. Paraquat treated *Sln1* mutant allele containing lines stained with DCFH-DA 4 h after treatment show a visual difference in H_2O_2 accumulation (Fig. 5.3b). *Sln1d* exhibit reduced fluorescence whilst *sln1c* showed increased fluorescence relative to *Sln1a* paraquat treated lines consistent with the pattern of O_2^- accumulation observed using NBT staining.

The blue staining of untreated control *sln1c* lines suggest that O_2^- levels may be endogenous high in these lines. To test this whole *sln1c* plants were stained with NBT. The staining confirms that O_2^- accumulates to high levels systemically, particularly in younger tissue (Fig. 5.4a). Staining of a developmental series of wild type plants grown at ML appears to suggest that O_2^- levels are altered dependent on developmental stage of the leaf. The newly emerged 3rd leaf and the 1st leaf are stained blue whilst the 2nd leaf is only stained at the cut end as a result of mechanical damage (Fig. 5.4b). Staining of *Sln1d* line was reduced relative to the *slnlc* line (data not shown).

5.3.3. **Influence of DELLA on PAMP triggered immunity**

One of the earliest host responses to pathogen attack is PTI. The recognition of a PAMP by the host triggers, among other things an oxidative burst. To test whether DELLA status influences the ROS burst resulting from PTI, barley lines differing in DELLA status were assessed for their PTI response. Flg22 and chitin were applied to Himalaya *Sln1a* lines. Flg22 elicited a ROS burst in barley but chitin did not elicit a response at the concentration tested (data not shown). The strength of the flg22 elicited ROS burst was then assessed in the *Sln1* mutant allele containing lines. The ROS burst elicited by flg22 is significantly reduced in *Sln1d* lines relative to *Sln1a* (Fig 5.5). Multiple assays were carried out with *sln1c* lines however the strength of the ROS burst relative to *Sln1a* lines was not consistent. In the majority of assays the intensity of the ROS burst was reduced relative to *Sln1a* but greater than *Sln1d*.

5.3.4. **DELLA and non-host resistance**

PTI is a component of non-host resistance. To test whether the reduction in the oxidative burst triggered by PAMP recognition, as observed in *Sln1d* lines, compromises non-host resistance, barley lines differing at the *Sln1* locus were challenged with an isolate of the inappropriate formae

speciales *B. graminis f. sp. tritici*. The susceptible wheat cultivar Cerco exhibits disease susceptibility (Fig. 5.6). None of the barley lines exhibited any macroscopic symptoms or evidence of disease development.

5.3.5. **Fluence and DELLA effect ROS**

The effects of DELLA on ROS metabolism have been described above and are likely to contribute to the biotroph-necrotroph resistance trade-off observed in the previous chapter. Fluence has also been shown to influence ROS metabolism so I next investigated the effect of the combination of fluence and DELLA on the outcome on pathogen resistance in three pathosystems representing each of the nutritional strategies investigated in the previous chapter (biotroph, hemibiotroph and necrotroph). Barley plants allelic at the *Sln1* locus were grown in three light environments from seed and sampled when high light grown seedlings were 10 days old. This set of experiments thus investigates the effect of SAA on the ability of a plant to respond to a subsequent biotic stress. In addition cross-over experiments were used to assess the effect of EEE on susceptibility to a subsequent biotic stress in the barley- *B. graminis f. sp. hordei* pathosystem.

5.3.5.1. Influence of fluence and DELLA on the expression of antioxidant enzymes and O_2^- accumulation

Superoxide accumulation (NBT staining) and the expression of genes encoding antioxidant enzymes was measured in *Sln1a* and *Sln1d* lines exposed to the three light treatments used in this study to evaluate their relative ROS accumulation and antioxidant activity. DELLA loss of function lines were not used in this portion of the study due to insufficient seed being available.

Visually the blue precipitation of formazan, which reports superoxide accumulation, was evident in a larger proportion of cells of leaves of both *Sln1a* and *Sln1d* lines in LL and HL grown plants relative to ML grown plants (Fig. 5.7a).

The effect of fluence and DELLA on the expression of a selection of antioxidant genes was measured (Fig. 5.7b). The expression of *CAT-1*, which encodes a catalase, was highly influenced by fluence. LL grown plants had greater expression of *CAT-1* compared to ML grown plants which in turn showed greater expression compared to HL grown plants. A DELLA effect was also evident, with increased *CAT-1* expression in *Sln1d* lines relative to *Sln1a* lines grown in ML and HL, however this is not observed at LL. Overall, the reduction in *CAT-1* expression with increasing fluence was greater in the *Sln1a* line than the *Sln1d* line. *Cu/ZnSOD* expression was reduced in HL relative to ML and LL grown plants. Again, the reduction in expression of Cu/ZnSOD with increasing fluence was greater in *Sln1a* than its dwarf, *Sln1d* counterpart. *Cu/ZnSOD* expression was significantly greater in *Sln1d* lines in HL grown plants. In contrast expression of *MnSOD* was not affected by fluence in either line.

5.3.5.2. Influence of fluence and DELLA on *B. graminis* susceptibility

To investigate the effects of fluence and DELLA on the disease outcome of a biotrophic pathogen barley *Sln1a* and *Sln1d* lines grown at three light intensities were challenged with *Blumeria graminis f. sp. hordei*. The Barley-*Blumeria graminis f. sp. hordei* pathosystem used previously required a detached leaf experimental design. However when used to assess the effect of pre inoculation light intensity HL grown plants exhibited accelerated senescence upon detachment. Senesing cells may affect the interaction with the fungus and for this reason the experiment was repeated using an attached leaf assay design. The attached leaf assay showed a highly significant (<0.001) genotype effect in which *Sln1d* plants were more susceptible to *Bgh* compared to *Sln1a* plants (Fig. 5.8) as previously shown. A significant (<0.001) effect of pre-inoculation fluence on susceptibility was also observed. ML grown plants were more susceptible than those grown in LL which in turn were more susceptible than those grown in HL. Pre-inoculation light level affected relative susceptibility but did not affect the increased susceptibility conferred by DELLA stabilisation, as shown by the interaction term (light level and genotype) being insignificant (P=0.517).

Cross over experiments, in which plants grown in LL or HL before inoculation were transferred to either LL or HL after inoculation were carried out. Significant effects of treatment (P<0.001), genotype (P<0.001) and a significant interaction (P<0.001) between treatment and genotype was detected (Fig. 5.9a). No effect of block (box) and replicate (side) was detected.

The results confirmed the finding that plants exposed to HL before inoculation were more resistant to infection compared to plants conditioned to LL prior to inoculation (Fig 5.9 b & c). When HL pre-conditioned leaves were returned to a HL environment after inoculation they were highly resistant. When HL preconditioned leaves were transferred to a LL environment after inoculation susceptibility increased relative to leaves exposed to HL post-inoculation. The exposure of LL preconditioned leaves to HL is expected to result in EEE. Transfer of leaves from LL to HL following inoculation resulted in an increase in susceptibility in check cultivar Golden Promise and the Himalaya *Sln1d* line. In contrast, no increase in susceptibility was observed in the Himalaya *Sln1a* or *sln1c* lines. *Sln1d* lines were however more susceptible to *Bgh* as compared to *Sln1a* in all of the conditions tested. No significant difference was detected between *Sln1a* and *sln1c* disease susceptibility in any of the treatments in this experiment.

5.3.5.3. Influence of fluence and DELLA on *R. collo-cygni* susceptibility

To assess the effects of fluence and DELLA on the disease outcome of a hemibiotrophic pathogen barley *Sln1a* and *Sln1d* lines grown at three light intensities were challenged with *R. collo-cygni*. Pre-inoculation light intensity has previously been demonstrated to influence the outcome of *R.*

collo-cygni infection (Makepeace & Brown, 2009). DELLA stabilised barley lines have been shown to be more susceptible to *R. collo-cygni* (Chapter 4; Fig. 4.5) postulated to reflect the relatively long biotrophic, endophytic phase the pathogen undertakes. *Sln1d* was significantly more susceptible ($P<0.05$) than *Sln1a* plants when preconditioned in ML (Fig. 5.10) confirming my earlier results. However, no difference in susceptibility was observed between *Sln1a* and *Sln1d* plants preconditioned to LL or HL resulting in a non-significant genotype effect overall. A significant ($P=0.036$) effect of pre-inoculation light intensity was observed with ML grown plants showing the greatest susceptibility followed by HL and LL grown plants (Fig. 5.10).

5.3.5.4. Influence of fluence and DELLA on *F. graminearum* susceptibility

In order to investigate the effects of SAA on necrotrophic disease susceptibility foliar disease spread from wound inoculation with *F. graminearum* was measured. Overall the genotype effect was highly significant (<0.001; Fig. 5.11). ML pre-conditioned *Sln1d* lines were significantly more resistant compared to *Sln1a* lines as previously reported. HL grown *Sln1d* plants were less resistant than ML grown plants resulting in a reduced genotype differential with respect to *Sln1a*. In contrast there was no significant difference in susceptibility between genotypes grown in LL. The pre inoculation light level to which the plants were exposed had a highly significant (<0.001) effect on susceptibility to subsequent infection with *F. graminearum*. HL preconditioned plants were most susceptible, followed by *Sln1a* ML grown plants then LL grown plants. *Sln1d* plants exhibited similar, high levels of resistance to lesion development when preconditioned under ML and LL fluence. A significant (0.022) light level x genotype interaction was observed due to the relative difference in DELLA conferred resistance depending on the pre-conditioning light level (Fig. 5.11).

5.4. Discussion

5.4.1. ROS inducing cell death is restrained by DELLA

In this study pharmacological inducers of ROS have been used to elucidate the role that DELLA confers in ROS-induced cell death. The chemicals used have been reported to induce ROS production in an organellar and ROS type specific manner (Bowler *et al*., 1991). The results show that superoxide inducing pharmacological treatments, paraquat and menadione, cause an altered cell death phenotype in a DELLA dependent manner, whilst no DELLA dependent differential was observed when treated with the hydrogen peroxide inducing chemical, alloxan. Therefore it can be postulated that DELLA controls O_2^- accumulation. Additionally it is possible that O_2^- generated signals are mediated, at least in part through DELLA-dependent pathways.

O_2^- production occurs in response to biotic and abiotic stress conditions. The main sources of O_2^- are chloroplasts and the plasma membrane localised NADPH oxidases. Plants have localised ROS detoxification systems that allow the cell to control ROS accumulation. Work in Arabidopsis has shown that DELLAs do not modulate NADPH-oxidase activity suggesting that O_2^- production itself is not DELLA regulated (Achard *et al.*, 2008b). However the expression of *CSD1* and *2*, which encode chloroplast localised Cu/Zn-SOD, are increased in DELLA accumulating Arabidopsis lines (*ga1-3*) and reduced in DELLA LoF lines (*ga1-3* quadruple DELLA) in comparison to WT plants (Achard *et al.*, 2008b). This was also shown for Cu/Zn-SOD protein and activity levels. Catalase activity was also shown to be enhanced in DELLA accumulating lines (*ga1-3*). The findings suggest that in Arabidopsis DELLA reduces ROS induced cell death by acting upon the ROS scavenging system. The finding, reported in the previous chapter, that DELLA GoF lines exhibit an enhanced deoxynivalenol-induced up regulation of the negative cell death regulators, *BI-1* and *CEO* (Fig. 4.10), suggest DELLAs observed role in negatively controlling cell death may be more complex than simply reducing ROS accumulation.

5.4.2. **ROS accumulation is reduced by DELLA**

The ROS staining protocols used in this study provide a qualitative and at best semi-quantitative measure of ROS accumulation. A quantitative assessment of formazan precipitation at a cellular level provides a better measure of O_2^- and crucially can also determine the subcellullar localisation of O_2^- accumulation. Superoxide accumulation was observed in paraquat treated *Sln1a* lines 2h after treatment throughout the leaf suggesting that the superoxide generated at the treatment site creates a signal systemically throughout the leaf initiating superoxide induction in distal cells. Although the systemic signal or source of the O_2^- is undetermined in this study, *Rboh* encoded NADPH oxidase is a strong candidate. NADPH oxidase has been demonstrated to propagate cell-to-cell signalling by producing apoplastic O_2^- (Joo *et al.*, 2005). The subsequent dismutation to H_2O_2 leads to an up regulation of the antioxidant system. Interestingly a localised suppression of O_2^- is observed around the treatment site, which may suggest an elicitation of localised antioxidant activity. While the disappearance of O_2^- by 4 h after treatment throughout the leaf suggests that the antioxidant system has been initiated systemically. Paraquat pre-treatment of cucumber has been shown to confer an increase in systemic resistance to *Colletotrichum lagenarium* (a necrotroph; Strobel & Kuc, 1995) suggesting paraquat induced chloroplastically localised O_2^- can induce a systemic increase in antioxidant systems consistent with the present study.

No paraquat induced superoxide accumulation was observed at the time points tested in *Sln1d* lines suggesting that DELLA is reducing paraquat induced superoxide accumulation, consistent with the findings above. Endogenous levels of O_2^- in *sln1c* containing lines are elevated compared to *Sln1a* and *Sln1d* lines evident in the control samples (Fig. 5.3a) and from whole plant staining (Fig. 5.4a).

This may result from a reduction of endogenous *CSD* gene expression and possibly the antioxidant system as a whole, as has been suggested in Arabidopsis DELLA LoF lines (Achard *et al.*, 2008b). This would result in an accumulation of ROS produced as a by-product of normal RET and PET activity. *sln1c* lines do however exhibit localised and systemic antioxidant activity at 2 and 4h respectively after paraquat treatment, albeit to a lesser extent relative to *Sln1a*. Therefore the systemic accumulation of O_2^- most likely in the apoplast, may be detoxified by antioxidant systems independent of DELLA.

The dismutation product of O_2^-, H_2O_2, was measured 4 h after paraquat treatment. H_2O_2 accumulation, reported by DCFH-DA fluorescence, was inversely proportional to DELLA accumulation. Achard *et al*. (2008b) demonstrated that catalase activity is increased in DELLA accumulating lines which would explain the pattern of H_2O_2 levels observed in GoF, wild type and LoF lines in this study. The Achard study did not however demonstrate that this is a result of DELLA regulating CAT transcript and protein levels. It may be that the activity of catalase is responsive to the elevated O_2^- levels.

Measurement of antioxidant gene expression in paraquat treated leaves allelic at the *Sln1* locus would be an interesting follow up study. Isolation of leaf material proximal and distal to the treatment site would be informative as to the differential activation of localised and systemic antioxidant systems in *Sln1* allelic lines. NBT and DAB staining, reporting O_2^- and H_2O_2 accumulation respectively, of a more detailed time course after treatment with paraquat would complement the expression data to provide a greater understanding of the differential ROS dynamics within the *Sln1* allelic series. Together the data may reveal a "wave" of ROS accumulation (O_2^- followed by H_2O_2 resulting from dismutation) and in its wake an up-regulation of the antioxidant coinciding with a decrease in ROS accumulation.

5.4.3. **DELLAs involvement in PTI**

In this study chitin, an important component of fungal cell walls, did not elicit a response in barley at the concentrations tested. Flg22, a 22 a.a. peptide derived from flagellin, elicits a PTI response in Arabidopsis similar to that of chitin and other fungal derived PAMPs and when tested in barley in this study elicited a ROS burst. For this reason, although not of great agronomic importance, flg22 was used to assess the influence of DELLA on the PAMP induced ROS burst. The flg22 induced ROS burst in DELLA stabilised lines was consistently reduced relative to wild type lines suggesting that DELLA plays a role in PTI. The DELLA LoF mutant exhibited a highly variable response possibly reflecting the general disruption of ROS homeostasis postulated in this mutant. It is not known from the present work whether DELLA is directly involved in PTI signal transduction

or whether the dampening of the ROS burst in *Sln1d* lines is due to DELLAs enhanced innate antioxidant system.

The non-host defence response involves a myriad of constitutive mechanisms such as wax layers, rigid cell walls and antimicrobial secondary metabolites (Thordal-Christensen, 2003). If the constitutive mechanisms are unsuccessful at staving off the potential pathogen the plant deploys inducible mechanisms such as the formation of cell wall appositions (CWA) and PTI. Barley lines allelic at the *Sln1* locus were inoculated with an inappropriate wheat pathogen *Blumeria graminis f. sp. tritici* isolate to test whether the reduction in the intensity of the PTI burst in DELLA GoF mutants would compromise non-host resistance. The results indicate that non-host resistance is not compromised in *Sld1d* lines with the isolate tested suggestive that CWA or constitutive defence responses were sufficient to prevent infection. Interestingly transient overexpression of *BI-1*, a suppressor of ROS inducing cell death has been shown to be compromised in non-host resistance (Eichmann *et al*., 2004), furthermore the authors show that overexpression of both *BI-1* and *Mlo* enhanced susceptibility to non-host *Bgt* even further. Other *Bgt* isolates could be tested to further investigate this interesting hypothesis. However it is worth noting that if the cell death response elicited by PTI is compromised in DELLA accumulating lines, a non-host pathogen will not necessarily cause disease due to other defence responses functioning normally as shown by Zurbriggen and colleagues (2009). Microscopic analysis of individual challenged cells may reveal an effect of DELLA on PTI, not visible at the macroscopic level.

A reduction in the levels of ROS accumulation conferred by DELLA acting on the ROS scavenging system results in the increased tolerance to paraquat-induced cell death in the present study. The reduced propensity to ROS induced cell death conferred by DELLA has previously been demonstrated to promote survival to abiotic stress (Achard *et al*., 2006) and resistance to necrotrophs in Arabidopsis (Achard *et al.*, 2008b). It will also have a significant part to play in the biotroph-necrotroph resistance trade-off demonstrated in monocotyledonous crop species in the previous chapter. These findings taken together form a model for DELLAs role in stress induced cell death (Fig. 5.12). Adapted from a model proposed by Mullineaux and Baker (2010) this model proposes that in a wild type (*Sln1a*) plant, increasing stress, be it biotic or abiotic causes a response in the form of increasing ROS production. A threshold exists below which ROS functions as a signalling molecule leading to acclimation or resistance to the stress and above which oxidative damage occurs followed by the initiation of cell death. In DELLA accumulating lines (*Sln1d*) an increase in ROS turnover means that more stress is required to produce sufficient ROS to meet the cell death threshold. In the case of abiotic stress and necrotrophic pathogens the consequence is greater tolerance/resistance whilst in the case of biotrophic pathogens an increase in susceptibility is observed due to reduced effectiveness of the hypersensitive cell death response. In contrast, the LoF line requires less stress to reach the cell death threshold and therefore exhibits increased

sensitivity/susceptibility to environmental adversity and necrotrophic pathogens and increased hypersensitive cell death in response to biotrophic pathogens resulting in an increase in resistance. The point at which the cell death threshold is set may be dependent on preconditioning of the plant. Both biotic (SAR) and abiotic (SAA) factors can influence the threshold, the latter is expanded upon below.

5.4.4. **The effect of fluence and DELLA on ROS homeostasis**

Reduced O_2^- accumulation was observed in ML preconditioned plants relative to LL and HL grown plants. It has been demonstrated that an increase in abiotic stress in a plant causes oxidative stress due to EEE and, in response to this, an increase in the antioxidant system is observed systemically. It is therefore conceivable that LL grown plants have little or no systemic antioxidant activity; ML preconditioned plants have undergone SAA leading to an increase in systemic antioxidant activity and the subsequent systemic reduction in O_2^- accumulation and HL pre-conditioned plants have an active systemic antioxidant system which is near saturation with respect to its ability to cope with the large amount of photo produced ROS, resulting in O_2^- accumulation. Therefore the light levels used in this study are differentially affecting ROS homeostasis, through the modulation of both production and detoxification. DELLA has been shown to influence ROS detoxification but no obvious visual differences were observed between the *Sln1a* and *Sln1d* lines. DELLA may not affect photo produced ROS levels due to the cellular compartmentalisation of DELLA conferred increases in antioxidant activity (perhaps intracellularly localised) and the light induced production and propagation of ROS (apoplastically localised).

In an attempt to examine the effect of light and DELLA on the activity of the antioxidant system the gene expression of two SOD encoding genes; Cu/ZnSOD and MnSOD (localised to the chloroplast and mitochondria respectively) and catalase was measured. The expected increase in antioxidant activity with increasing light was not observed in any of the genes quantified, in fact catalase and Cu/ZnSOD expression decreased with increasing light. Cold treatment of wheat and barley lines resulted in a decrease in catalase activity (Janda *et al*., 2003). The reduction in catalase transcription observed with increasing light in this study is consistent with a reduction in catalase activity in response to cold treatment which can also lead to EEE and SAA (Okuda *et al*., 1991). Salicylic acid, which increases with light fluence, has been implicated in inhibiting catalase activity (Sanchez-casas & Klessig, 1994; Janda *et al*., 1999) which may in turn perturb catalase expression.

Endogenous expression of Cu/ZnSOD encoding genes have previously been demonstrated to be more abundant in Arabidopsis DELLA accumulating lines compared to WT (Achard *et al*., 2008). In the present study a significant increase in expression of Cu/ZnSOD encoding genes was observed in HL preconditioned *Sln1d* plants relative to *Sln1a*, with a similar but not significant

trend also evident in LL and ML preconditioned plants. This may suggest that the DELLA conferred increase in Cu/ZnSOD is exaggerated under adverse conditions.

APX2 encodes ASCORBATE PEROXIDASE 2, a cytosolic localised peroxidase, which has been shown to be transcriptionally up-regulated in high light induced SAA using Arabidopsis *APX2*-luciferase reporter lines (Karpinski *et al*., 1999). *APX2* expression has also been demonstrated to increase in response to drought (Rossel *et al*., 2006) and high temperature (Kotak *et al*., 2007) both of which initiate EEE. Analysis of publicly available Arabidopsis microarray data at Genvestigator (Zimmermann *et al*., 2004) substantiate these findings, showing that *APX2* is highly up regulated in conditions that promote EEE, whereas the perturbations of catalase and SOD encoding genes in the same experiments were minimal. These findings together suggest that APX2 expression provides a marker for EEE. Due to insufficient time and the absence of a fully annotated barley genome an *APX2* gene specific expression assay was not developed and implemented. If time permitted it would have been interesting to investigate whether APX2 expression and resulting activity in barley is influenced by stress and/or DELLA.

In order to fully interpret the outcomes of the pathology data that follows a greater understanding of the extent of fluence and DELLA on SAA in the material would be required. APX2 expression and activity have been demonstrated to provide a useful marker for SAA (Karpinski *et al* 1999). Chlorophyll fluorescence provides an indicator of photosynthetic performance and photoinhibition (Baker, 2008) and may provide evidence of any differential effects conferred by DELLA.

In addition to altered ROS homeostasis conferred by SAA, a complex network of signalling transduction pathways are influenced including SA, JA and ethylene leading to common gene sets being elicited to those induced by biotic stress (Sharma *et al.*, 1996; Borsani *et al.*, 2001; Kunkel & Brooks, 2002; Kangasjarvi *et al.*, 2005). Further investigation should therefore include the quantification of characterised SAA induced defence responses to see if they are altered in a DELLA and light level dependent manner in the plant material used. For example PR1, GPX7 and GST6 have each been demonstrated to be up regulated during SAA in Arabidopsis (Mullineaux *et al*., 2000) and all three participate in biotic stress responses (Alvarez *et al*., 1998).

5.4.5. **Influence of fluence and DELLA on *B. graminis* susceptibility**

Plants grown in medium light exhibit the greatest susceptibility to *B. graminis f. sp. hordei* infection. If, as suggested above, ML preconditioned plants have undergone SAA and associated increase in the antioxidative system then it could be postulated that ROS production initiated by the recognition of the pathogen maybe detoxified. A reduction in the contribution of pathogen derived

ROS in addition to the reduced steady state levels of ROS would result in total ROS levels in the challenged cell being insufficient to exceed the cell death threshold leading to pathogen establishment. Plants pre-conditioned in high light exhibit the least susceptibility to *Bgh* infection. It could be postulated that the high levels of environmental derived ROS, which exceed the levels that the antioxidative system can cope with, together with the ROS production elicited by the pathogen exceeds the cell death threshold thus resulting in increased frequency of hypersensitive cell death reactions leading to an increase in resistance. Microscopic analysis of individual challenged cells would be informative as to the contribution that hypersensitive cell death is conferring to the altered disease outcomes observed. Ozone initiates EEE and subsequent SAA, Arabidopsis plants exposed to ozone prior to inoculation with a virulent isolate of *Pseudomonas syringae pv. maculicola* were more resistant than plants grown in ambient air (Sharma *et al*., 1996). This may have resulted from a combination of a greater propensity to cell death conferred by an increase of environmental derived ROS and an increase in SA driven defence mechanisms which were demonstrated to be up regulated in ozone treated plants.

The transfer of LL grown plants to HL, expected to result in EEE, combined with the simultaneous inoculation with *Bgh* resulted in an increase in susceptibility compared to plants that were kept at low light post inoculation (Fig. 5.9). Plants exposed to EEE induce localised increase in antioxidant activity to cope with the increase in oxidative stress. The antioxidative system may initially be highly active in order to reduce oxidative stress caused by EEE resulting in a reduction in environmental ROS to an extent that pathogen induced ROS is insufficient to exceed the cell death threshold. This contrasts with the increased resistance conferred by plants that have been grown in HL prior to inoculation, possibly suggesting that over time the antioxidative system is reduced as plants become acclimatised to the HL.

This suggests that the timing of the initial abiotic stress in relation to the subsequent biotic stress is an important consideration, also demonstrated in a study by Mittler *et al*. (1999). In that study tobacco plants were inoculated with an incompatible bacterial strain whilst simultaneously exposed to elevated oxygen pressure, which causes oxidative stress, resulting in a stimulation of HR leading to resistance as expected. Conversely, when plants were exposed to elevated oxygen pressure prior to inoculation with an incompatible bacterium strain the HR was suppressed, supposedly as a result of the induced antioxidant capacity dampening the pathogen induced ROS burst. The latter finding correlates with the ML preconditioned plants in the present study exhibiting increased susceptibility. The altered disease outcome of HL pre-conditioned plants, which is postulated to swamp the antioxidative system, highlights that the disease outcome is dependent on the degree of the applied abiotic stress treatment in addition to the timing of inoculation in relation to the timing of a preceding abiotic stress treatment (discussed above) and the nutritional lifestyle of the pathogen (continued below).

5.4.6. **Influence of fluence and DELLA on *R. collo-cygni* susceptibility.**

R. collo-cygni has a strong association with abiotic stress; disease establishment in the field tends to follow a period of full sun preceded by rain near the end of the growing season (Brown, pers. comm.). In this study plants pre-conditioned in ML show the greatest level of susceptibility consistent with the findings of Makepeace and Brown (2009). The finding is also consistent with the results with the obligate biotroph, *B. graminis f. sp. hordei* above, and likely reflects the long biotrophic growth phase that is characteristic of *R. collo-cygni*. Unlike the barley- *B. graminis f. sp. hordei* pathosystem, the DELLA conferred increase in resistance is not evident in plants preconditioned in LL or HL. This erosion of the DELLA conferred differential may result from the fungus adapting the point at which it makes the switch to necrotrophic nutrition in reaction to the physiological state of the plant. *R. collo-cygni* has been demonstrated to exhibit symptoms of necrotrophic nutrition at the end of the season when, in combination with optimum environmental conditions for the fungus, the antioxidant system of the host plant is degraded (Schutzendubel *et al*., 2008). Similarly, Wei *et al.* (1997) demonstrated that senescence of mallow plants greatly accelerated symptom development of the hemibiotroph *Colletotrichum gloeosporioides f. sp. malvae,* whilst treatment with glutathione, an antioxidant, lengthened the biotrophic stage and delayed symptom development. In the present study, although seedlings have been challenged, the increase levels of ROS in LL and HL preconditioned plants, maybe sufficient to trigger necrotrophic growth, thus shortening the biotrophic phase. *Sln1d* lines have been shown to be more resistant to necrotrophic growth (Chapter 4), resulting in an erosion of the resistance differential between *Sln1a* and *Sln1d* lines.

5.4.7. **Influence of fluence and DELLA on *F. graminearum* susceptibility**

HL preconditioned plants exhibit the greatest level of susceptibility to *F. graminearum*, which is the reverse outcome of the biotrophic pathosystem. The addition of pathogen induced ROS to the already elevated steady state levels of ROS in HL grown plants may lead to more extensive cell death which confers susceptibility to necrotrophs but leads to resistance to biotrophs. Environmental turnover, conferred by preconditioning to ML, alone is insufficient to moderate the ROS induced by *F. graminearum* infection to levels below the cell death threshold in *Sln1a* plants, resulting in a similar level of resistance to that observed in HL grown *Sln1a* plants. Whilst DELLA conferred turnover in addition to environmental turnover in *Sln1d* plants reduces cell death and disease development. The overall trend resembles the model proposed in Fig 5.12 in which DELLA GoF mutants are able to tolerate more stress induced ROS production before exceeding the cell death threshold. In light of the data presented here, it could be concluded that a preceding abiotic stress, by altering the ROS homeostasis of a plant, affects the disease outcome of a second biotic stress. It is not known whether DELLA is modulating just the pathogen derived ROS or both the

pathogen and environmentally derived ROS in this present work. To address this the antioxidant system up regulated in response to EEE, of which APX2 has been identified as a major contributor, could be quantified in the *Sln1* allelic lines.

5.4.8. **Chloroplast as central abiotic and biotic response node**

Apoplastic generation of ROS by NADPH oxidases has been considered as the major contributor to host defence responses to pathogens but the chloroplast is emerging as an important component in the initiation of ROS signalling. The full manifestation of the HR requires light, and is delayed or abolished in the dark (Montillet *et al.*, 2005; Chandra-Shekara *et al.*, 2006), strongly implicating chloroplastically derived ROS in the initiation of HR. Liu *et al.* (2007) suggest that pathogens elicit a MAP kinase pathway (SIPK/Ntf4/WIPK) which signals a reduction in carbon fixation in the chloroplasts resulting in excess excitation energy, in plants grown in the light, and the resulting generation of ROS in the chloroplasts. The authors propose that the initial chloroplast derived ROS burst subsequently activates NADPH oxidase action leading to a second more substantial extracellular ROS burst. Further investigation by Liu *et al.* (2007) demonstrate that increasing light intensity further exacerbates the excess excitation energy condition in chloroplasts, leading to a more intense ROS burst and accelerated cell death.

Ozone exposure elicits responses that resemble biotic defence including a biphasic oxidative burst and induction of the HR and SAR (Conklin & Last, 1995). Joo *et al* (2005) demonstrate that the first oxidative burst is confined to the chloroplast of guard cells, from where the signal was propagated to neighbouring cells and tissues in a NADPH oxidase dependent manner. Flavodoxin (Fld) is an electron shuttle present in prokaryotes and algae and when artificially expressed in chloroplasts of plants acts as a general antioxidant, preventing the formation of different types of ROS in chloroplasts (Tognetti *et al*., 2006). Fld-expressing plants infiltrated with a non-host pathogen resulted in lower ROS accumulation in chloroplasts compared to non-transformed plants, preventing hypersensitive cell death (Zurbriggen *et al*., 2009). The studies reported by Joo *et al* (2005) and Zurbriggen *et al.* (2009) further highlight the importance of chloroplast derived ROS in both abiotic and biotic stress responses.

There are multiple sources of ROS production in plants, including cell wall peroxidases, amine oxidases, NADPH oxidases and intracellular oxidases and peroxidases in chloroplasts, mitochondria, peroxisomes, and nuclei (Allan & Fluhr, 1997; Bolwell & Wojtaszek, 1997; Bowler & Fluhr, 2000; Corpas *et al.*, 2001; Laurenzi *et al.*, 2001; Bolwell *et al.*, 2002). DELLA has been implicated in modulating the levels of chloroplastic derived ROS through the ROS scavenging system, however whether DELLA influences other ROS sources directly or whether a reduction in chloroplast derived ROS influences downstream signalling is yet to be determined.

5.4.9. **Open questions as to how DELLA is influencing ROS homeostasis and resulting cell fate**

Growth and stress are often opposed, evident from growth retardation in response to abiotic and biotic stress. Many hormones have been demonstrated to affect DELLA action, either directly or indirectly, including auxin, ethylene, cytokinin and ABA (Achard *et al.*, 2003; Fu & Harberd, 2003; Achard *et al.*, 2006; Achard *et al.*, 2007; Weiss & Ori, 2007; Zentella *et al.*, 2007). Making DELLA an attractive candidate for an integrator of signals provided by hormones to prioritise either growth or stress responses.

DELLA have been shown to be positive regulators of ROS detoxifying enzymes in Arabidopsis, I further speculate that DELLA may also regulate the expression of genes which control cell death. Due to the lack of a recognisable DNA binding domain, DELLA is unlikely to regulate these genes directly. DELLA have been demonstrated to interact with PHYTOCHROME INTERACTING FACTORS to negatively regulate the transcription of their growth promoting gene targets. It may be that PIF proteins are negative regulators of the ROS detoxifying enzymes, thus enabling accumulation of growth-promoting ROS.

DELLA accumulation has been shown to positively correlate with increasing light intensity (Achard *et al.*, 2007) whilst, PIF accumulation has been shown to negatively correlate with increasing light intensity due to the action of PHYB marking PIFs for proteasomal degradation (Chen *et al.*, 2004). This raises the question of whether the shifting DELLA:PIF ratio with increasing light may also influence ROS homeostasis.

Previously I have shown that DELLA confers a resistance trade off in monocot crop species to pathogens with contrasting nutritional strategies. The work carried out in this chapter has provided insights on how the DELLA conferred resistance trade off is expressed in an adverse environment in which field grown crops are likely to have to contend, i.e. frequently experiencing conditions which promote EEE. This will particularly occur towards the end of the growing season of UK winter and spring cereal crops when high light and drought can be prevalent. I have shown that there is an affect of environment on disease resistance trade off and this is at least in part a result of an altered ROS homeostasis which is further exacerbated by DELLA. There remain many gaps in this study but the preliminary work carried out forms a basis for further exciting research.

6. General discussion

6.1. **Aims of study**

The main aim of this thesis was to gain a greater understanding of DELLA (RHT) in wheat. This is of significance because of the widespread use of GoF mutant alleles in modern semi-dwarf cultivars. To this end a homoeologue-specific qRT-PCR assay was developed to monitor the transcriptional regulation of DELLA in wheat. The application of the assay enabled the study of previously uncharacterised dwarfing alleles and revealed that transcriptional control of DELLA contributes in part at least to GA mediated growth and development in wheat. A central theme of the thesis was translating findings from the dicot model system, Arabidopsis, to monocotyledonous crop species. The influence of DELLA was studied in diploid (barley) and polyploid (wheat) crop species, allowing the influence of polyploidy to be examined as well as the effect of LoF mutation in the diploid species. In doing so a role for *Rht* in disease response in monocotyledonous temperate crop species has been defined along with insights into interactions with abiotic factors.

6.2. **Translation from dicot model system**

The profound differences that exist between monocots and dicots means that mechanisms functioning in one system cannot necessarily be inferred in the other. Much of the fundamental research on DELLA has been undertaken in the model dicot system, *Arabidopsis thaliana*, a species distantly related to wheat. Rice has been the subject of a lot of DELLA related findings, but although a monocot, rice is still relatively distantly related to wheat. Understanding DELLA related processes in wheat by direct means is therefore an important objective, not least due to the widespread use of mutant alleles in modern cultivars.

Wheat is a hexaploid species and as such contains three DELLA encoding genes. Therefore a GoF mutation in one of the genes leaves two functional wild type genes in the background. In this respect, wheat GoF mutations are very different to those of Arabidopsis, barley or rice which have diploid genomes. In some respects, however, the presence of five DELLA genes in Arabidopsis might, where they exhibit functional redundancy, be expected to mirror the effect of polyploidy. The effect of the polyploid genome was particularly evident in Chapter 4 in which wheat and barley lines were challenged with the biotrophic pathogen, *Blumeria graminis*. The GoF mutation of *SLN1* in the diploid genome conferred a striking increase in susceptibility, while GoF mutations of *Rht* in wheat, which cause a similar degree of dwarfing, conferred no significant increase in susceptibility. However, when double GoF mutants were challenged, in which two of the three *Rht* homoeologues were mutated, a significant increase in susceptibility was observed compared to wild type. Thus, an effect of background wild type homoeologues was observed in the polyploid wheat which was not evident in the diploid genome of barley.

Two seminal studies in Arabidopsis demonstrate a role for DELLA in disease response; in Chapter 4 the translation of these findings into monocotyledonous crop species is discussed. Navarro and colleagues (2008) showed that the *ga1-3* mutant (a GA biosynthesis mutant in which all DELLA proteins are stabilised) confers an increased susceptibility to biotrophic pathogens and an increased resistance to necrotrophic ones and conversely that the quadruple DELLA mutant (in which four of the five DELLA genes are missing) were more resistant to biotrophs and less susceptible to necrotrophs. The authors demonstrate that DELLA is influencing the relative strength of SA and JA hormone signalling required for resistance to biotrophs and necrotrophs respectively. The second study, (Achard *et al*., 2008) again demonstrates that the *ga1-3* mutant confer increased resistance to necrotrophic challenge. In this second study the authors demonstrate that DELLA confers increased resistance to necrotrophs by modulating the levels of ROS. In the present study a general resistance trade-off of pathogenic lifestyle was observed in wheat and barley GoF mutants. Furthermore, using barley as a model, the effects of LoF DELLA mutations could be assessed in monocots. The LoF mutant showed the converse to the GoF. Barley is a particularly good model due to the presence of a single DELLA encoding gene in a diploid genome thus avoiding issues of gene redundancy in Arabidopsis (which has a DELLA gene family) and wheat (which is polyploidy).

The influence of hormone dependent resistance was beyond the scope of this present study. However, the findings of Navarro *et al*. (2008), revealed the effect of DELLA on the relative strength of the SA and JA pathways. The barley model would provide a useful tool to assess the influence of DELLA on hormone signalling in monocots. In addition to direct measurement of hormone levels, assessment of hormone signalling pathways can be inferred using previously reported markers, such as PR1 and PDF1.2 for SA and JA signalling respectively. Relative expression levels for these genes could be measured in the GoF and LoF lines before, and during a time course subsequent to, challenge using the biotroph (*B. graminis*) and necrotroph (*F. graminearum*) pathosystems discussed in Chapter 4.

Whereas dicot species such as Arabidopsis encode multiple DELLA proteins, monocot species such as wheat and barley can undertake all GA-mediated processes with only one. It is not known how monocot species can achieve this. Chapter 2 investigated the possibility that differential regulation of *Rht* homoeologues in different tissues and developmental stages might provide the flexibility of growth control to wheat that is afforded by the five DELLA proteins in Arabidopsis. Analysis of *Rht* homoeologue contribution to the *Rht* transcriptome at different developmental stages and in different tissue types revealed that homoeologue contribution was similar in wild type plants in all instances examined. However all tissues and developmental stages were not investigated and so differential regulation remains a possibility.

Previous studies had suggested the existence of additional DELLA genes within the wheat genome. Chapter 3 describes how, based upon this information a search was undertaken to isolate a candidate *Rht*-like gene from the wheat genome. A wheat BAC library was probed with sequence from a putative gene located on chromosome 7D which had high homology to the group 4 *Rht* genes. Unfortunately, my search for this gene was ultimately unsuccessful. Database searches of the rice genome confirmed the presence of a single DELLA encoding gene, however two additional genes were revealed, SLRL1 and 2, which have high homology to DELLA but lack the N terminal domain (Itoh *et al.*, 2005). The genes were shown to result from partial duplication events of *Slr1*, and it is postulated that they may function as a fine control mechanism of GA mediated processes (Itoh *et al.*, 2005). SLRL1 has also recently been implicated in submergence tolerance (Fukao & Bailey-Serres, 2008). In the present study database searches of the *Brachypodium distachyon* and *Sorghum bicolour* sequenced genomes suggest the presence of DELLA-like genes based on homology to SLRL sequence, therefore strengthening the possibility that there are additional genes involved in GA mediated processes within the wheat genome. With the dawn of the fully assembled wheat genome approaching this will be an interesting query to validate the presence of a putative *Rht* or *Rht*-like gene on 7D and elsewhere in the wheat genome. An alternative approach may be successful in isolating this elusive gene. PCR screening of a pooled BAC library (Febrer *et al.*, 2009), was successfully used to isolate group 4 *Rht* containing BACs (Wilhelm, NIAB, Cambridge, UK), and it may be possible to isolate the putative gene on 7D using a similar strategy.

However, it should be borne in mind that the difference in the number of DELLA genes in monocots and dicots may simply be because duplication of the DELLA genes occurred in dicots after divergence from monocots, with subsequent subfunctialisation occurring (Gallego-Bartolome *et al.*, 2010).

6.3. **Linking genotype with phenotype**

Peng *et al.* (1999) characterised the semi-dwarfing *Rht-B1b* and *Rht-D1b* GoF alleles revealing that they contained nucleotide substitutions which introduced premature stop codons at distinct but similar locations in the N-terminal domain. The authors postulate that re-initiation of translation results in a truncated product which contains the C-terminal growth repressing domain but lacks the N terminal domain essential for GA induced degradation, thus conferring a GA insensitive phenotype. Other GA insensitive alleles remained uncharacterised including the severe dwarf alleles, *Rht-B1c* and *Rht-D1c*. Both alleles confer a similar level of dwarfing however using the homoeologue specific qRT-PCR assay, in Chapter 2 I reveal that the mechanisms of dwarfing are very different.

Rht-B1c lines had a reduced level of transcription of *Rht-B1* and yet paradoxically have a reduced plant height phenotype. An insertion was identified in *Rht-B1* DNA sequence which was subsequently corroborated by two laboratories. Chandler *et al*. (CSIRO, Canberra, Australia) identified and sequenced a gDNA insert whilst Pearce (RRes, Harpenden, UK) identified a cDNA insert. A region of 90bp of common sequence bordered by predicted splice sites suggested splicing results in a 30aa insertion in the DELLA domain of the RHT-B1c protein. Yeast-2-hybrid experiments carried out by Pearce (RRes, Harpenden, UK) demonstrate complete abolishment of the interaction between GID1 and the RHT-B1c protein. Interestingly complete abolishment of the interaction between GID1 and the RHT-B1b and RHT-D1b C-terminally truncated products was also observed. Pearce suggests that the milder dwarfing phenotype observed may be a result of a lower rate of translation, due to the efficiency of ribosomal reinitiation (Pearce, RRes, Harpenden, UK, pers. comm.). The RHT-B1c protein is therefore hypothesised to be stabilised, presumably resulting in negative feedback on further *Rht-B1c* expression, which was observed in these lines. Interestingly, negative feedback in *Rht-B1c* lines was specific to *Rht-B1* transcription suggesting independent transcriptional control of each *Rht* homoeologue.

In contrast *Rht-D1c* lines had an increase in the level of *Rht-D1* transcript (Chapter 2). I subsequently present evidence that suggests that *Rht-D1c* contains multiple copies of the *Rht-D1b* allele possibly resulting from several gene duplication events. The presence of *Rht-D1b* polymorphism in *Rht-D1c* would suggest that *Rht-D1c* may have arisen from gene duplication in a *Rht-D1b* containing line. An *Rht-D1c* BAC library has been created (Jia, CAAS, Beijing, China) offering the potential to screen for confirmation of multiple copies of the gene.

One of the questions arising from the two contrasting mechanisms of dwarfing in the severe dwarf lines, *Rht-B1c* and *Rht-D1c* is the effect conferred on downstream stress tolerance. No differences in tolerance have been observed in response to salt or heat shock treatments in experiments (Boulton and Korolev, JIC, Norwich, UK, pers. comm.), however it has been suggested that the treatments may be too severe to detect differences. It is conceivable that *Rht-D1c* may respond more quickly to the onset of adversity and recover growth processes more quickly after treatment removal, due to the greater flexibility of transcriptional control. The response of these lines to milder stress treatments and monitoring their recovery after treatment removal could be studied to examine this possibility.

With reference to biotic stress, the resistance conferred by the severe dwarf lines, *Rht-B1c* and *Rht-D1c* were indistinguishable upon challenge with *Oculimacula* spp. The assessment of resistance to *F. graminearum* was only carried out in the *Rht-B1* allelic series owing to the presence of a *Fusarium* susceptibility factor which has been shown to be linked to *Rht-D1b* (Srinivaschry *et al*., 2008). If *Rht-D1c* was derived from *Rht-D1b* as is speculated above then *Rht-D1c* may also contain

the susceptibility factor. Depending on the size of the duplication unit surrounding *Rht-D1*, multiple copies of the susceptibility factor may also be present. The DELLA-conferred resistance has been demonstrated to be dosage dependent (discussed below), depending on whether the susceptibility factor is also dosage dependent may determine whether the increased resistance conferred by DELLA is cancelled out by an increase in susceptibility factor. The size of the duplicated region is currently unknown but synteny between wheat and other grass species for which the full genome sequence is available has allowed the identification of genes that probably flank the *Rht* locus. In addition, BAC sequence approximately 100 kb upstream and downstream of each *Rht* homoeologue is available (Wilhelm, NIAB, Cambridge, UK). Using a similar approach to that used in Chapter 2 to identify multiple copies of *Rht-D1* in *Rht-D1c*, the candidate genes could also be quantified relative to *Rht-D1b* susceptibility factor. This approach is dependent on sequence from the homoeologues of the candidate genes being sufficiently dissimilar to permit design D-homoeologue specific primers. Ideally isolation and sequencing of *Rht-D1* containing BACs from the aforementioned Chinese *Rht-D1c* BAC library would validate duplication of the *Rht-D1* gene and reveal the size of the duplication unit. The *Fusarium* bioassays described in Chapter 4 could be carried out to assess whether the DELLA conferred resistance is compromised in *Rht-D1c* lines compared to *Rht-B1c* lines, so indicating whether the duplicated region also contains the *Fusarium*-susceptibility factor.

Additional experiments suggest that the influence of DELLA on growth is tissue specific and strongly influenced by temperature. For example. the dwarfing phenotype conferred by *Rht* mutant alleles is clearly evident in the coleoptile of seedlings grown at 25˚C and yet is not expressed in the roots of the same plants. In fact the roots of lines containing the *Rht-B1b* and *Rht-D1b* semi-dwarfing alleles are longer than those of wild type. Similarly, the effect of the dwarfing alleles diminishes with decreasing temperature, to the extent that semi-dwarf lines are taller than wild type lines when grown at 5˚C. Both of these effects may be a results of reduced levels of DELLA expression: expression in roots was less than that in shoots and expression in shoots grown at 5˚C was less than in shoots grown at 25˚C. These results indicate that an effect on phenotype will, as expected, only be observed in tissues and under conditions where DELLA is being expressed.

The link between genotype and phenotype was also examined in relation to diurnal growth. In Chapter 2 I showed that *Rht* expression was diurnally modulated. However measurement of coleoptiles over time revealed that growth was linear irrespective of the light environment. This contrasts markedly with findings in Arabidopsis in which diurnally regulated hypocotyl growth has been reported (Nozue *et al.,* 2007). My findings suggest that, in wheat, other components of the GA signalling pathway may be buffering the effects of altered *Rht* transcription. In order to gain further insight into the role of DELLA in this process, additional assays are required to enable

quantification of DELLA protein and GA levels in order to fully elucidate the link between genotype and phenotype.

I also observed an association between the influence of DELLA on growth habit (dwarfing) and response to biotic stress. Lines carrying *Rht-B1c* are significantly shorter than those carrying *Rht-B1b* and, as I showed in Chapter 4 this DELLA 'dose' effect was also evident in the disease response. *Rht-B1c* mutants show an exaggerated susceptibility/resistance phenotype to type 1 and type 2 components of *F. graminearum* resistance respectively compared to *Rht-B1b*. Additionally, the finding that double, but not single, GoF mutations confer an increase in susceptibility to *B. graminis* may suggest a DELLA threshold need be meet to confer the susceptibility phenotype.

6.4. **DELLA as a node to growth or stress response**

Semi-dwarfing alleles were introduced into wheat varieties to reduce plant height. However their introduction had the unexpected side effect of influencing the plants response to abiotic and biotic stress. Growth reduction during the onset of stress suggests that resources are diverted from growth processes to stress responses. Wild type DELLA accumulation in response to abiotic stress exposure confers increased tolerance (Achard *et al.*, 2006) and flg22 treatment has been demonstrated to delay GA-mediated degradation of DELLA in Arabidopsis resulting in flg22-induced growth inhibition (Navarro *et al.*, 2008). Together this suggests DELLA is a strong candidate for having a function in controlling the resource distribution between growth and stress responses. Through the introduction of GoF mutant alleles in wheat cultivars to repress growth the inadvertent result has been an altered response to stress.

DELLA has been implicated in two major mechanisms influencing response to stress in Arabidopsis, namely hormone signalling and ROS accumulation. In the present study an emphasis has been made on examining potential DELLA-conferred effects on ROS accumulation in monocots. It was demonstrated in Chapter 5 that GoF *Sln1* barley lines exhibit greater resistance to paraquat and menadione induced cell death, whilst LoF mutant lines show increased susceptibility. Both of these treatments induce production of O_2^-. In contrast, treatment with a H_2O_2 inducing chemical, alloxan, showed no DELLA-dependent differential cell death response. It is therefore postulated that DELLA is involved in the negative regulation of O_2^- induced cell death, although further work is required to substantiate this hypothesis. In the Arabidopsis model system it has been demonstrated that DELLA modulates ROS levels through an effect on the ROS scavenging system, but it may be that DELLA, directly or indirectly, influences cell death by other means. For example, in Chapter 4 data is presented which shows that deoxynivalenol, a *Fusarium* virulence factor which induces cell death, causes an enhancement of expression of two putative cell death regulators, *BI-1* and *RCD1/CEO*. The enhanced expression is greater in DELLA GoF lines compared to wild type. Interestingly, the spreading lesion phenotype of Arabidopsis *rcd1* lines is

induced by ozone and extracellular O_2^-, but not H_2O_2 (Overmyer *et al.*, 2000) raising the possibility that DELLA functions to modulate elements that control O_2^--induced cell death.

Biotrophic pathogens require host cells to remain alive in order to derive nutrients from them whilst necrotrophs tend to kill the host cell before deriving nutrients from them. Thus plants in which cell death processes are altered generally exhibit a trade-off of increased resistance to one class of pathogens and susceptibility to the other. Two such examples of genes where a trade-off situation has been observed in the null or over-expressing condition are *BI-1* and *MLO*.

HvBI-1 suppresses defence responses and resistance to *B. graminis* f. sp. *hordei* and enhances resistance to the necrotrophic fungus, *F. graminearum* when over-expressed in barley (Babaeizad *et al.*, 2009). Down-regulation of *HvBI-1* by transient- or virus-induced gene silencing was demonstrated to reduce susceptibility to *B. graminis* f. sp. *hordei*, suggesting that HvBI-1 is a susceptibility factor toward *B. graminis* f. sp. *hordei* (Eichmann *et al.*, 2010). The ectopic expression of HvBI-1 in carrots resulted in increased resistance to the cell death-inducing pathogens, *B. cinerea* and *Chalara elegans* (Imani *et al.*, 2006). Whilst LoF BI-1 mutants in Arabidopsis show accelerated cell death when treated with the PCD-inducing fungal toxin fumonisin B1 (FB1; Watanabe & Lam, 2006).

MLO has been reported to be involved in at least three trade-off scenarios. For the LoF (*mlo*), the positive effect in each instance is the increased resistance conferred towards *B. graminis*. The first reported negative effect is an increase in necrotic leaf spotting promoted by exposure to abiotic stress (Bjornstad & Aastveit, 1990). Another trade-off reported is an enhanced susceptibility of *mlo*-mutants to necrotrophic pathogens. For example, barley *mlo* lines are more susceptible to *Magnaporthe oryzae*, *F. graminearum* and the toxic culture filtrate of *Bipolaris sorokinana* (Jarosch *et al.*, 1999; Kumar *et al.*, 2001; Jansen *et al*., 2005). The third reported trade-off of *mlo* is a decrease in susceptibility to the arbuscular mycorrhizal fungus, *Glomus mossae* (Ruiz-Lozano *et al.*, 1999). The defence mechanisms conferred by *mlo* that prevent *B. graminis* infection are also likely to be effective against the biotrophic symbiont *G. mossae*, thus reducing colonisation. In light of the increased susceptibility to *B. graminis* conferred by DELLA GoF mutations it could be postulated that they may also enhance colonisation by *G. mossae*. Symbiosis with mycorrhizal fungi such as *G. mossae* is potentially very beneficial as it aids the capture of nutrients such as phosphorus from the soil. Therefore, plants that permit increased colonisation may benefit with a resulting increase in yield. If colonisation by arbuscular mycorrhizal fungi is greater in DELLA GoF mutants than their tall counterparts this may represent a contributing factor to the increased yields observed for the *Rht* semi-dwarf mutants of wheat.

DELLA, through an alteration in the propensity for a cell to undergo cell death, is also associated with a resistance trade-off in Arabidopsis (Achard *et al.*, 2008b; Navarro *et al.*, 2008) and the

monocot crop species, wheat and barley (Chapter 4). HvBI-1 over-expression in barley *mlo* lines results in an increase in susceptibility to *B. graminis* f. sp. *hordei* suggesting that *mlo* is functionally complemented by *BI-1* over-expression (Huckelhoven *et al.*, 2003). It would therefore be interesting to test whether *mlo* might be similarly complemented by DELLA GoF mutations.

The semi-dwarfing alleles, *Rht-B1b* and *Rht-D1b*, in wheat and *mlo* alleles in barley are ubiquitous in modern cultivars. However, while both provide invaluable traits to improve yield they also simultaneously confer deleterious effects. By understanding the nature and magnitude of the potentially negative traits conferred by these alleles, breeders will be able to maximise the positive aspects. For example, in *mlo* carrying lines necrotic spotting resulting from exposure to abiotic stress can lead to a reduction in yield (Bjornstad & Aastveit, 1990). Breeders have successfully alleviated the detrimental effects of necrotic spotting in *mlo* lines by selecting background genes which suppress this phenotype while retaining the mildew resistance (Brown, 2002). While the pleiotropic effect of GoF *Rht* mutants in conferring broad spectrum necrotroph resistance and abiotic tolerance is beneficial to breeders, they will need to balance this by the introduction of genes conferring enhanced resistance to biotrophic pathogens. Although the polyploidy genome of wheat seems to negate the negative aspect of the trade-off, resulting in a similar level of resistance in semi-dwarf lines compared to their tall counterpart upon challenge with *B. graminis* and potentially other biotrophic pathogens.

The effects of environment on the DELLA conferred resistance trade-off were assessed in Chapter 5. The effect of the external environment is particularly important to consider due to its influence on ROS homeostasis. Light in particular is a well recognized inducer of EEE and SAA both of which affect ROS homeostasis. EEE and SAA are also promoted by ozone, drought, salinity and extremes of temperature. Thus relative light intensity was used in this study to assess how a preceding abiotic stress through its influence on ROS homeostasis affects a subsequent biotic stress. It was postulated that the external environment influences the point at which the cell death threshold is set through alterations in ROS homeostasis. The altered cell death threshold will have differential effects on disease outcome dependent on the nutritional lifestyle of the pathogen. A reduction in the cell death threshold, as was postulated to be the case in LL and HL preconditioned plants, reduces the DELLA conferred biotroph-necrotroph resistance trade-off as illustrated in figure 6.1.

Based on the findings in Arabidopsis, that DELLA influences SA/JA signalling, and the suggestion by Love *et al.* (2008) that SA promotes apoptosis-like PCD while JA and ethylene promote autophagic PCD another component can be added to the model proposed in Chapter 5. Presented in figure 6.1 the model proposes that in addition to DELLA conferred ROS modulation affecting the amount of stress required before the cell death threshold is reached and the environmental influence

on the level at which the threshold is set, the DELLA conferred hormone alterations may influence the mode of PCD (apoptotic or autophagic) that cells undergo. This is consistent with unreported observations of increased pathogen-associated leaf tip senescence in DELLA GoF barley lines and a decrease in LoF lines compared to wild type.

In this study the understanding of DELLA related processes acquired in the model diploid dicot, Arabidopsis, have been translated to the diploid and polyploidy monocot temperate cereals, barley and wheat. In doing so a DELLA conferred biotroph-necroroph resistance trade-off has been defined in these species. These studies were undertaken in a controlled environment in which plants were grown in optimal conditions contrasting from conditions in the field where the plant interacts with, often adverse, abiotic factors. Thus a constant adjustment of the cell death threshold might be necessary for a plant to survive in a constantly fluctuating environment. It has been demonstrated in this study that as a consequence the plants response to biotic stress is affected and the DELLA conferred ressistance differential is reduced. By gaining a better understanding of the growth mechanisms of GoF *Rht* alleles and their plieotropic effects in wheat the breeder can be better informed of the optimum utilisation of the mutant alleles in the field.

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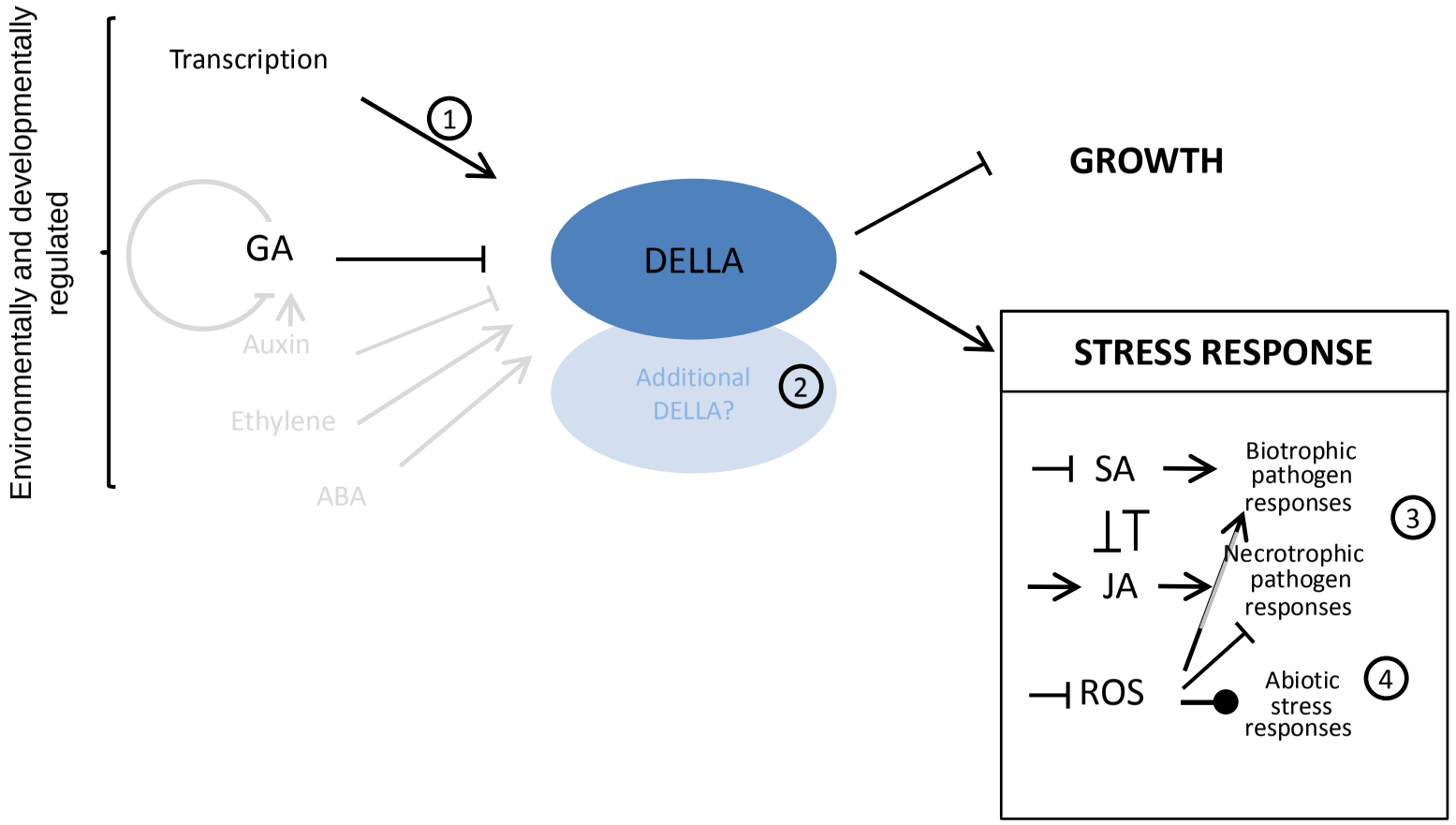


Figure 1.8. An overview of the thesis aims. The central themes of the study are based on findings in the model dicot, Arabidopsis with the aim of translating the findings to monocot crop species. Numbers refer to different components of the study and are expanded in the main text.

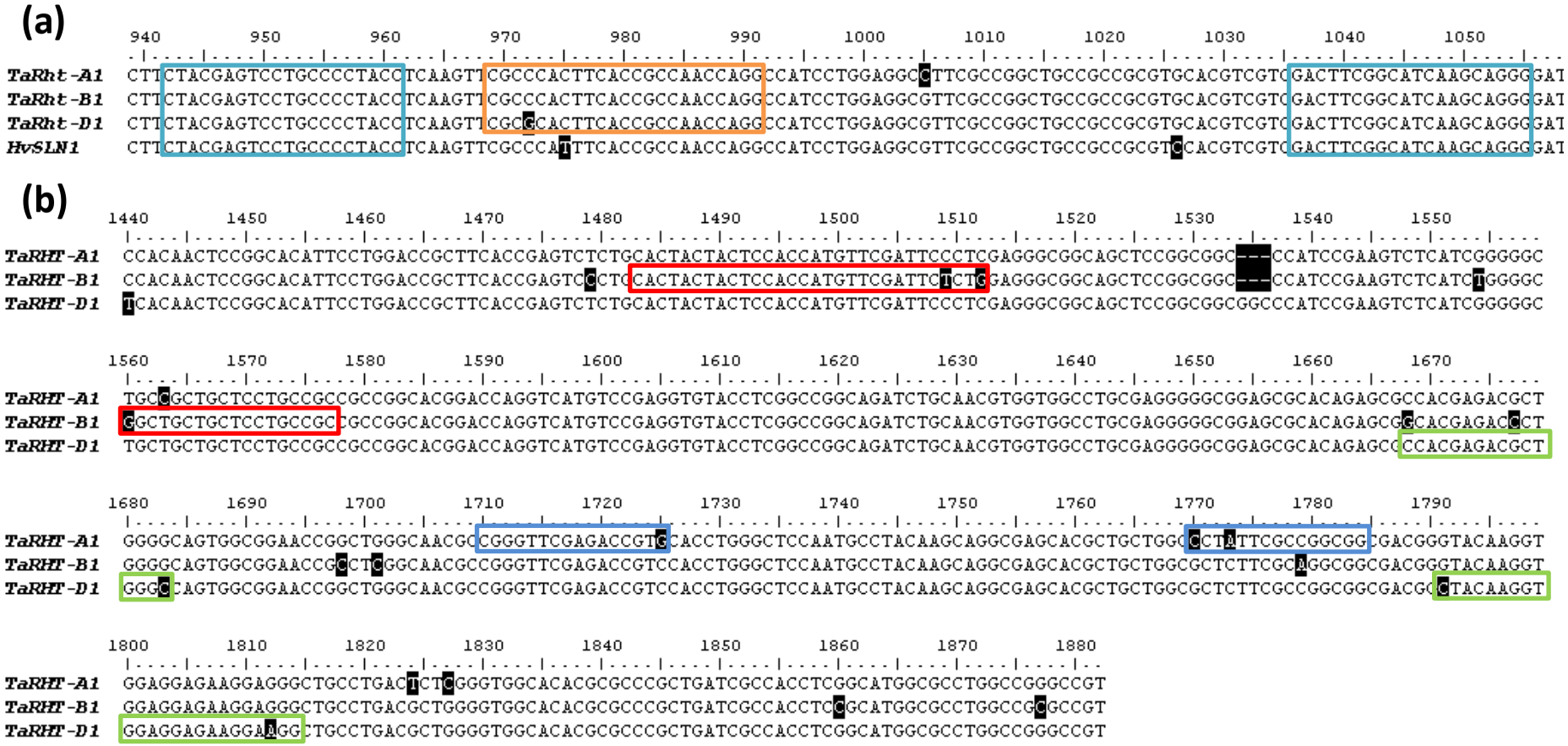


Figure 2.1. Sequence alignments with primer binding sites. (a) a portion of *Triticum aestivum* (*Ta*) group 4 *Rht* homoeologues and the *Hordeum vulgare* (*Hv*) DELLA orthologue, *Sln1* is aligned. Probe (orange) and primer (turquoise) binding sites for a generic Taqman assay are boxed. These primers have been used also for a generic SYBR assay. (b) an alignment of the 3' region of the *Rht* homoeologues. Homoeologue specific primer binding sites are boxed; *Rht-A1* (blue); *Rht-B1* (red); *Rht-D1* (green). In both cases the numbers refer to the *Rht* gene co-ordinates with the first nucleotide of the ATG start codon being +1. Black infilled regions highlight polymorphisims.

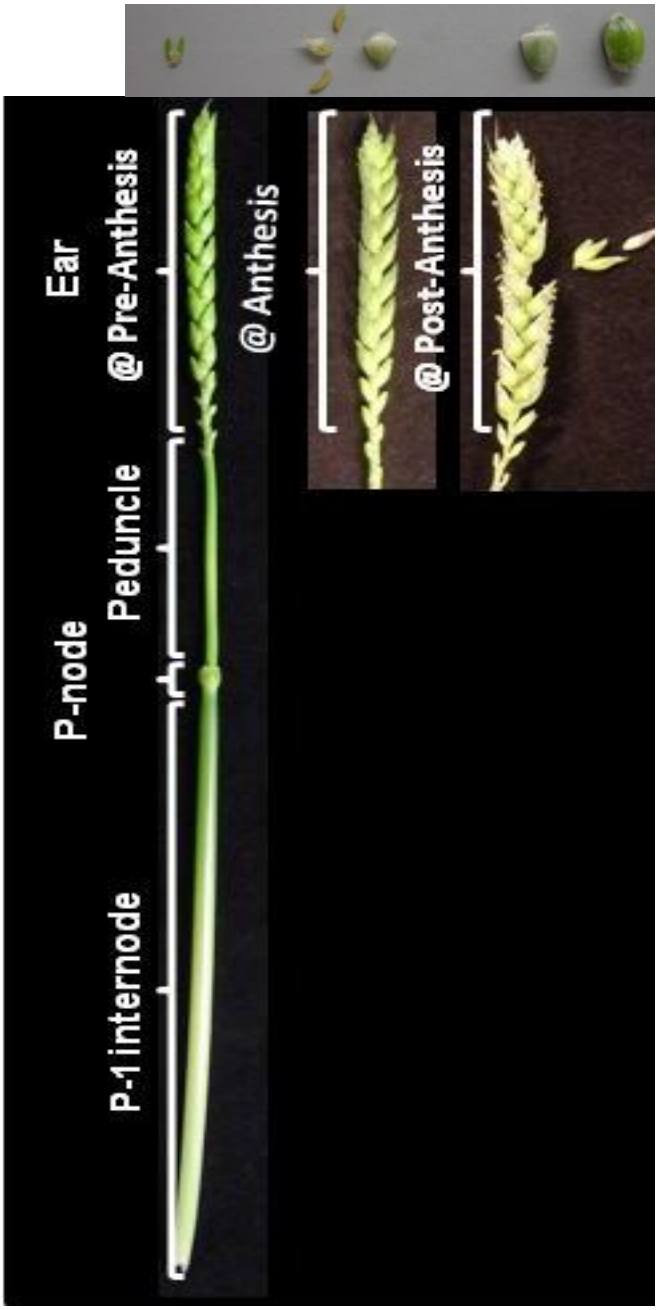


Figure 2.2. The tissues sampled for expression analysis of *Rht* in mature wheat plants. The top panel illustrates the stages of seed development sampled at pre-anthesis, anthesis and post-anthesis respectivley.

(a)

Primer set	Forward	Reverse
A	GTTGTTGGATTTGATCATCGG	GTCAACATGGAAAAAAATCCCA
B	GCCGGCTATATTTAAGACACGTG	GGATTTGATCATAGGTAGACAAGTT
C	TTGCATGGCTTCAAAGTTTCC	CCTTTGTTTCTTGTTCCTCCAGG
D	GAATAAATAAACAGAAGTACGTTTTTTC	CTGTAGTACCCTGGCCG

(b)

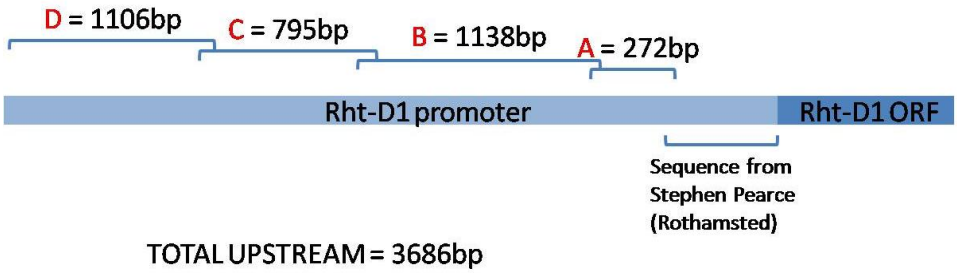


Figure 2.3. Amplification of sequence upstream of the *Rht* ORFs of the Rht-D1 allellic series. (a) *Rht-D1* specific primers designed to amplify blocks of promoter sequence (b) from *Rht-D1a*, *Rht-D1b* and *Rht-D1c*. The primers (A-D) allowed sequence to be obtained up to 3686 bp upstream of the *Rht-D1* ATG of the *Rht* open reading frame. The sizes of the amplified and sequenced products are shown above the infilled boxes.

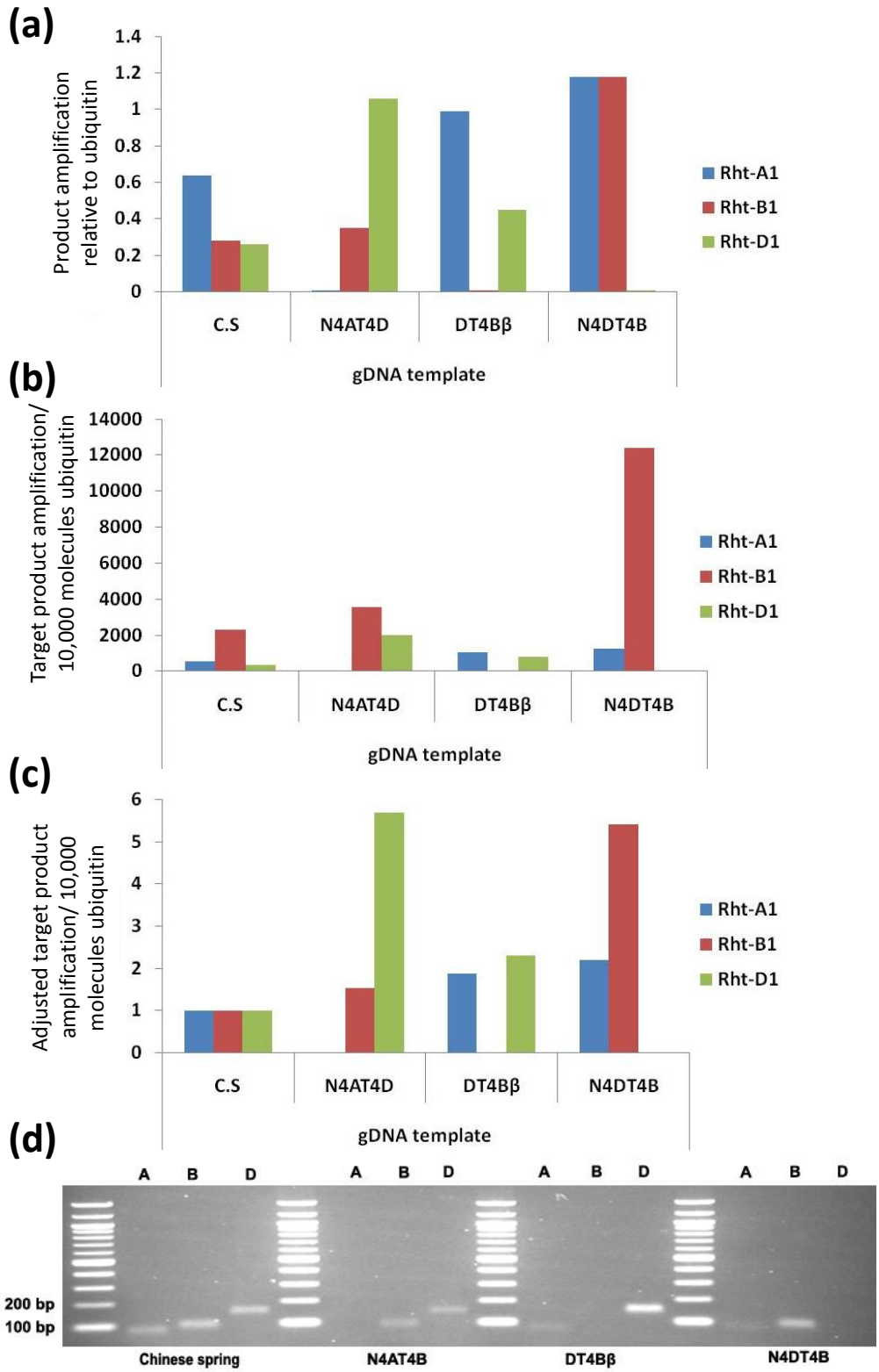


Figure 2.4. Validation of the *Rht* homoeologue specific qPCR assays. (a) validation data for each assay presented relative to ubiquitin. (b) validation data presented as number of target molecules/10,000 molecules of ubiquitin (c) data as in (b) is corrected so that C.S had a 1:1:1 contribution from each homoeologue, as is expected to be the case. (d) the endpoint products visualised following electrophoresis through an agarose gel containing ethidium bromide. Each assay was tested on respective templates and the products of each assay are shown separated by a 100 bp ladder. C.S Chinese spring, the euploid control; N4AT4B, nullisomic 4A tetrasomic 4B; DT4Bβ, ditelosomic 4Bβ; N4DT4B, nullisomic 4D tetrasomic 4B.

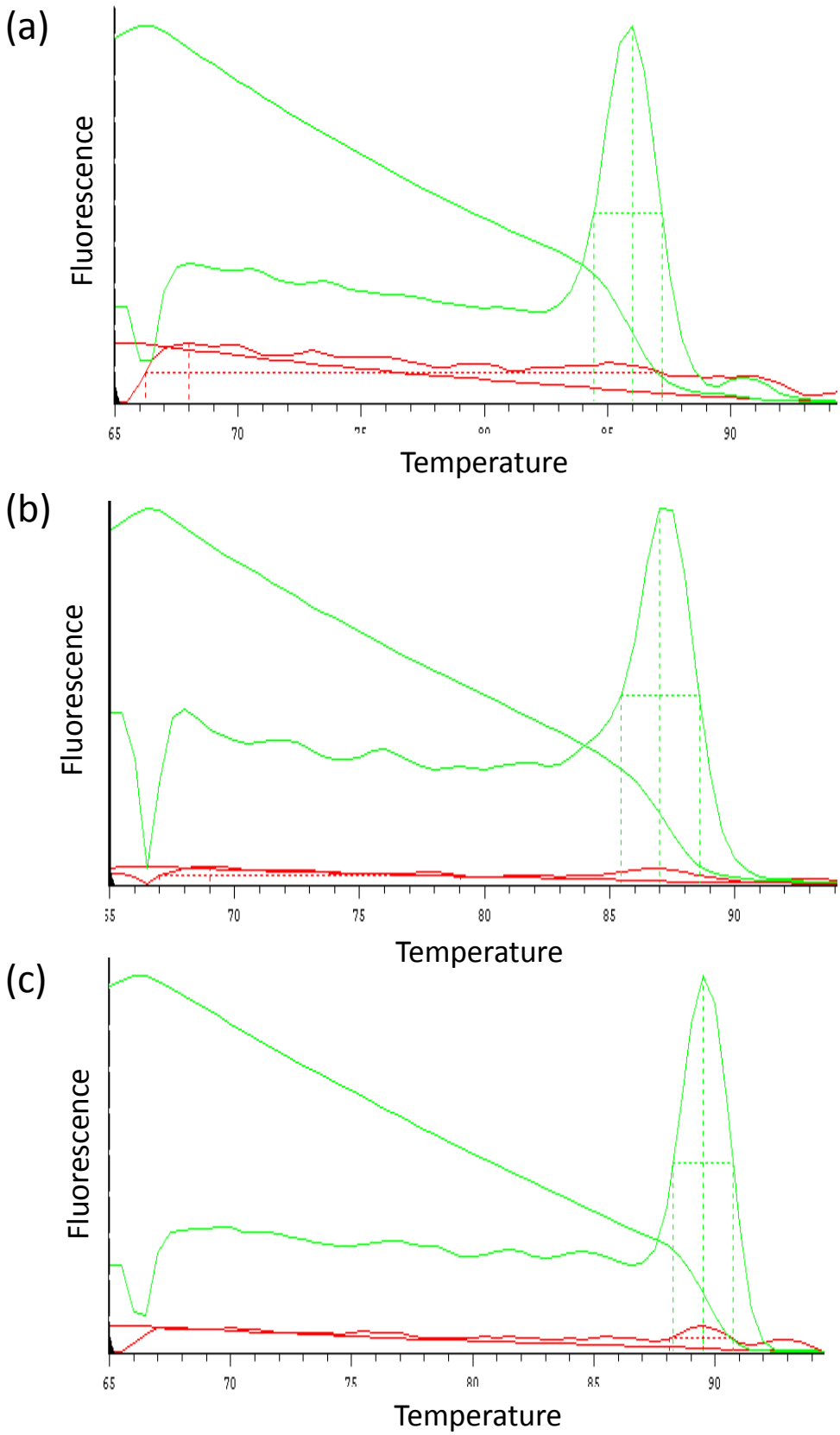


Figure 2.5. Meltcurve analysis of the qPCR validation assays of each of the *Rht* homoeologue specific primer sets. (a) *Rht-A1*, (b) *Rht-B1* and (c) *Rht-D1.* In each case Chinese Spring was used as the positive template (green) and anuploid template (lacking the respective gene as described in figure 2.4.) was used as the negative template (red). Both intensity and -dI/dT traces are displayed.

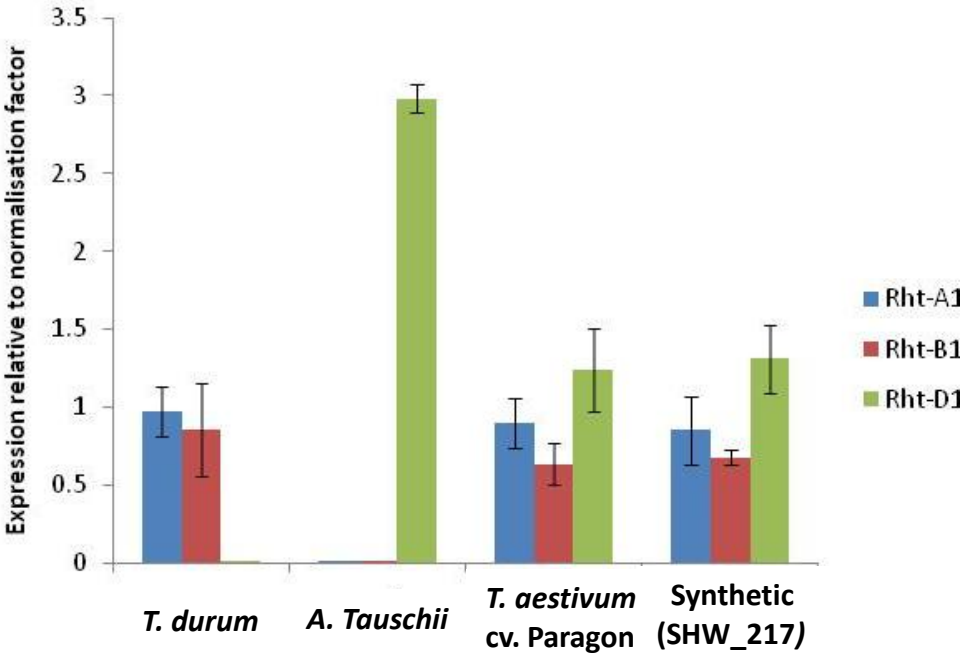


Figure 2.6. qRT-PCR to determine whether *Rht* expression is affected by ploidy level. Homoeologue specific expression in *Triticum durum* (tetraploid; AABB), *Aegilops tauschii* (diploid; DD), *Triticum aestivum* (hexaploid; AABBDD) and a newly generated hexaploid line, SHW_217. Expression presented relative to normalisation factor (based on GAPDH and EF1α). Bars: 1 SEM.

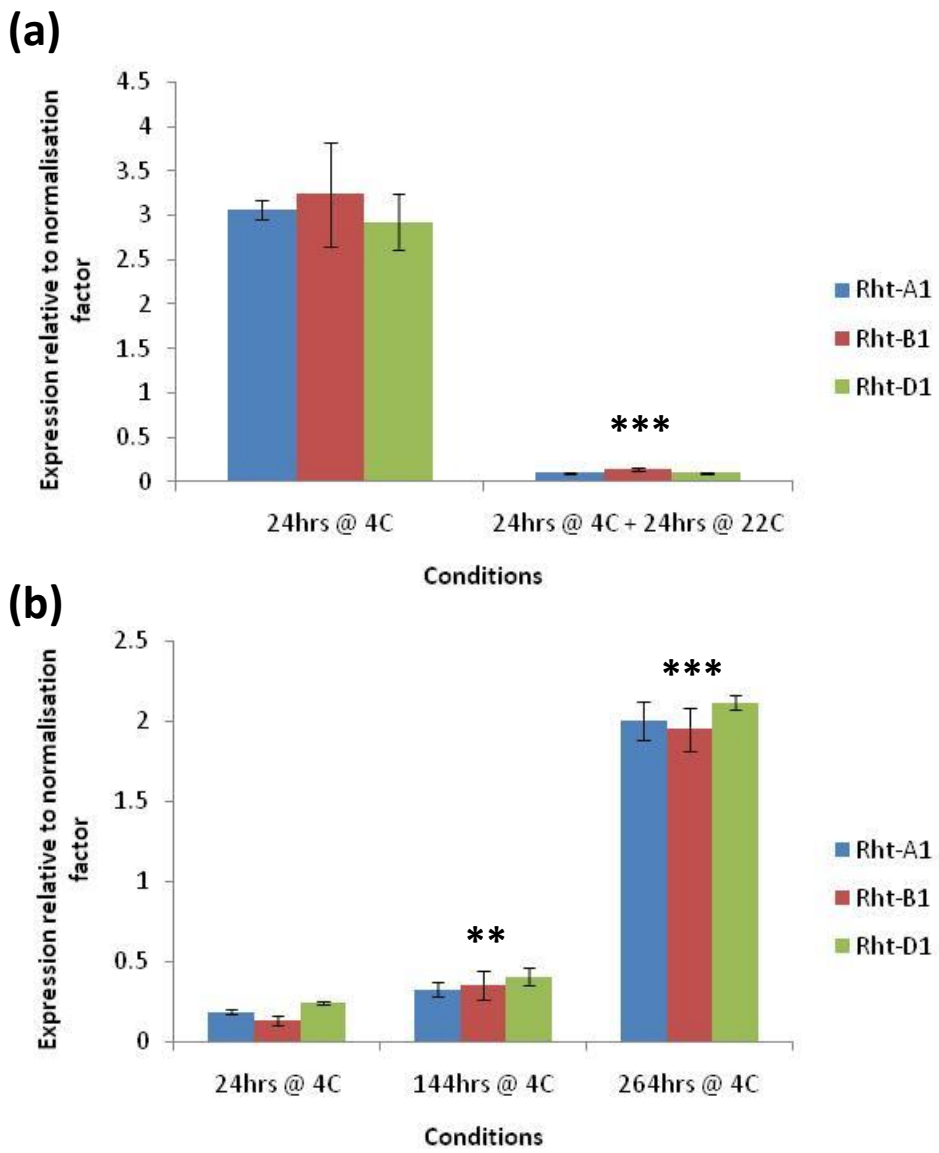


Figure 2.7. *Rht* expression increases during imbibition and is reduced upon germination. (a) *Rht* expression in *rht-tall* seed is significantly reduced when seeds are transferred into the warmth, coinciding with germination. *** = significant difference (P=<0.001) from 24h @4˚C (b) *Rht* expression increases during imbibition in the cold. ** = significant difference (P=<0.01) from 24h @4˚C; *** = significant difference (P=<0.001) from 144h @4˚C. Expression presented relative to normalisation factor (based on GAPDH and EF1α). Bars: 1 SEM.

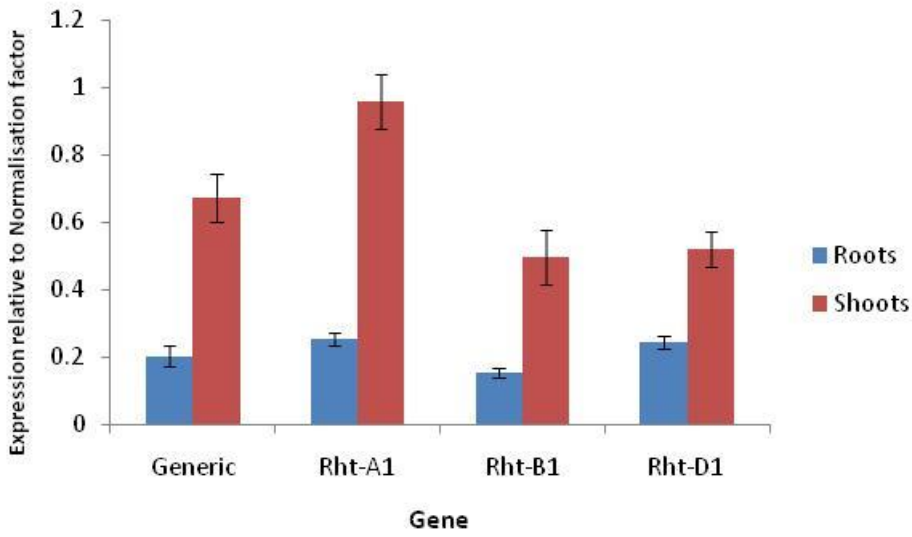


Figure 2.8. *Rht* expression is lower in roots than the shoots of young *rht-tall* wheat seedlings. qRT-PCR data for each homoeologue, and for all three homoeologues (generic assay) is presented relative to normalisation factor (based on GAPDH and EF1α). Bars: 1 SEM.

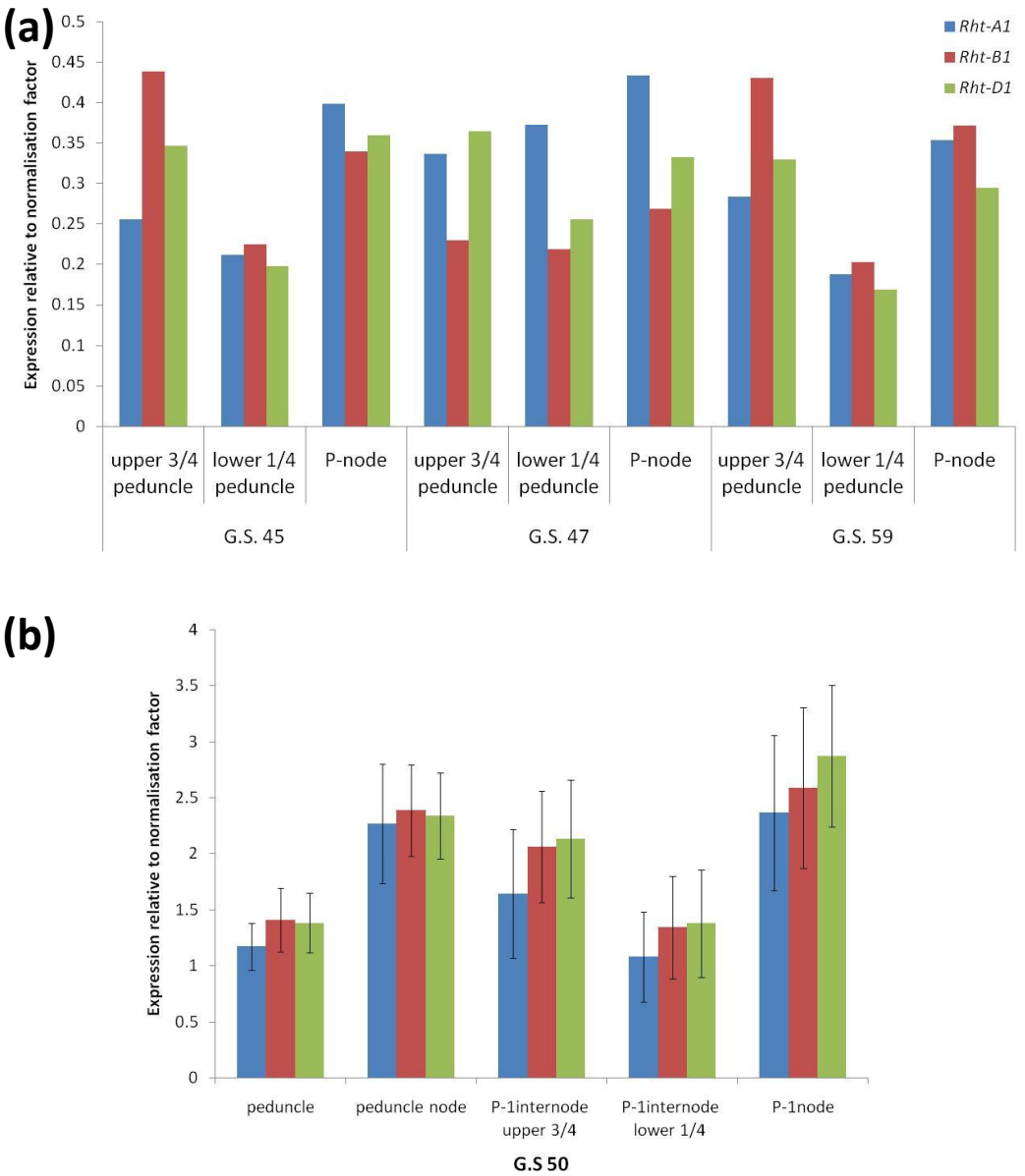
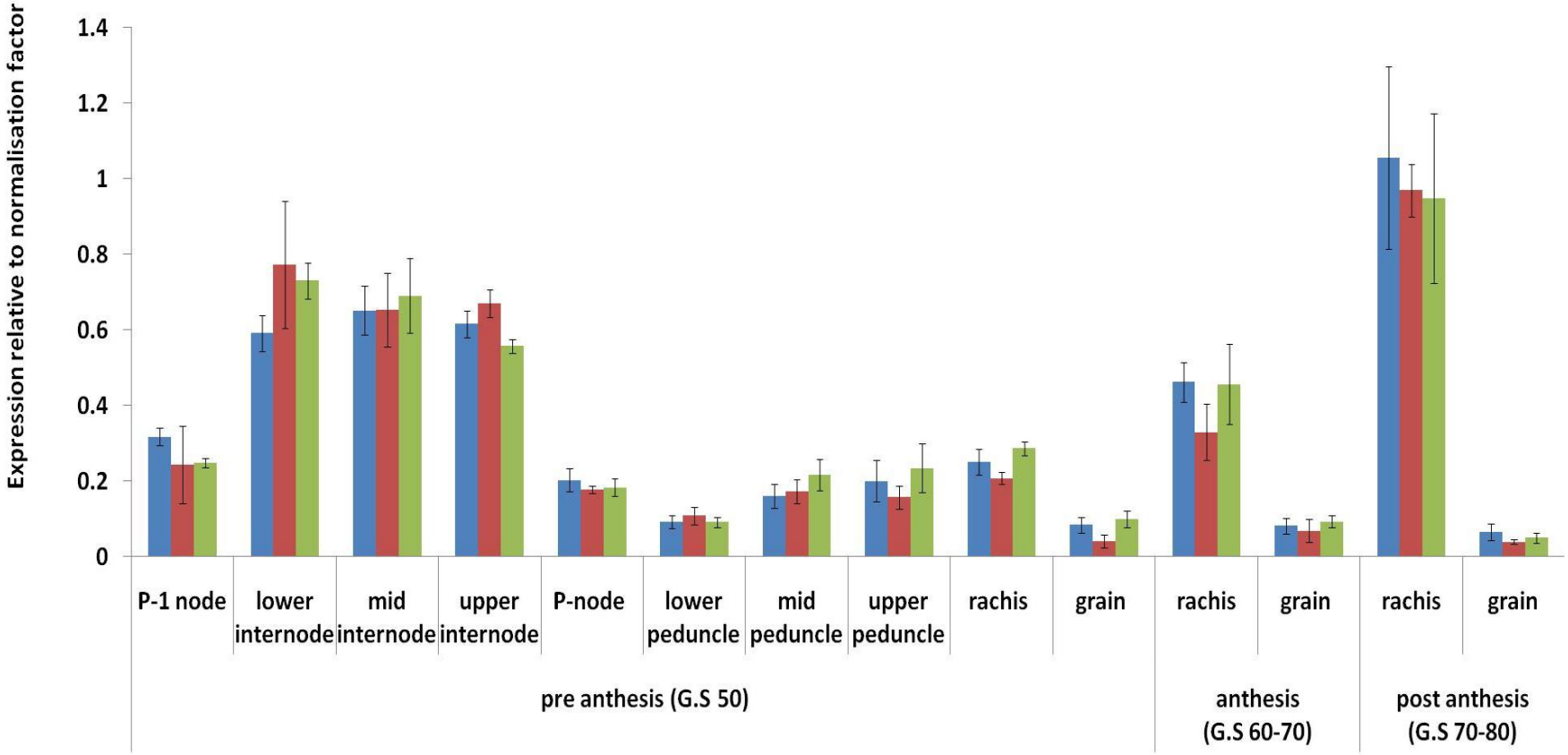


Figure 2.9. Comparison of *Rht* expression in the elongating stem and ear of *rht-tall* wheat plants. Homoeologue-specific expression was measured in three independent experiments (a), (b) and (c). The tissues sampled are shown in Fig. 2.2. Expression presented relative to normalisation factor (based on GAPDH and EF1α). Bars: 1 SEM.

(c)


Expression relative to normalisation factor
1.4
1.2
1
0.8
0.6
0.4
0.2
0
P-1 node
lower internode
mid internode
upper internode
P-node
lower peduncle
mid peduncle
upper peduncle
rachis
grain
rachis
grain
rachis
grain
pre anthesis (G.S 50)
anthesis (G.S 60-70)
post anthesis (G.S 70-80)

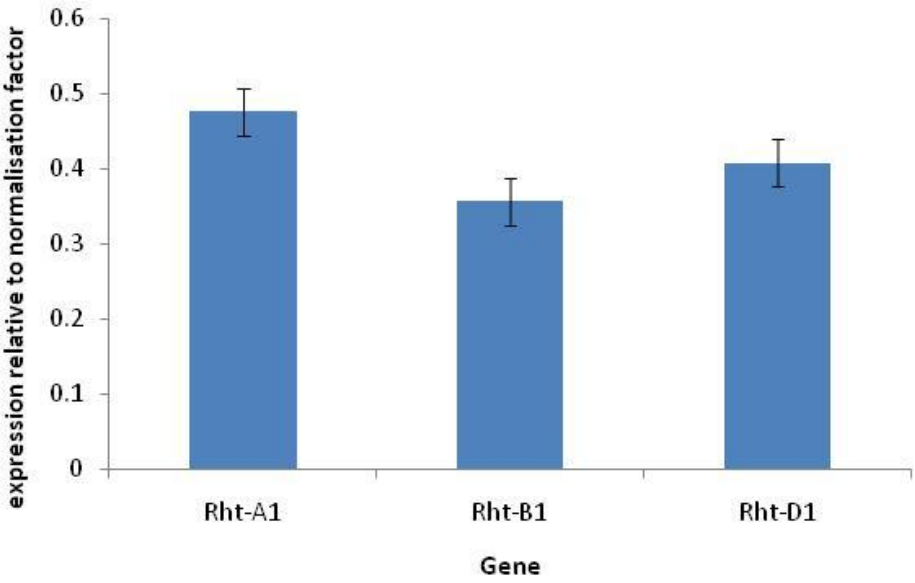


Figure 2.10. *Rht* expression in pre-dehiscent stamens is not predominated by any homoeologue. The qRT-PCR data were obtained using four replicates of at least 40 florets for each sample. Expression presented relative to normalisation factor (based on GAPDH and EF1α). Bars: 1 SEM. This experiment was repeated with similar results.

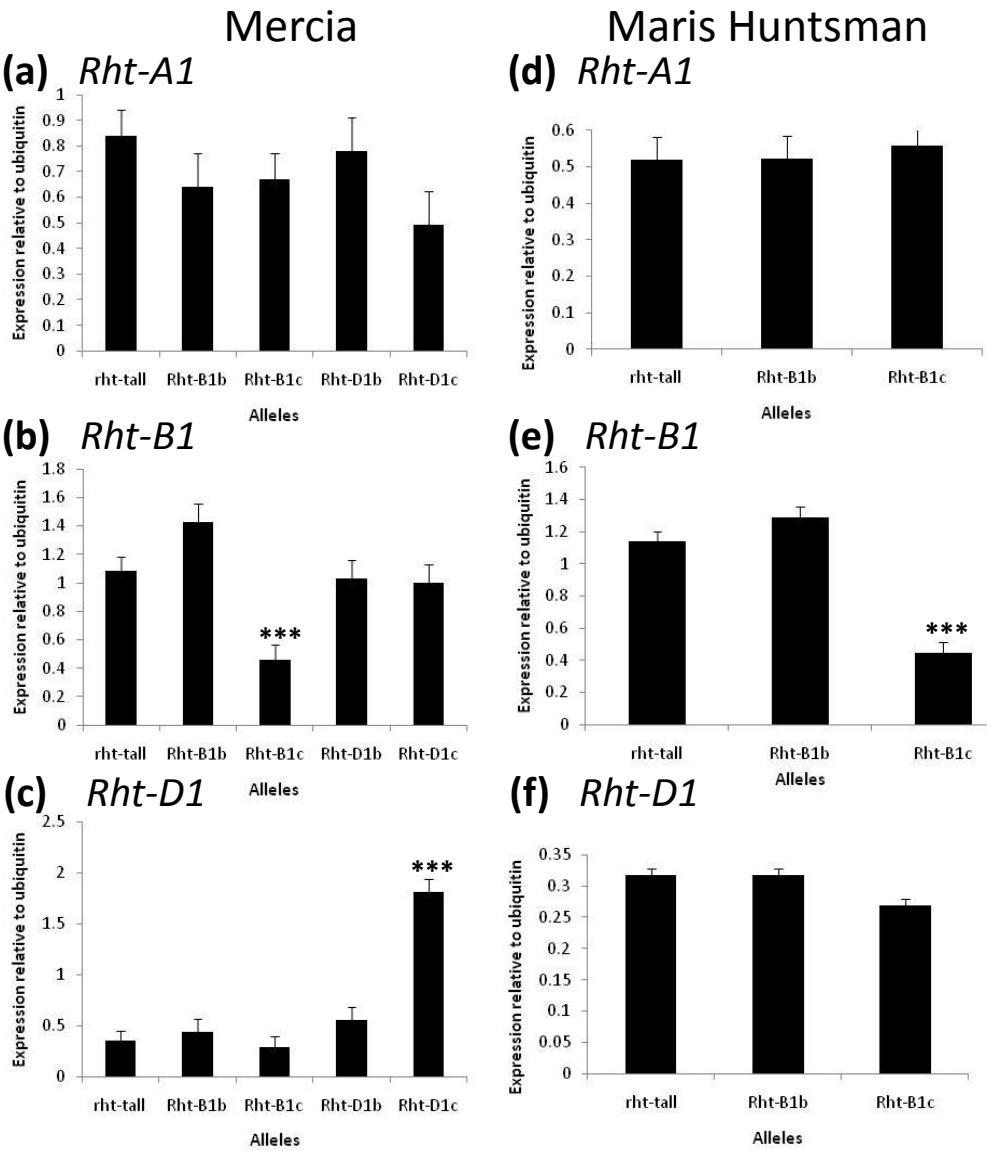


Figure 2.11. The effect of mutant alleles on *Rht* homoeologue-specific expression. Expression was investigated using qRT-PCR in two sets of near isogenic lines; Mercia (a-c) and Maris Huntsman (d-f). *Rht* expression was measured for each homoeologue; *Rht-A1* (a & d); *Rht-B1* (b & e) and *Rht-D1* (c and f). The data shows a specific decrease in *Rht-B1* transcript in *Rht-B1c* lines and an increase in *Rht-D1* transcript in *Rht-D1c* lines. No significant differences were observed in *Rht-A1* expression across the lines analysed. *** = significant difference (P=<0.001) to respective *rht-tall* lines within the same cultivar/homoeologue-specific primer set. Expression presented relative to ubiquitin. Bars: 1 SEM.

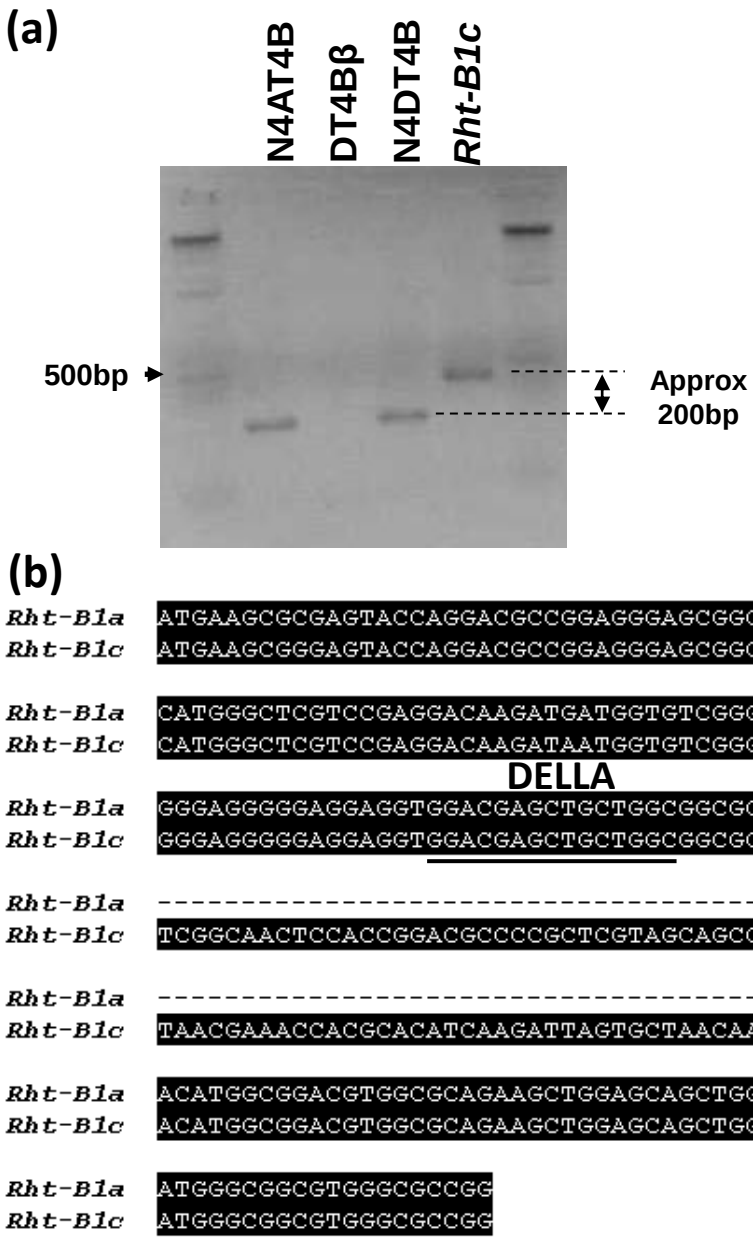


Figure 2.12. *Rht-B1c* contains an insertion within the DELLA region. (a) products, amplified from genomic DNA template with *Rht-B1* specific primers spanning the DELLA domain, separated on an agarose gel. DNA template from aneuploid lines confirms B-homoeologue specificity; N4AT4B, nullisomic 4A tetrasomic 4B; DT4Bβ, ditellosomic 4Bβ; N4AT4B, nullisomic 4D tetrasomic 4B (b) The cDNA sequences of *Rht-B1* from *Rht-B1a* and *Rht-B1c* are aligned (Pearce, Rothamsted). A 90 bp insertion is present in the *Rht-B1c* mutant sequence. The nucleotides encoding the DELLA amino acid motif are underlined.

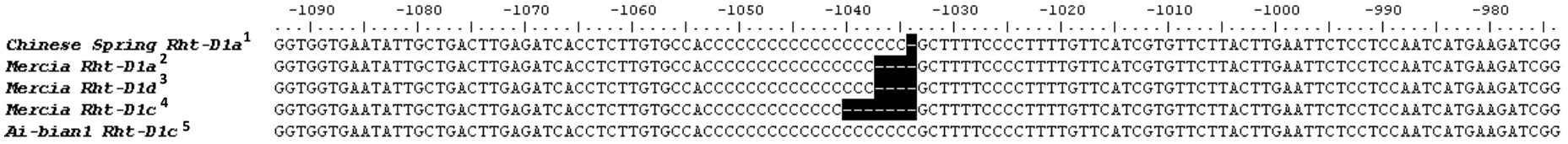


Figure 2.13. Alignment showing the variable cytosine repeat in *Rht-D1* promoter sequences; 1=C.S *Rht-D1a* BAC sequence (from E. Wilhelm, NIAB), 2,3,4=*Rht-D1a –D1b* and –D1c promoter sequence obtained in the present study, 5= Ai-bian *Rht-D1c* BAC sequence (from J. Jia, Beijing). Numbers refer to the co-ordinates upstream of the *Rht-D1* ORF with the first nucleotide of the ATG start codon being +1. Displayed is a portion of the full 3686bp of sequence attained upstream of the *Rht-D1* translation start site, the rest is in appendix 7.1.

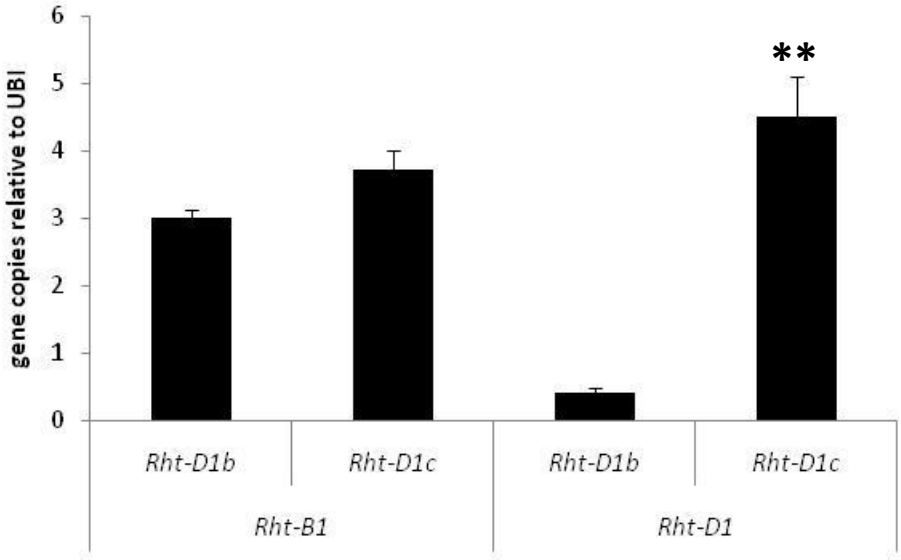


Figure 2.14. *Rht-D1c* appears to be a copy number variant of *Rht-D1b*. Genomic DNA of *Rht-D1b* and *Rht-D1c* was amplified using *Rht-B1* and -*D1* specific assays and the amplification signals were normalised to ubiquitin. *Rht-D1* amplification is greater than that of the *Rht-D1b* template. **= significant difference (P=<0.01) from *Rht-D1b*. Bars: 1 SEM.

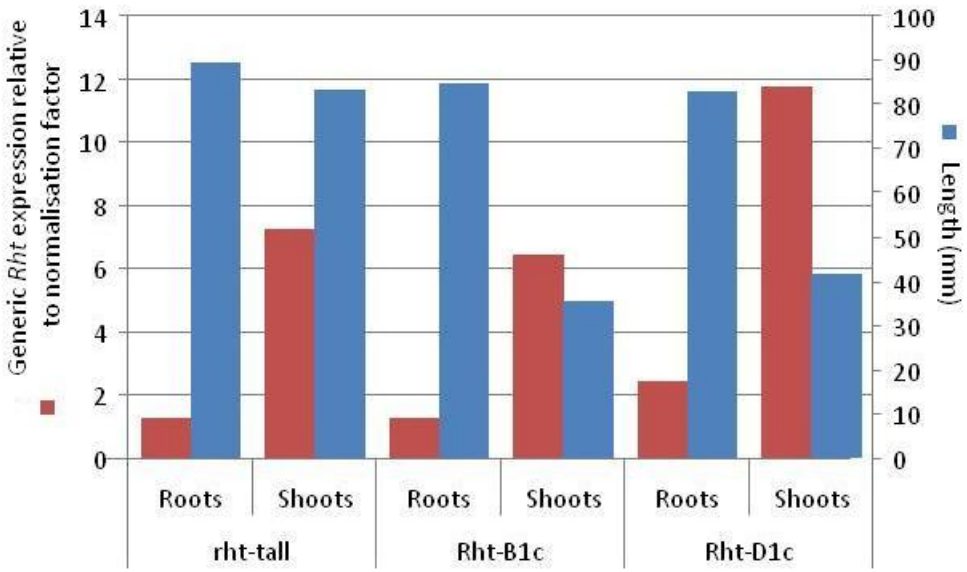


Figure 2.15. Comparison of generic *Rht* expression and root and shoot growth phenotypes. Expression of *Rht* (shown as red bars) was determined in shoots and roots of plants containing the *rht-tall*, and severe dwarfing (*Rht-B1c* and *Rht-D1c*) alleles by qRT-PCR. Shoot height and root length are shown as blue bars. Expression data is presented relative to normalisation factor (based on GAPDH and EF1α).

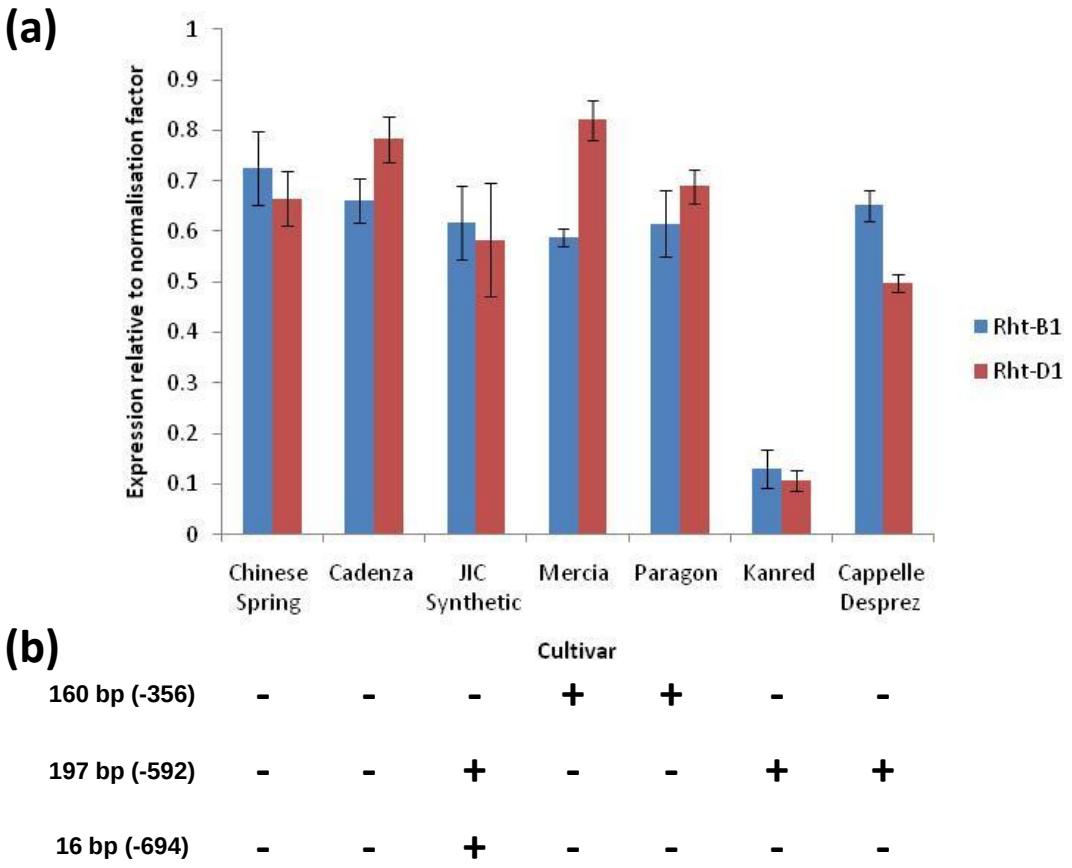


Figure 2.16. *Rht-B1* and *Rht-D1* expression in wheat cultivars containing *Rht-B1* promoter insertions. *Rht-B1* expression (blue bars) is equivalent to *Rht-D1* expression (red bars) within each promoter haplotype. Expression presented relative to normalisation factor (based on GAPDH and EF1α). Bars: 1 SEM. The *Rht-B1* promoter haplotype classifications for each cultivar are shown in panel (b), the sizes of the insertion, and the position upstream of the *Rht* ORF are shown. + denotes the presence of an insertion.

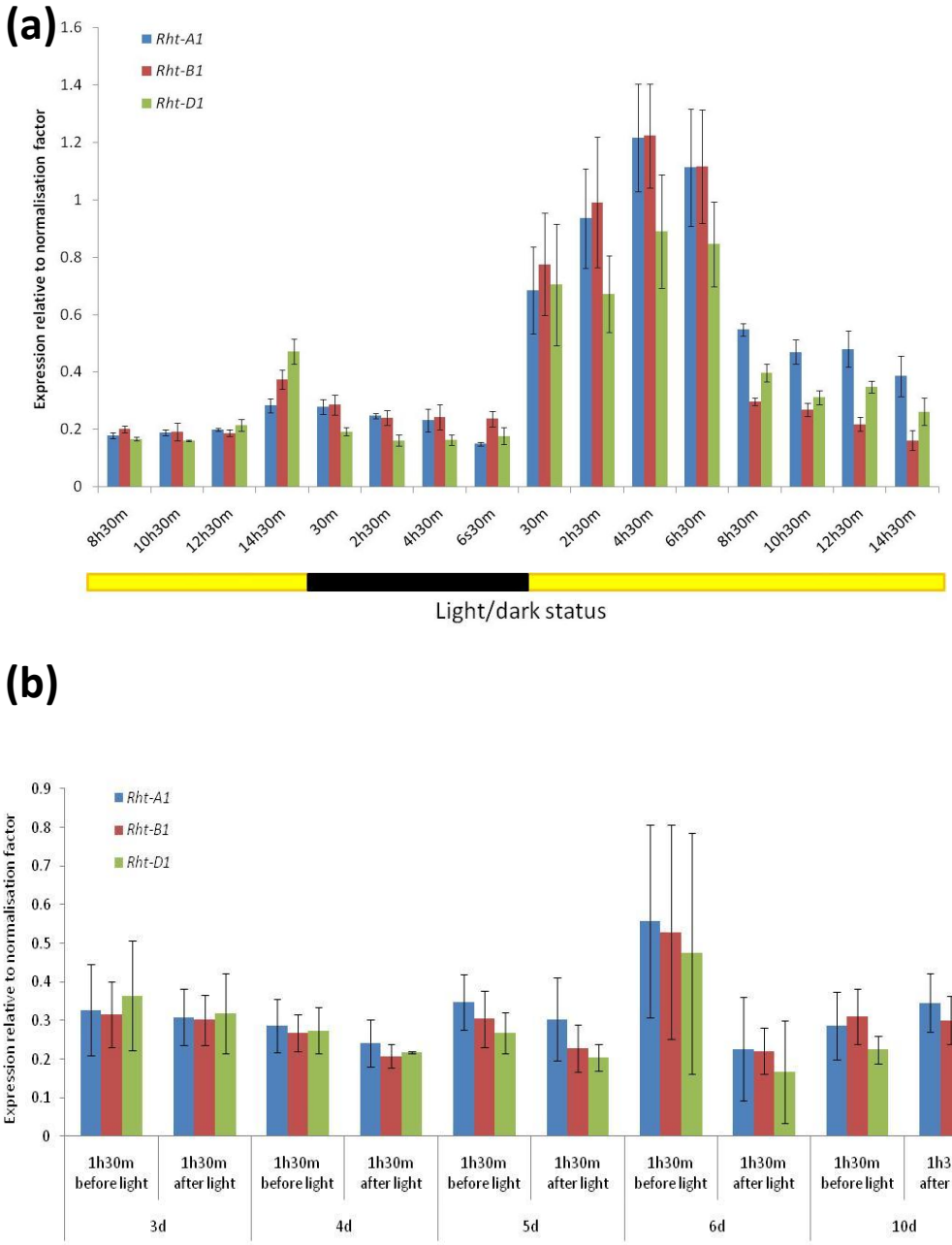


Figure 2.17. *Rht* expression in response to light/dark cycles (a) *Rht* expression was measured over a 36h period in seedlings grown in a 16/8h light/dark cycle. Light status is indicated on the X-axis (yellow; light, black; dark). (b) *Rht* expression was measured on several days 1h 30m before and after the lights were switched on to monitor changes in homoeologue contribution over time. Expression presented relative to normalisation factor (based on GAPDH and EF1α). Bars: 1 SEM.

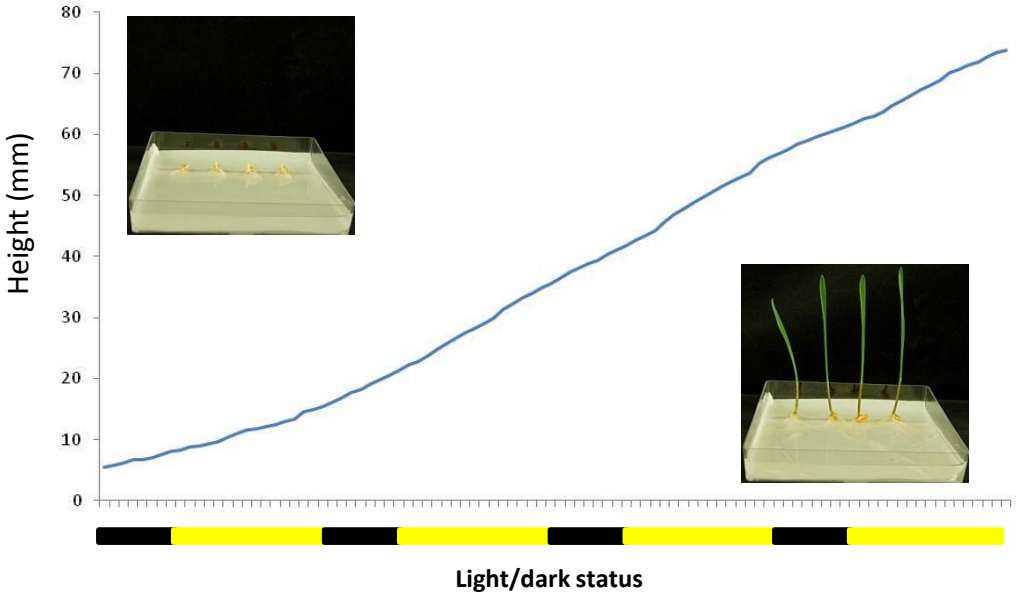


Figure 2.18. Growth rate is not effected by diurnal or *Rht* transcript fluctuations. The growth rate of *rht-tall* seedlings grown under a 16h photoperiod was measured over 4 days. Picture insets show the seedlings at the start and end of the experiment.

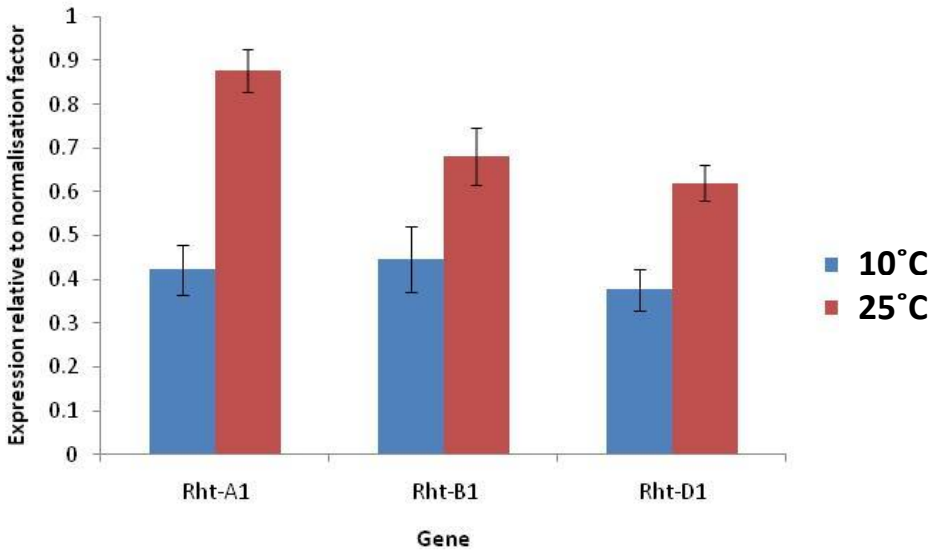


Figure 2.19. *Rht* expression is lower in *rht-tall* seedlings grown at 10˚C in the dark relative to those grown at 25˚C in the dark. Samples were collected after 100 degree days. Expression presented relative to normalisation factor (based on GAPDH and EF1α). Bars: 1 SEM.

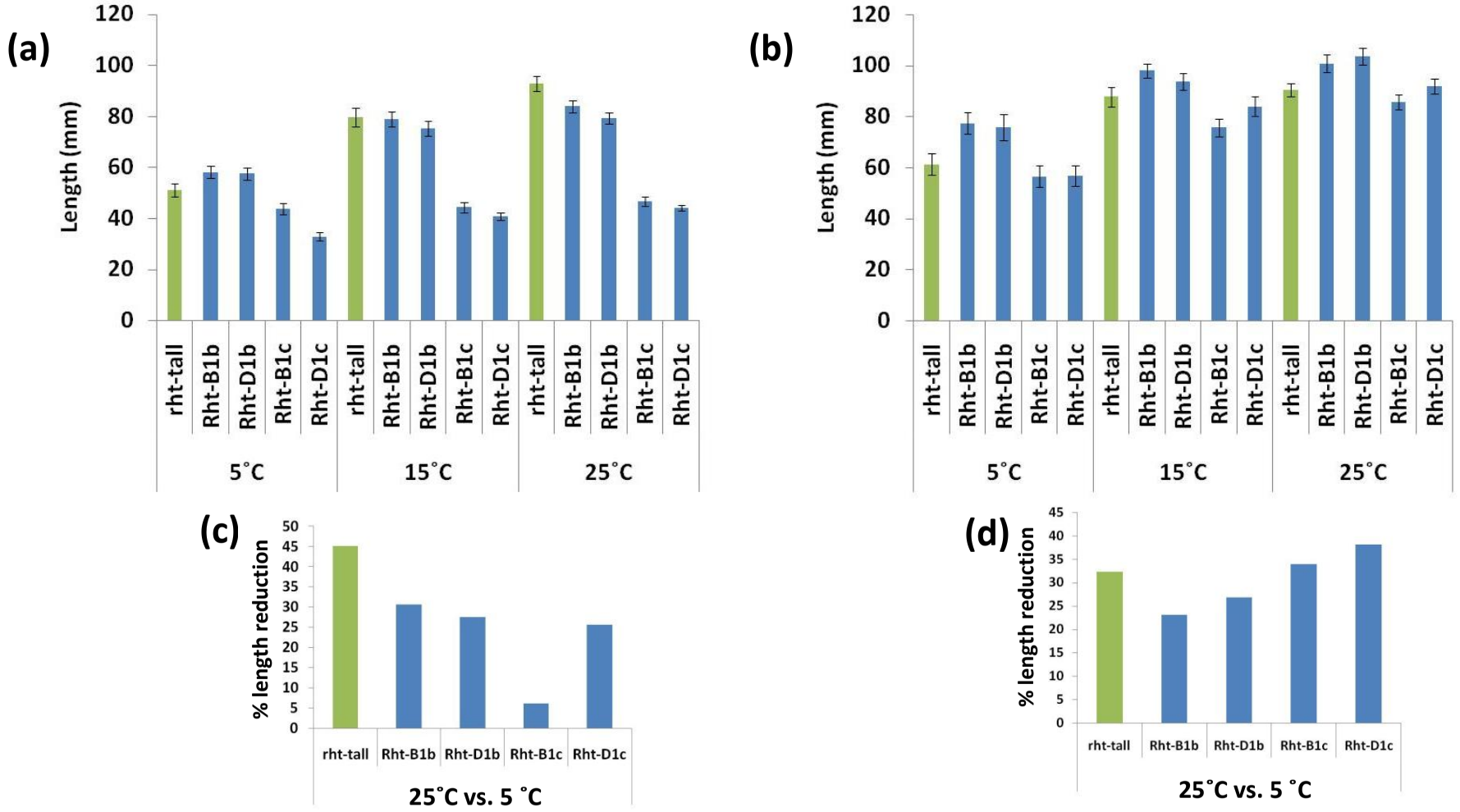


Figure 2.20. *Rht* NILs are affected to different extents in response to growth at low temperature. *Rht*-NILs (*rht-tall* (green), *Rht-B1b*, *Rht-D1b*, *Rht-B1c* and *Rht-D1c* (blue)) were grown in the dark at three temperatures (5˚C, 15˚C and 25˚C) and the shoots (a) and roots (b) were measured after an equivalent time period of 100 degree days. Inset graphs show % growth reduction in shoots (c) and roots (d) grown at 5˚C compared to 25˚C . Bars: 1 SEM.

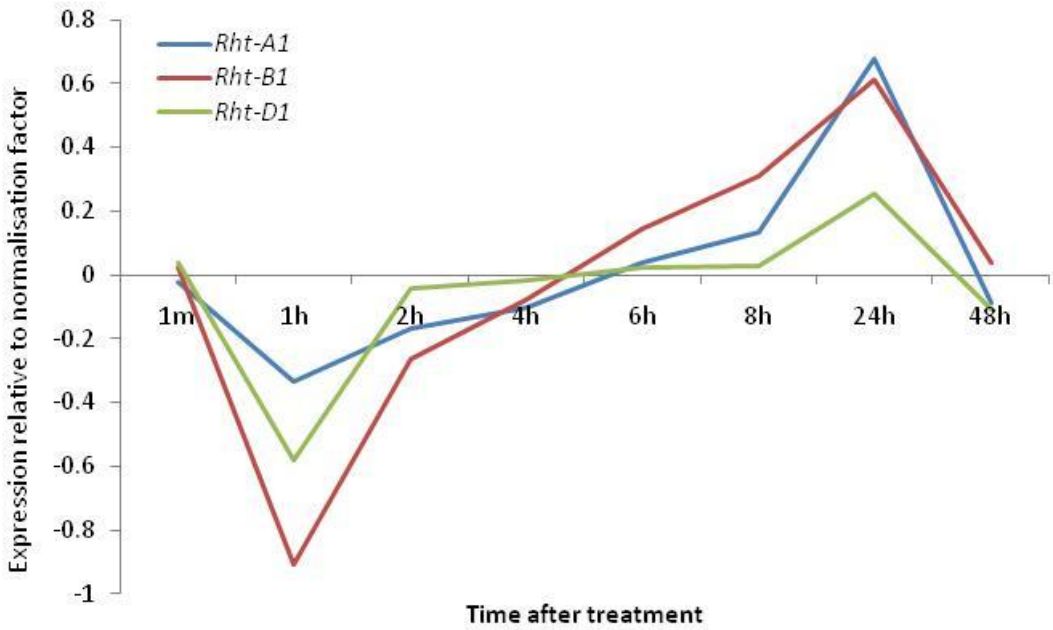


Figure 2.21. Heat stress results in a rapid down regulation of *Rht* transcription. *Rht* expression of *rht-tall* plants was measured at several time points after treatment and presented as treated minus unstressed control. Expression presented relative to normalisation factor (based on GAPDH and EF1α).

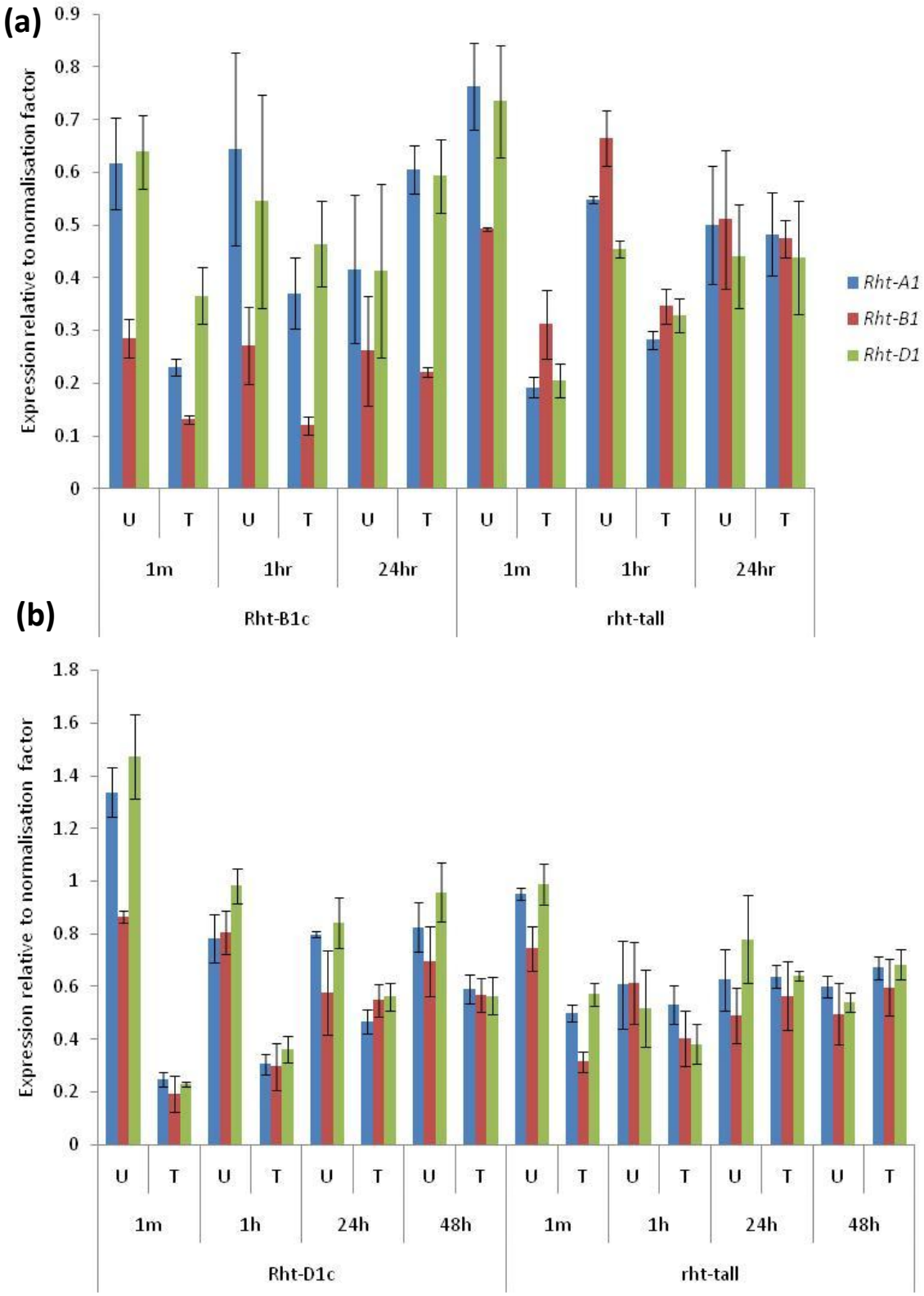


Figure 2.22. Plants containing the *Rht* severe dwarfing alleles show an aberrant heat shock response compared to the wild-type (*rht-tall*). Expression of the three *Rht* homoeologues relative to normalisation factor (based on GAPDH and EF1α) is shown (a) *rht-tall* and *Rht-B1c* expression. (b) *rht-tall* and *Rht-D1c* expression. 1m = 1 minute after heat shock. Bars: 1 SEM.

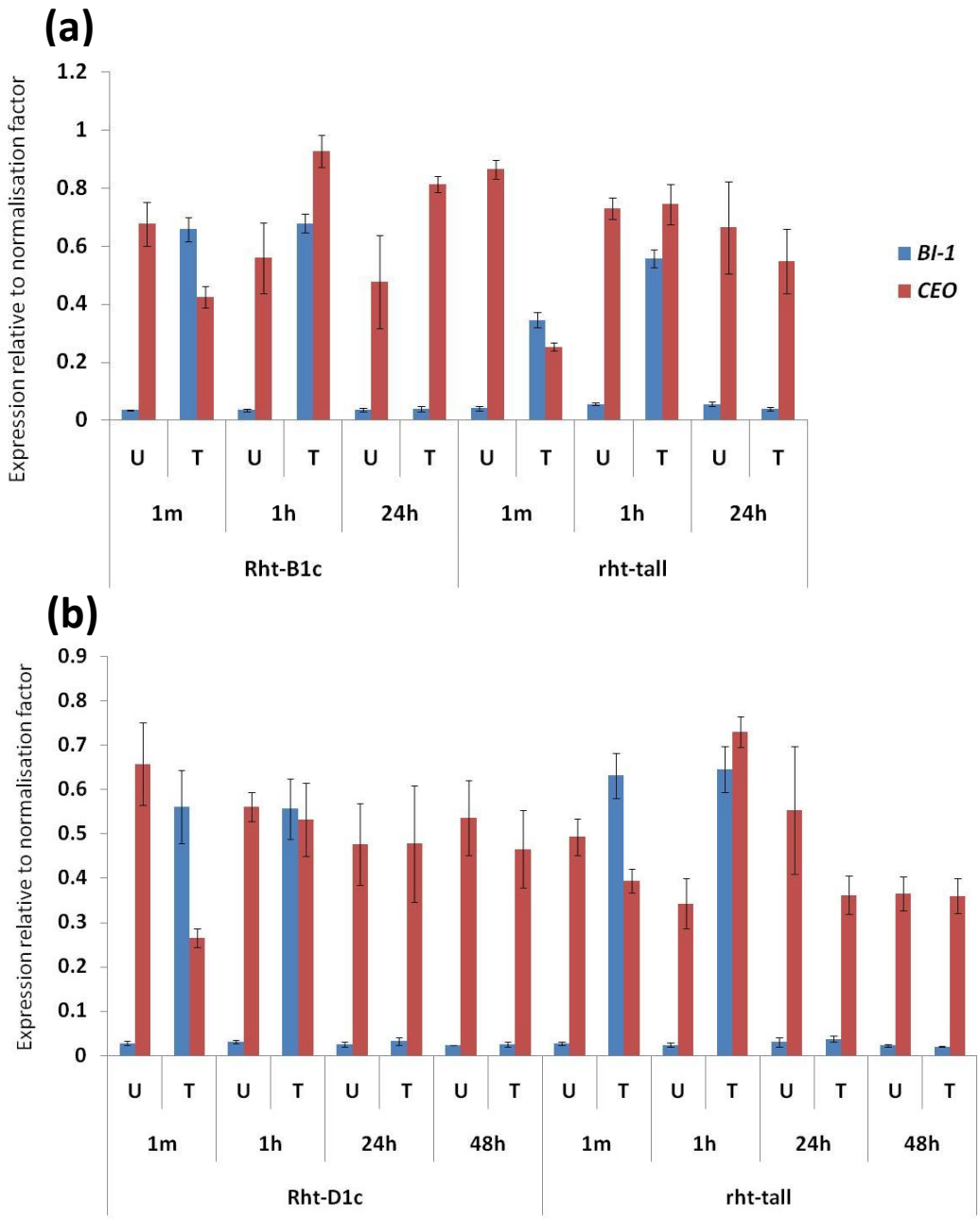


Figure 2.23. Expression of *BAX INHIBITOR-1* (*BI-1*; blue bars), but not *CLONE EIGHTY ONE* (*CEO*; red bars), both of which play a putative role in the negative regulation of cell death, is affected by heat stress. (a) compares *BI-1* and *CEO* expression in *Rht-B1c* and *rht-tall*. (b) compares *BI-1* and *CEO* expression in *Rht-D1c* and *rht-tall.* Expression presented relative to normalisation factor (based on GAPDH and EF1α). Bars: 1 SEM. 1m; 1 minute after treatment removal.

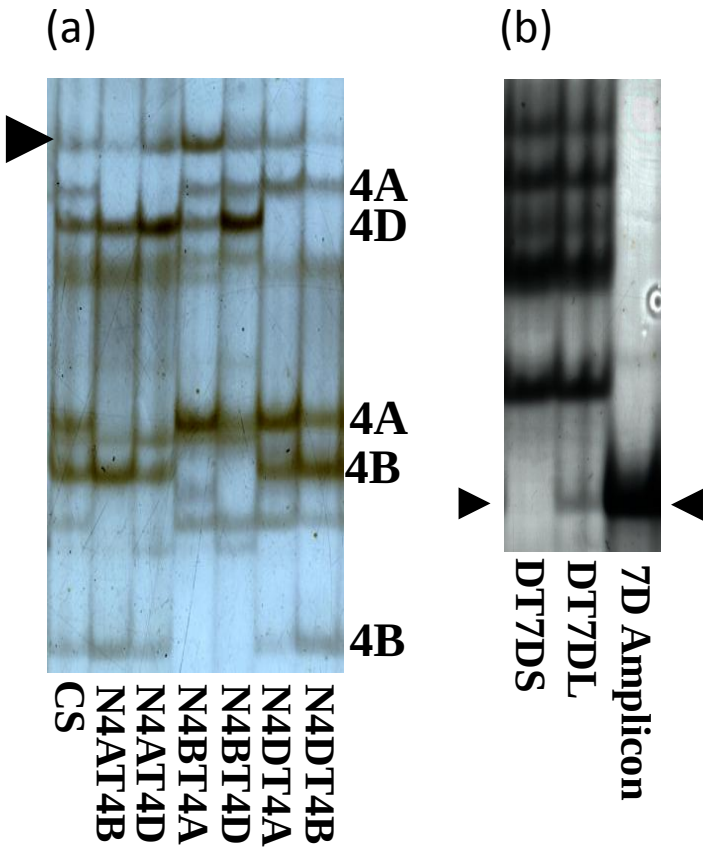


Figure 3.1. SSCP gels suggest the presence of an additional *Rht* family member on chromosome 7D. (a) Generic *Rht* were used to amplify the *Rht* genes in each of the group 4 chromosome nullisomic lines and Chinese Spring euploid control (CS). The *Rht* bands were not amplified in the respective nullisomic-tetrasomic lines, however one band remained in all lines (arrowed). (b) Representative gel showing the *Rht*-specific amplification products obtained using DNA from ditelosomic lines DT7DS and DT7DL. The product identified in panel (a) was located to the long arm of chromosome 7D (arrowed). Gels were provided by A.Bottley (JIC).

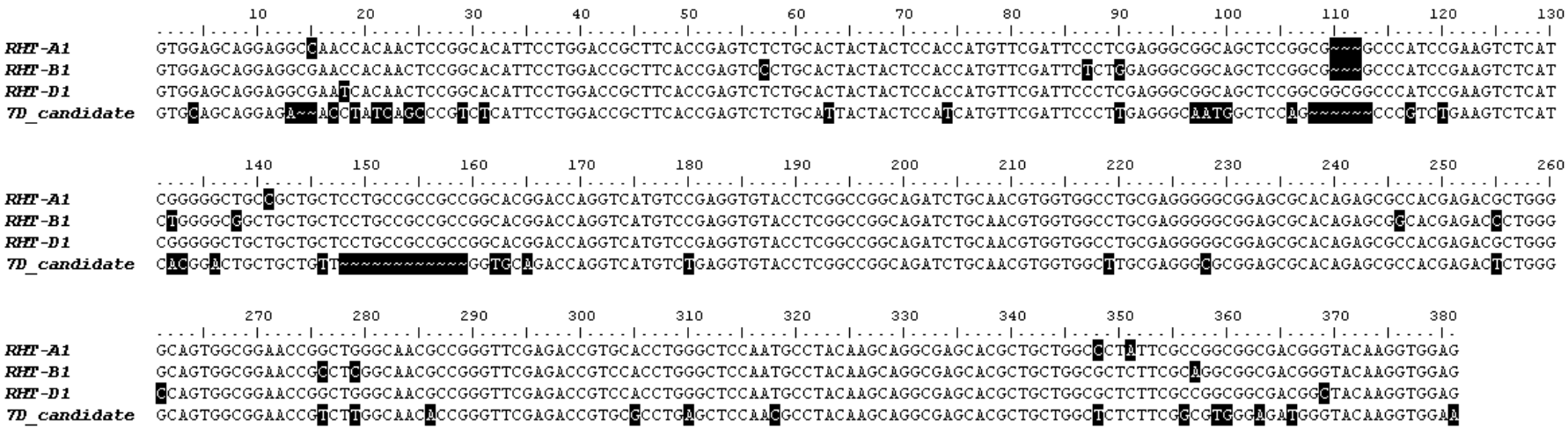
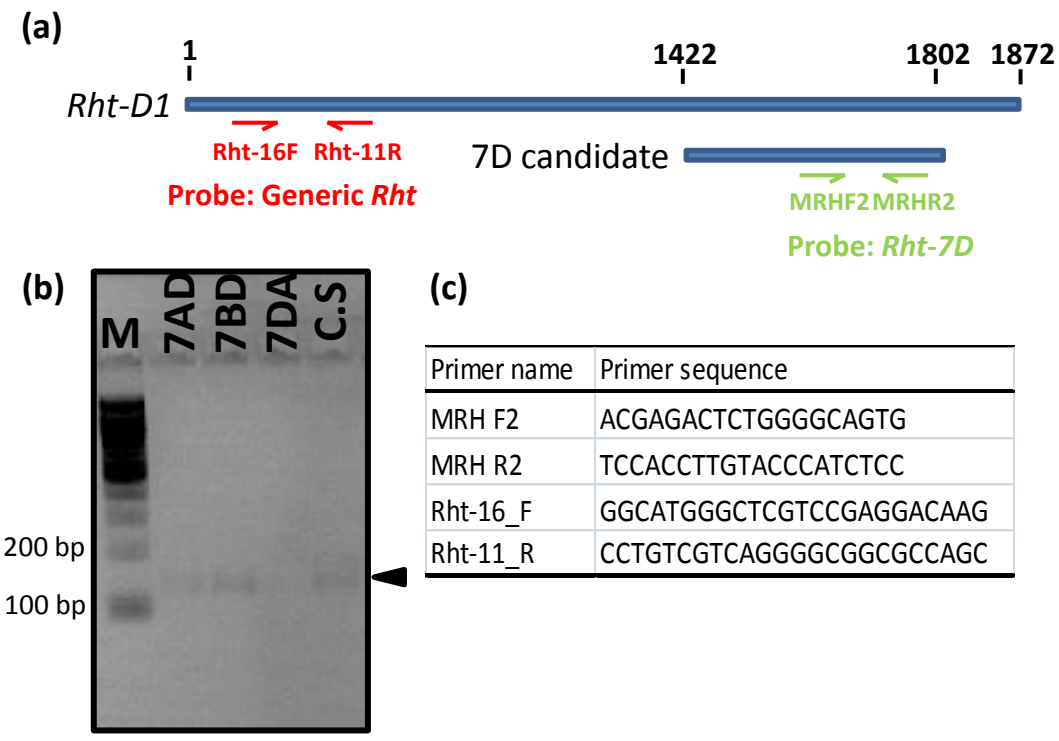


Figure 3. 2. A conservation plot of a section of the group 4 *Rht* gene sequence aligned to the 7D candidate gene sequence. The homology across the group 4 *Rht* gene homoeologues is 96-98%. The homology between the group 4 *Rht* gene homoeologues and the 7D candidate gene is 87-88%. Black infilled regions highlight polymorphisims. ~ denotes deletion.



Primer name	Primer sequence
MRH F2	ACGAGACTCTGGGGCAGTG
MRH R2	TCCACCTTGTACCCATCTCC
Rht-16_F	GGCATGGGCTCGTCCGAGGACAAG
Rht-11_R	CCTGTCGTCAGGGGCGGCGCCAGC

Figure 3.3. Probes and primer sets used to screen the BAC libraries. (a) Schematic of the *Rht-D1* and 7D alignment. Numbers refer to the *Rht-D1* gene co-ordinates with the first nucleotide of the ATG start codon being 1. Generic DELLA primers, Rht-16_F and Rht-11_R (red), amplify a 195 bp product referred to as Generic *Rht* whereas the 7D candidate gene specific primers, MRH F2 and MRH R2 (green), amplify a 133 bp product referred to as *Rht-7D*. (b) Products (arrow) amplified with the MRH primer set from group 7 nullisomic-tetrasomic templates separated on an agarose gel. C.S = Chinese Spring; M = 100 bp molecular weight marker. The absence of a product from the nullisomic 7D template proves primer specificity. (c) Sequences of primers used to screen the BAC libraries.

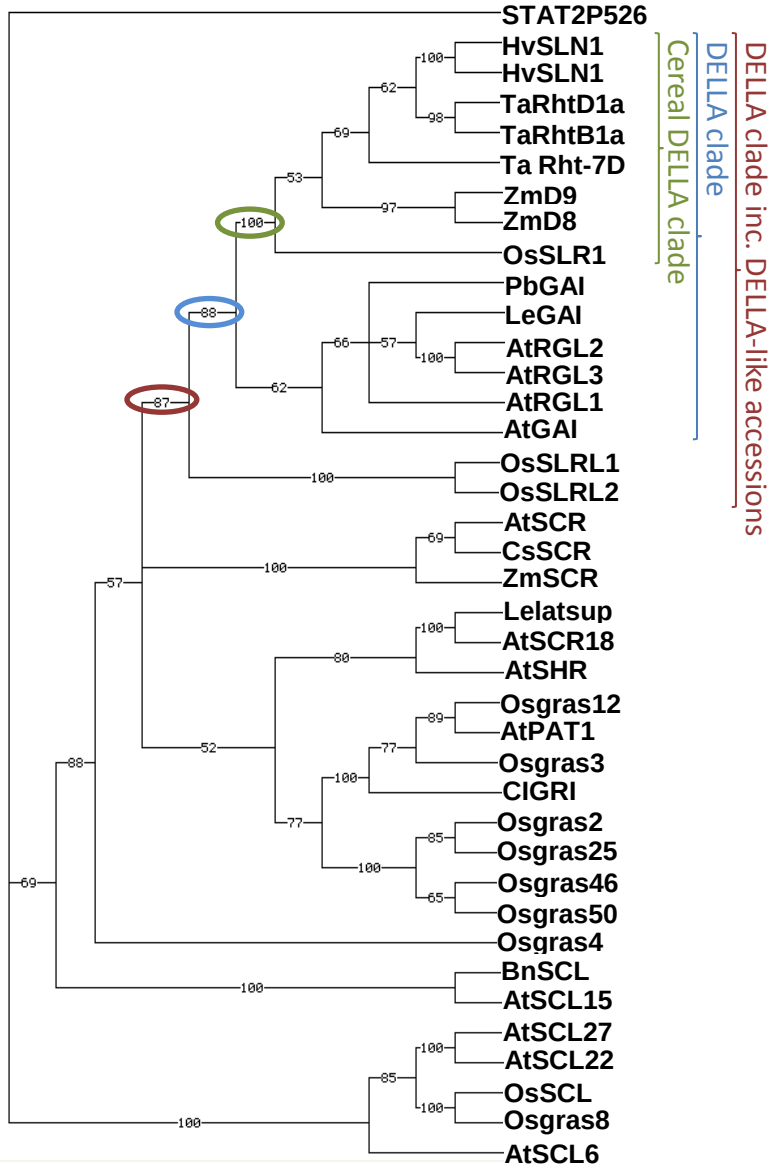


Figure 3.4. Phylogenetic tree of GRAS genes from a variety of plant species; At, Arabidopsis; Hv, barley; Le, tomato; Os, rice; Pb, poplar; Ta, bread wheat; Zm, maize. Sequence from the C-terminal region of the GRAS genes that aligned to the sequence for TaRht-7D (Fig. 3.2) were used to construct the tree. The DELLA clade, with and without DELLA-like accessions, and the cereal DELLA sub-clade are highlighted, respective bootstrap values are 87, 88 and 100. The 7D candidate gene (arrowed) falls within the cereal DELLA sub-clade. The tree was rooted using the C-terminal region of a member of the signal transducers and activators of transcription (STAT) family of proteins (STAT2P526), which are structurally and functionally similar to the GRAS proteins (Richards *et al*., 2000).

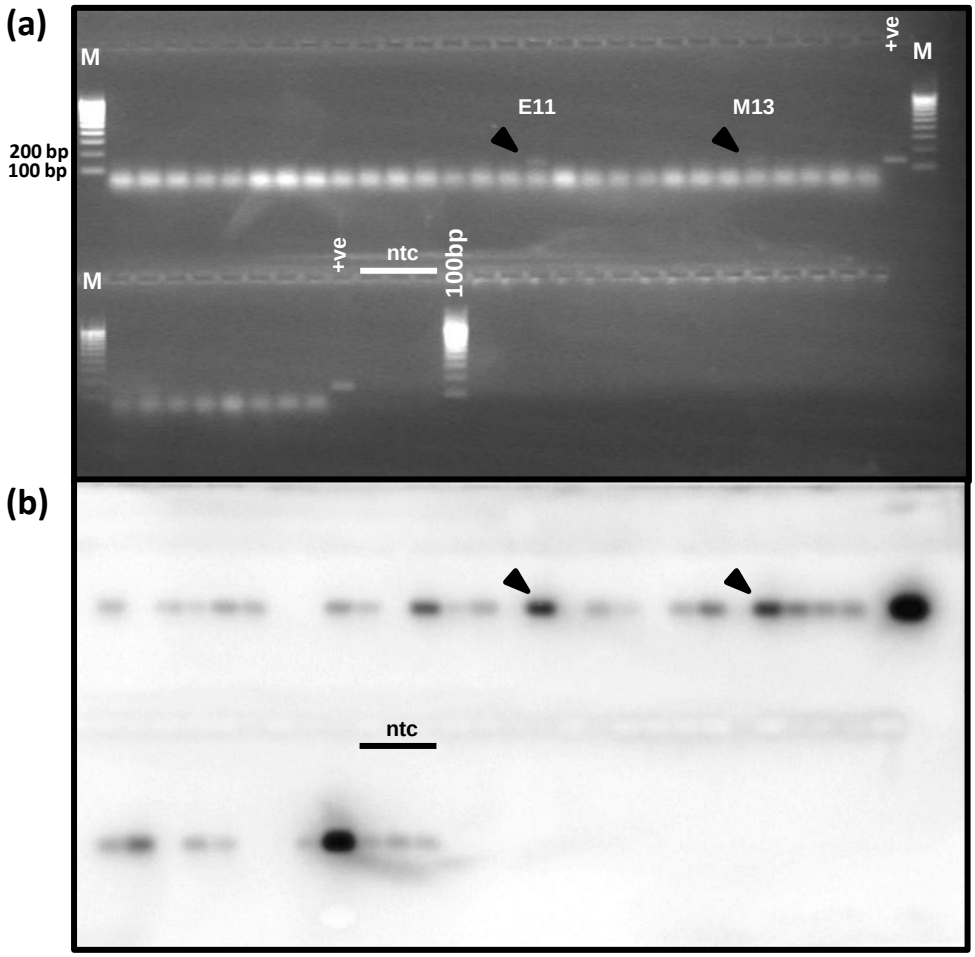


Figure 3.5. Screening of generic *Rht* probe selected BACs. (a) Products amplified with MRH primers from minipreps of BAC clones that had hybridised to the generic *Rht* probe were separated by gel electrophoresis. Clones from which product is amplified are highlighted. The positive template was a clone of the available 7D sequence. M = 100 bp molecular weight marker.(b) DNA from (a) was transferred to a nylon membrane by Southern blotting and hybridised with the generic *Rht* probe. (ntc; non-template control)

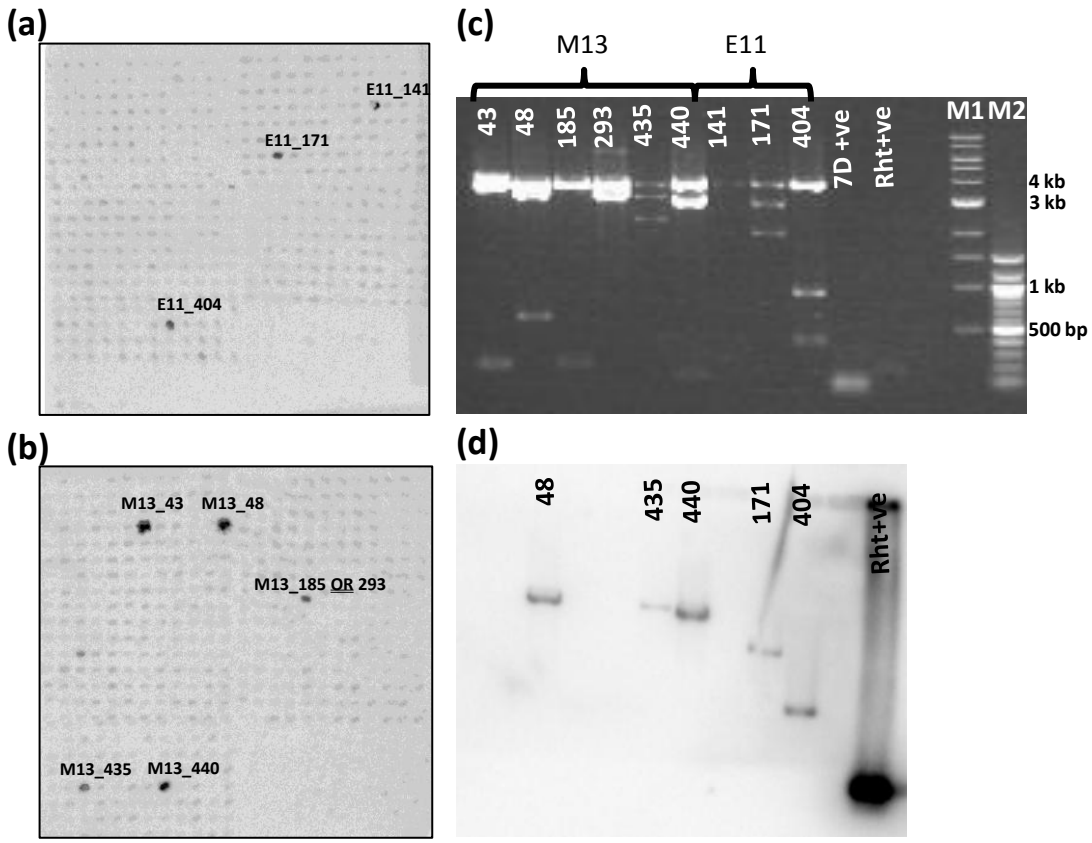


Figure 3.6. Screening of the sub-clone library for clones containing Rht-7D sequence. Colony hybridisation of the E11 (a) and M13 (b) sub-clone libraries with the generic *Rht* probe. The sub-clone IDs are shown for clones that produced a hybridisation signal. Overlapping plates during transfer of the M13 subclones meant that one positive was not possible to call therefore both clones (M13_185 and M13_293) were tested (c) The plasmid DNA of the positive clones from (a) and (b) were digested with EcoR1 and separated by gel electrophoresis and visualised by ethidium bromide in order to estimate insert size. M1 and M2 = 1 kb and 100 bp molecular weight marker respectivley (d) Southern hybridisation of the gel shown in panel (c) using the generic *Rht* probe. Apart from the 7D positive control the *Rht-7D* probe did not produce a hybridisation signal on duplicate blots of either a, b or d.

Table 3.#.
In excel file

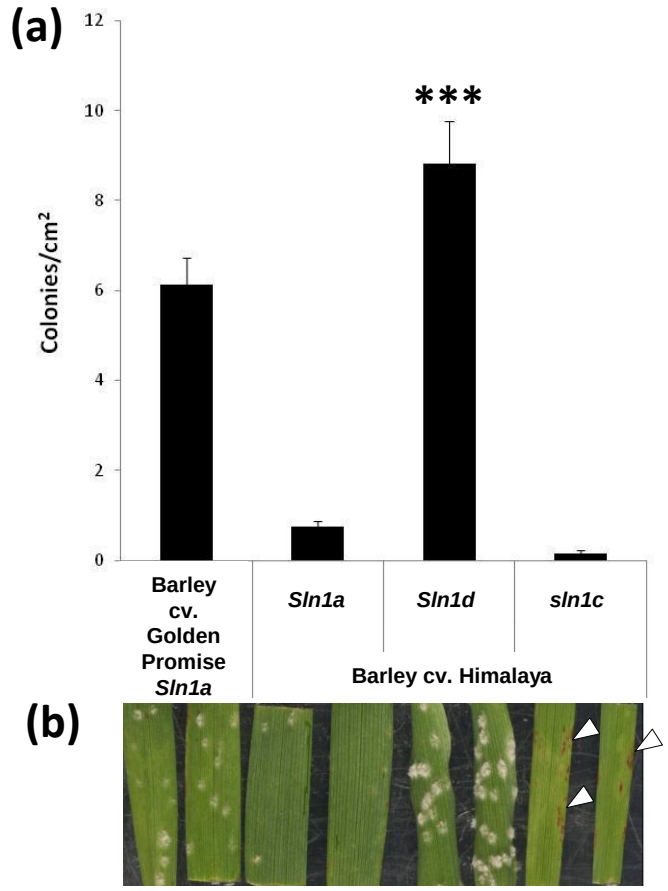


Figure 4.1. The effect of *Sln1* alleles on infection of barley with *B. graminis* f. sp. *hordei*. (a) Leaves of barley cvs. Golden Promise (susceptible control) and Himalaya allelic at the *Sln1* locus inoculated with *Bgh*. The number of colonies per leaf area (cm^2) were measured 8 dpi. Bars; 1 SEM.(b) Representative disease phenotypes, white arrow heads denote the hypersensitive response in *sln1c*. *** = significant difference (P=<0.001) to Himalaya *Sln1a*.

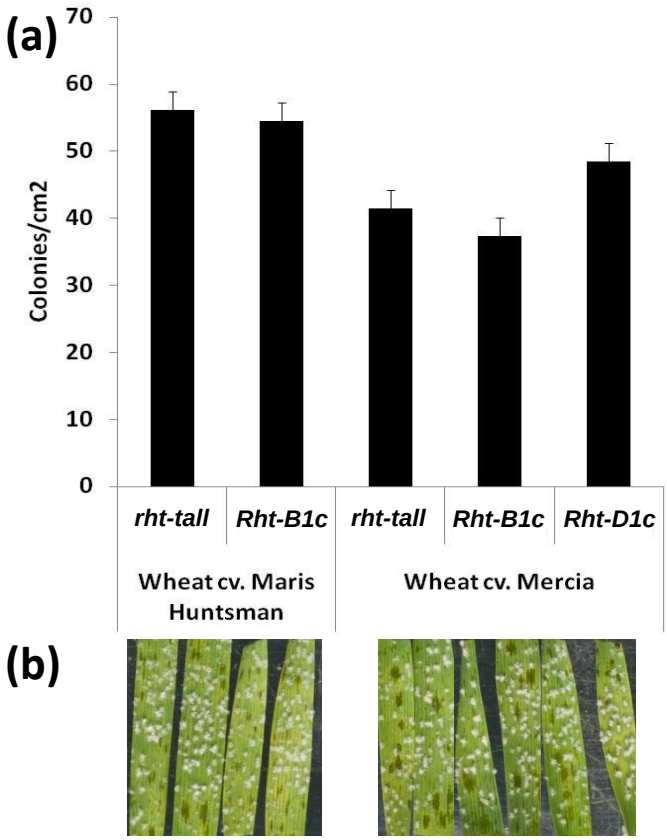


Figure 4.2. The effect of single GoF *Rht* alleles on infection of wheat with *B. graminis* f. sp*. tritici* Wheat cv. Maris Huntsman and Mercia *rht-tall* (wild-type) and severe dwarf NILs inoculated with *Bgt*. (a) The number of colonies per cm^2 at 8dpi. Bars; 1 SEM. (b) Representative disease phenotypes.

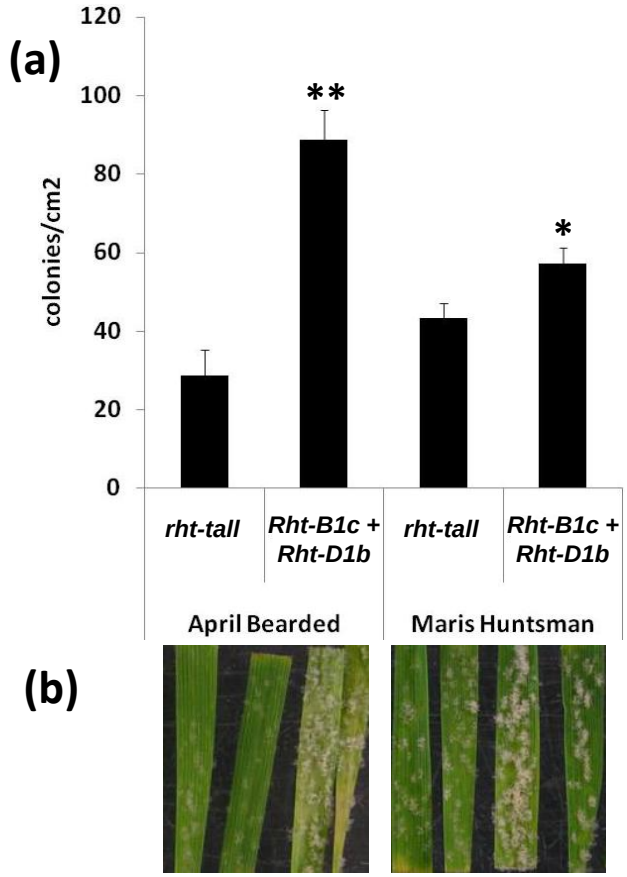


Figure 4.3. The effect of double GoF *Rht* alleles on infection of wheat with *B. graminis* f. sp*. tritici.* Wheat cv. April Bearded and Maris Huntsman, *rht-tall* (wild-type) and double mutant (*Rht-B1c* + *Rht-D1b*) NILs inoculated with *Bgt*. (a) The number of colonies per cm^2 at 8 dpi. Bars; 1 SEM. (b) The representative disease phenotypes. * and ** = significant difference (P=<0.05 and <0.01) to respective wild type lines.

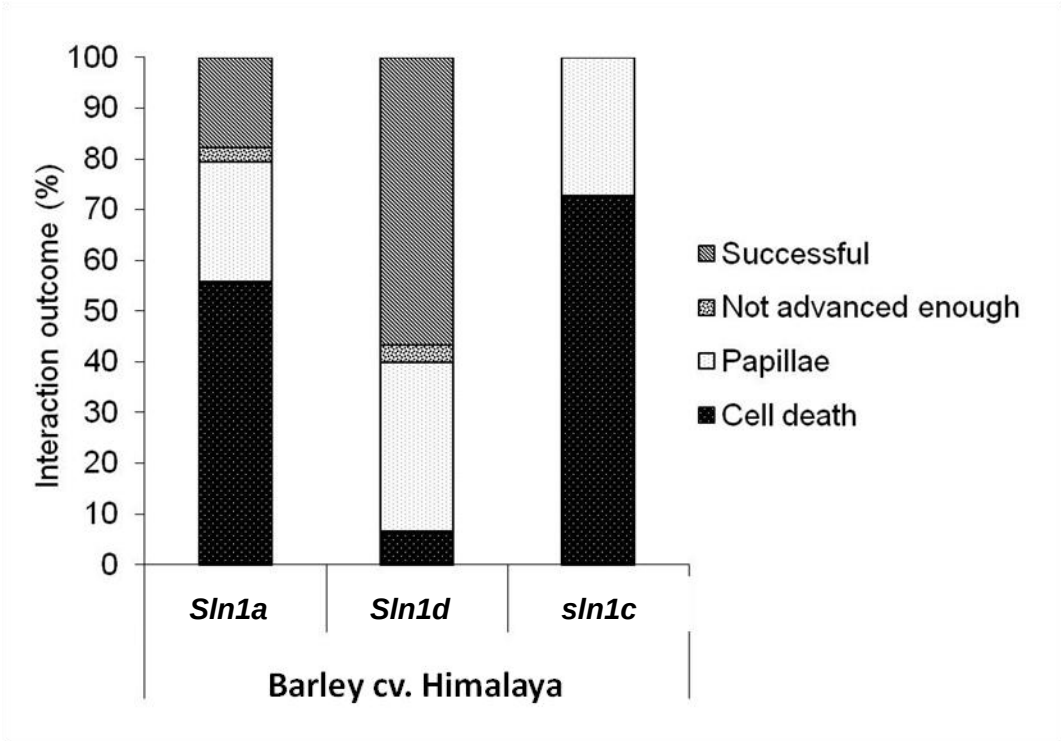


Figure 4.4. Cytological analysis of the effect of Sln1 alleles on infection of barley with *B. graminis f. sp. hordei*. The interaction outcome of 100 spores was analysed at 60 hpi. Spores which produced a haustorium were defined as successful. Unsuccessful interactions were attributed to either host cell death, papillae response or because spores were not advanced enough to elicit a response.

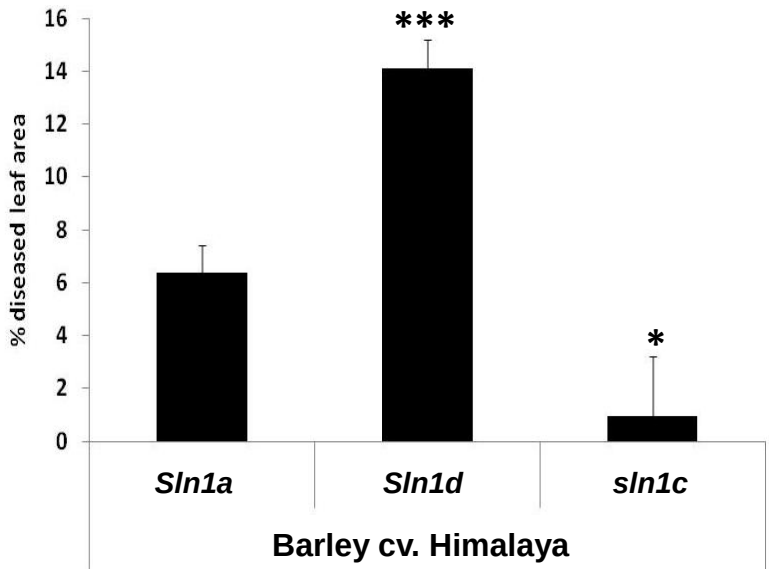


Figure 4.5. The effect of *Sln1* alleles on suceptibility of barley to *R. collo-cygni.* Barley cv. Himalaya lines allelic at the *Sln1* locus inoculated with *R. collo-cygni* scored for disease (% diseased leaf area) 15 dpi. Data presented are cumulative results from 3 experiments for *Sln1a* and *Sln1d* lines; *sln1c* line was only included in a single experiment. * and *** = significant difference (P=<0.05 and <0.001) to Himalaya WT. Bars; 1 SEM.

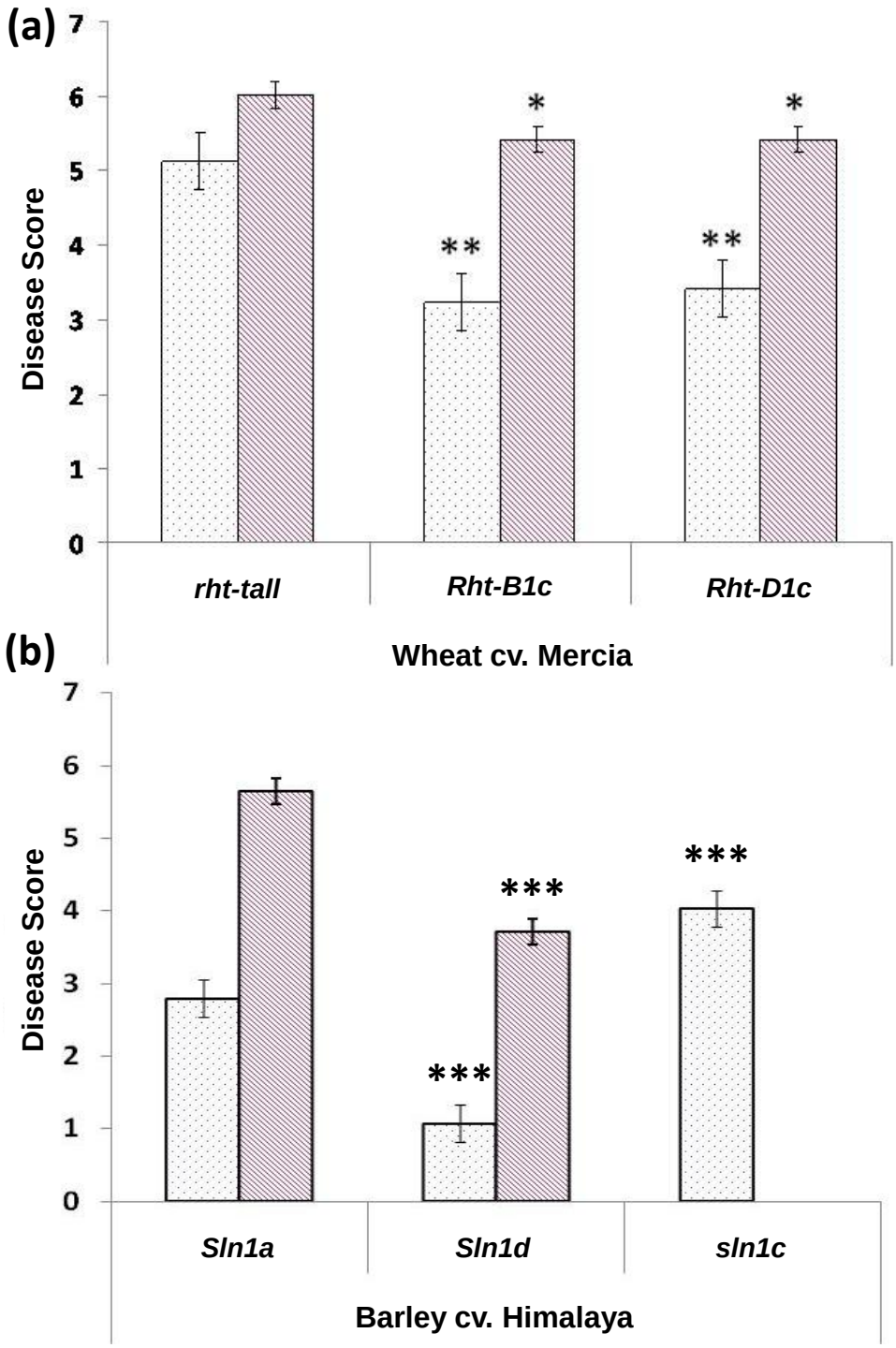


Figure 4.6. The effect of *Rht and Sln1 alleles on resistance of w*heat and barley to *Oculimacula* species. (a) Wheat cv. Mercia *rht-tall* (wild type) and severe dwarf NILs inoculated with either *O. acuformis* ( ) or *O. yallundae* ( ) were scored for disease severity using a scale devised by Scott (1971). * and ** = significant difference (P=<0.05 and <0.01 respectively) to Mercia *rht-tall.* (b) Barley cv. Himalaya *Sln1a, Sln1d* and *sln1c* were scored and presented as above. The *sln1c* line was not inoculated with *O. yallundae.* *** = significant difference (P=<0.001) to Himalaya *Sln1a*. Bars; 1 SEM.

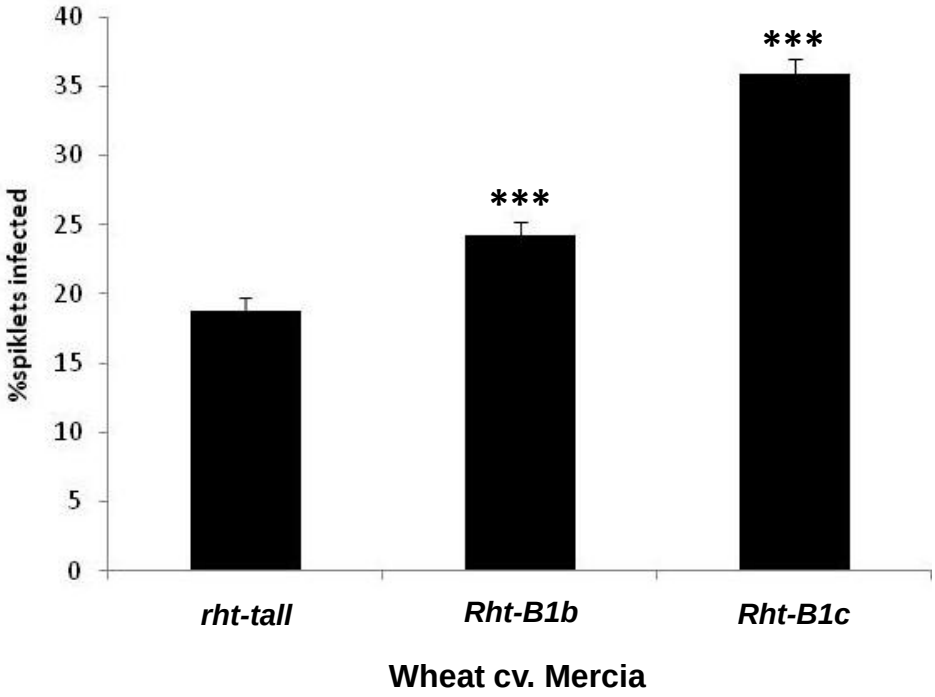


Figure 4.7. The effect of *Rht-B1* alleles on type 1 resistance to Fusarium head blight. Plants of wheat cv. Mercia NILs allelic at the *Rht-B1* loci were spray inoculated with *F. graminearum* to assess their resistance to initial infection. The percentage of spikelets infected were measured 14dpi. ***= significant difference (P=<0.001) to *rht-tall.* Bars; 1 SEM.

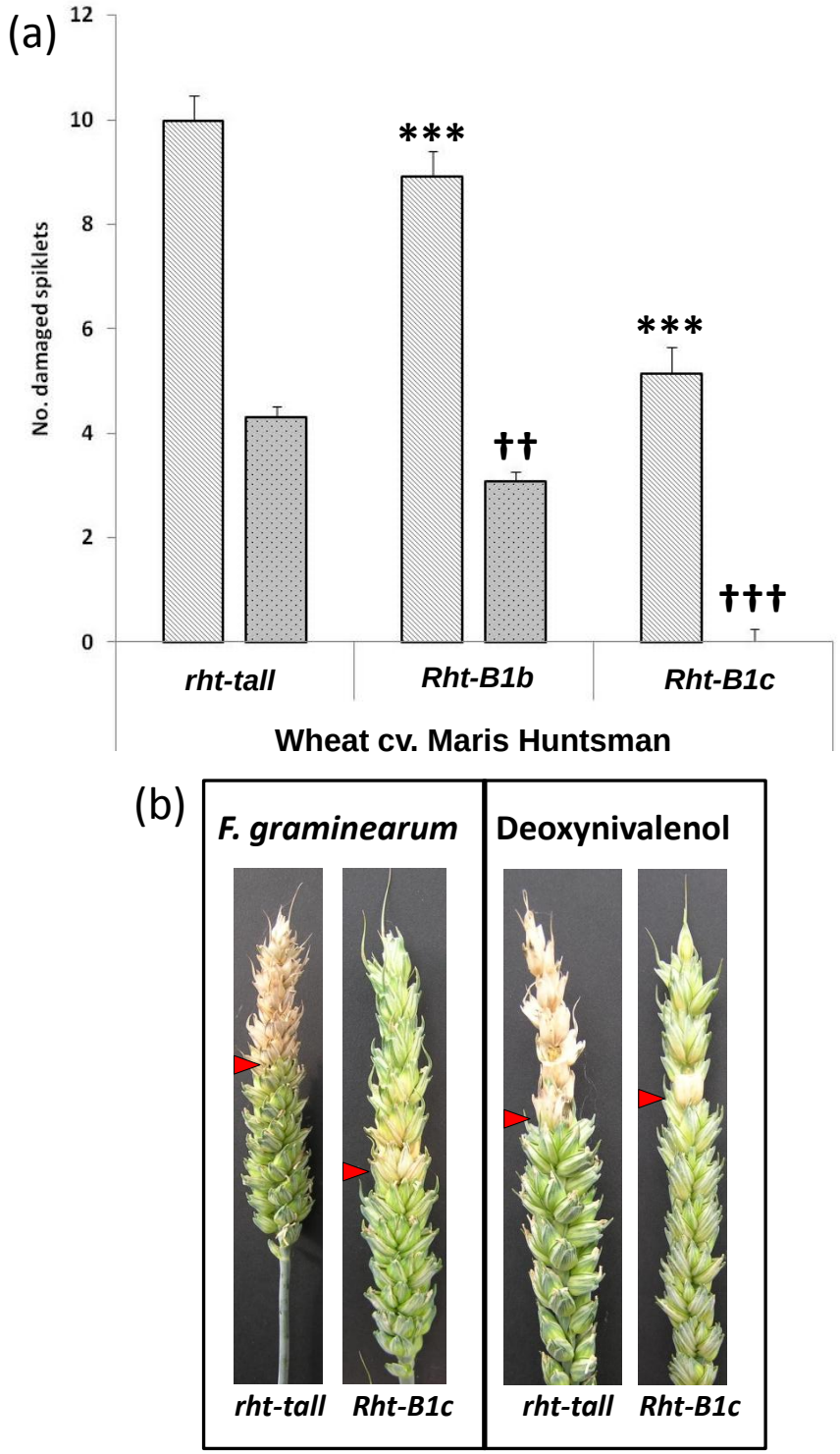


Figure 4.8. The effect of *Rht-B1* alleles on type 2 resistance to Fusarium head blight and deoxynivalenol. Point inoculation with either *F. graminearum* or deoxynivalenol (DON) of the ears of wheat cv. Maris Huntsman NILs allelic at the *Rht-B1* loci 14 dpi. (a) Number of damaged spiklets. Hatched bars represent *F. graminearum* inoculated ears ***= significant difference (P=<0.001) to r*ht-tall*. Dotted bars represent DON treated ears †† and †††= significant difference (P=<0.01 and <0.001 respectivley) to *rht-tall.* Bars; 1 SEM.(b) Picture of typical symptom spread and contained phenotype in *rht-tall* and *Rht-B1c* lines. Injected spikelet is arrowed.

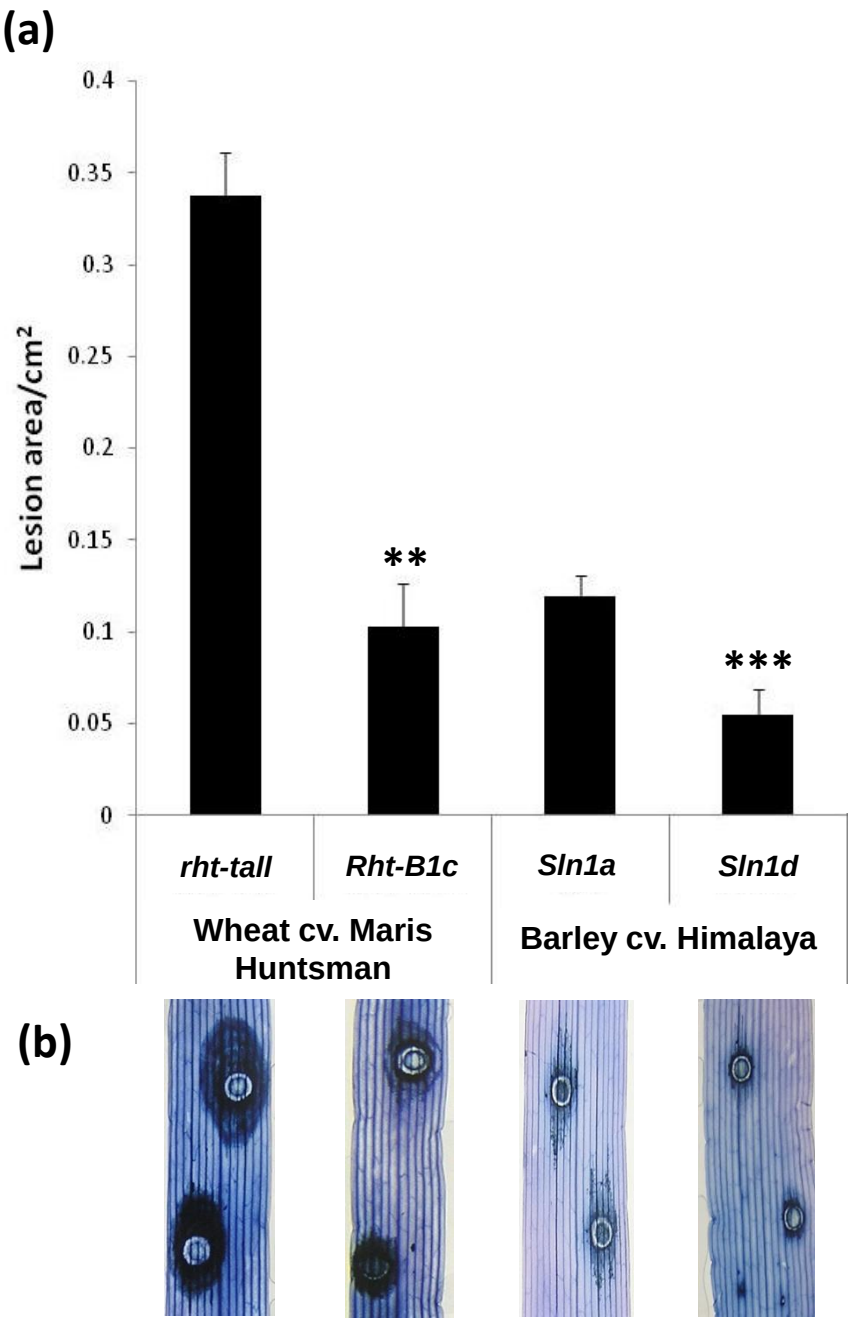


Figure 4.9. Assessment of GoF *Rht* and *Sln1* mutant alleles for resistance to *F. graminearum* in wheat and barley. (a) Mean cell death lesion area (cm^2) 6 dpi. ** and ***= significant difference (P=<0.01 and <0.001) to corresponding wild-type. Bars; 1 SEM. (b) Representative lesions on leaves of wild type and mutant plants stained with trypan blue to detect cell death. The central circles show inoculation points.

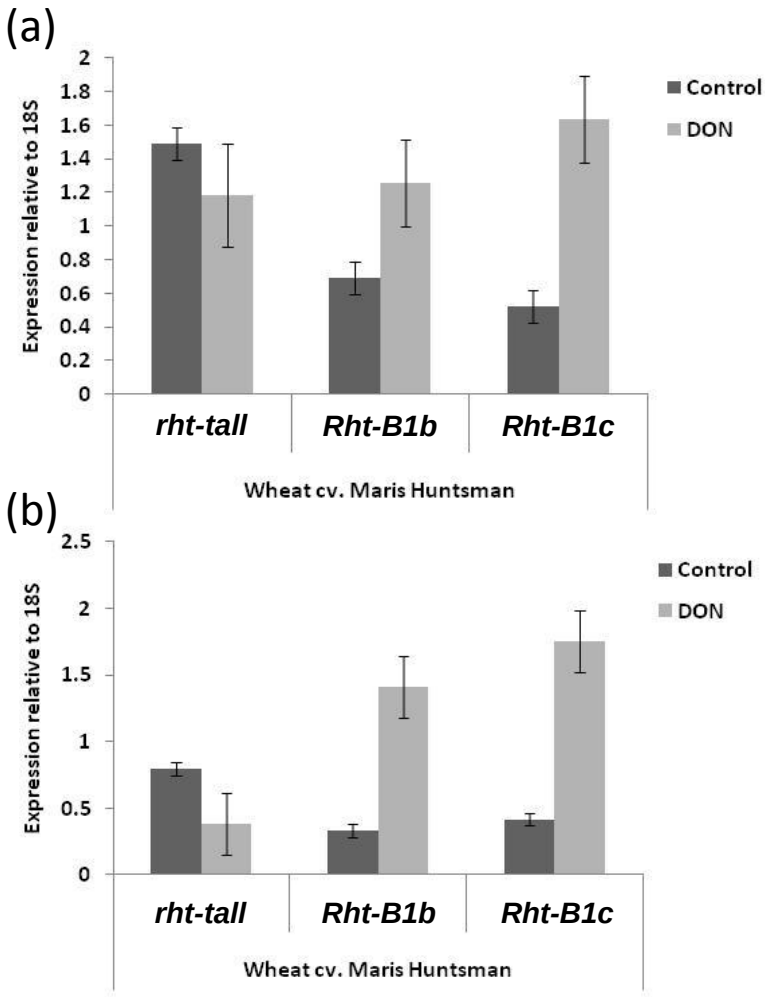


Figure 4.10. The effect of deoxynivalenol on the expression of genes involved in the negative regulation of cell death. *BAX INHIBITOR-1* (a) and *CLONE EIGHTY ONE* (b) expression was measured in the root tissue of wheat cv. Maris Huntsman NILs allelic at the *Rht-B1* locus treated with water (control) or deoxynivalenol (DON). Target gene expression was normalised to 18S rRNA expression. Bars; 1 SEM.

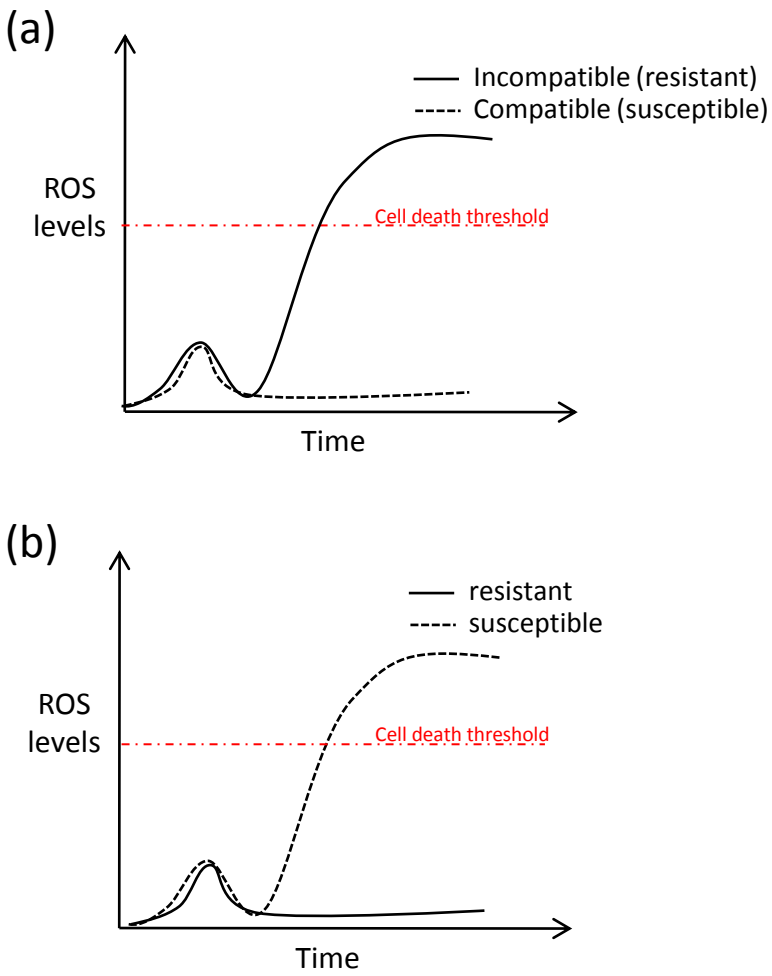


Figure 5.1. A schematic of host ROS burst disease outcome scenarios dependent on pathogenic lifestyle. (a) host ROS levels during incompatible and compatible plant pathogen interactions to biotrophic pathogens. The dotted red line represents a cell death threshold. If pathogen-elicited ROS levels exceed this threshold, cell death is initiated resulting in resistance. If the pathogen is not recognised by the host or the pathogen delivers effectors to reduce the host response then ROS levels remain beneath the threshold leading to susceptibility. Adapted from Grant and Loake (2000) (b) ROS levels in resistant and susceptible hosts in response to necrotrophic pathogen challenge. Resistant lines dampen pathogen promoted ROS burst keeping levels below the cell death threshold. Extrapolated from data presented by Able (2003) who investigated barley host ROS response to *Rhynchosporium secalis* and *Pyrenophora teres* challenge.

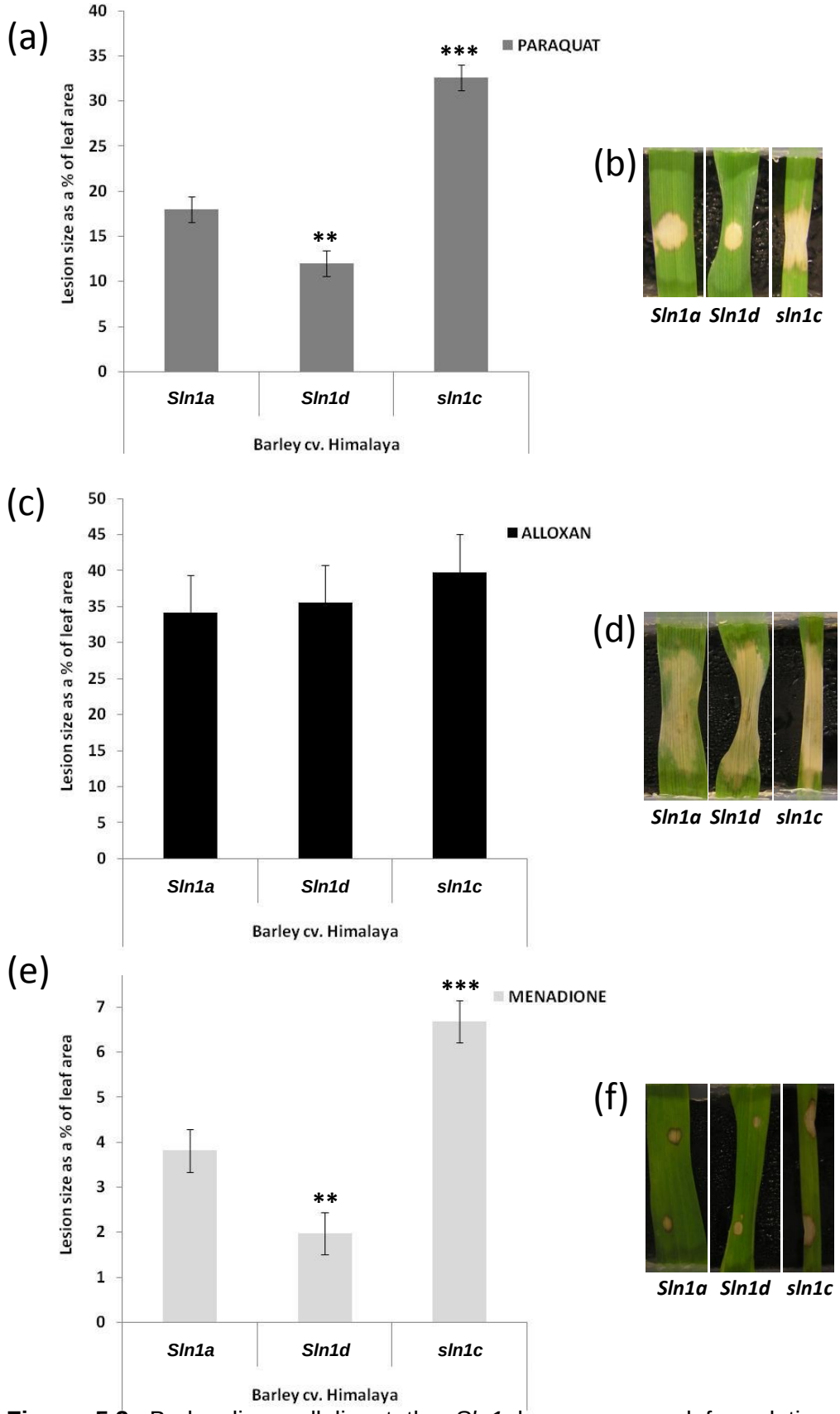


Figure 5.2. Barley lines allelic at the *Sln1* locus assessed for relative resistance to pharmacological ROS inducers. Resistance (measured as cell death lesion size as a percentage of leaf area) of *Sln1a* (wild type), *Sln1d* (GoF), and *sln1c* (LoF) lines to paraquat (a), alloxan (c) and menadione (e) is presented. ** and *** = significant difference (P=<0.01 and <0.001 respectively) from *Sln1a*. Bars: 1 SEM. Representative pictures of treated leaves are displayed to the side of each graph (b, d and f)

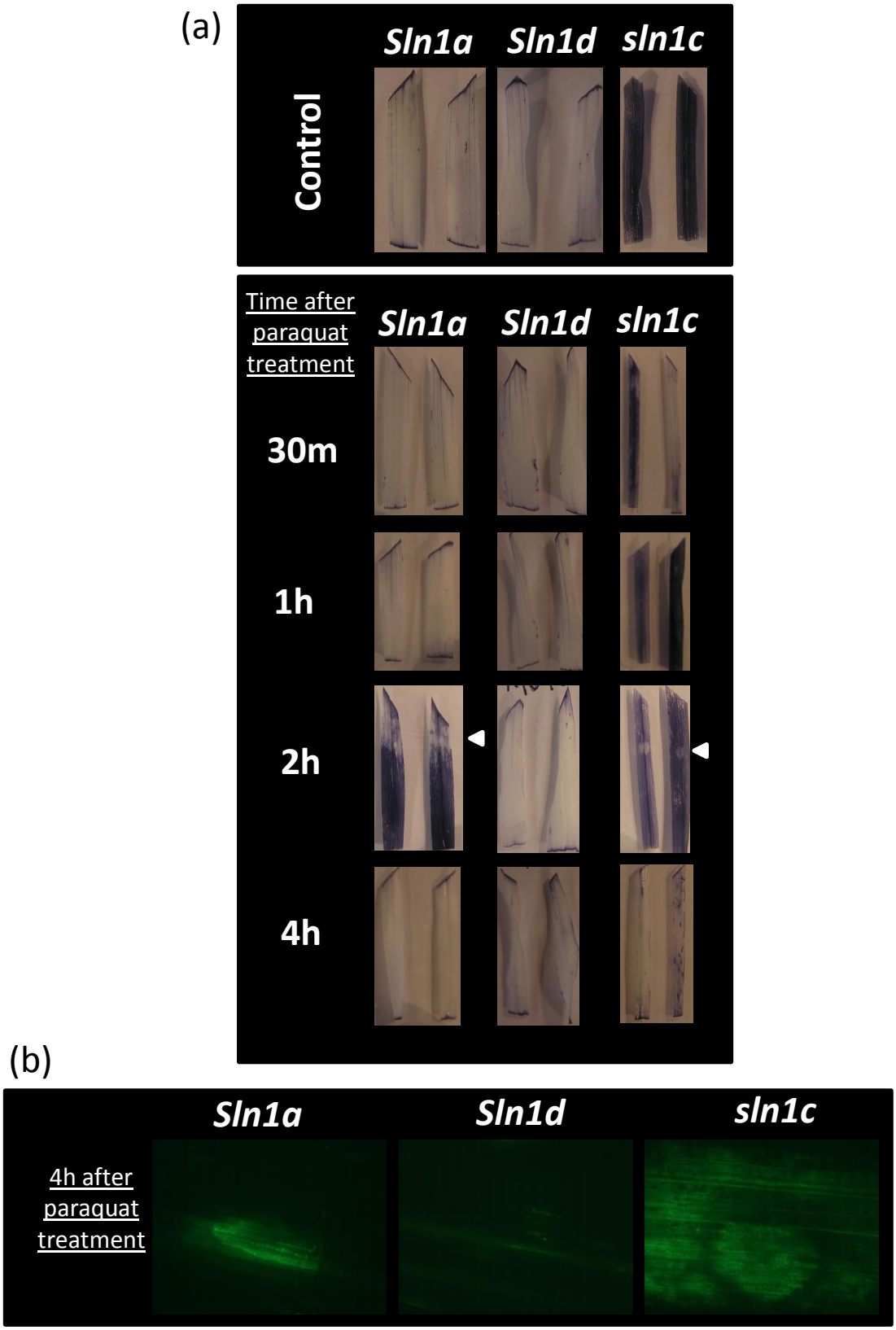


Figure 5.3. The detached leaves of barley lines allelic at the *Sln1* locus treated with paraquat were stained for ROS; (a) O_2^- (nitroblue tetrazolium [NBT] stain - blue). Untreated (control) leaves and leaves treated with paraquat through a time course (30m, 1h, 2h and 4h) were stained with NBT. White arrows denote localised clearing of staining at 2h after treatment. (b) H_2O_2 (2', 7' Dichlorfluorescein-diacetate [DCFH-DA] stain – green flourescence). Leaves were stained for H_2O_2, the dismutation product of O_2^- , 4h after treatment with paraquat. Leaves are photographed at 2x magnification through a FITC filter.

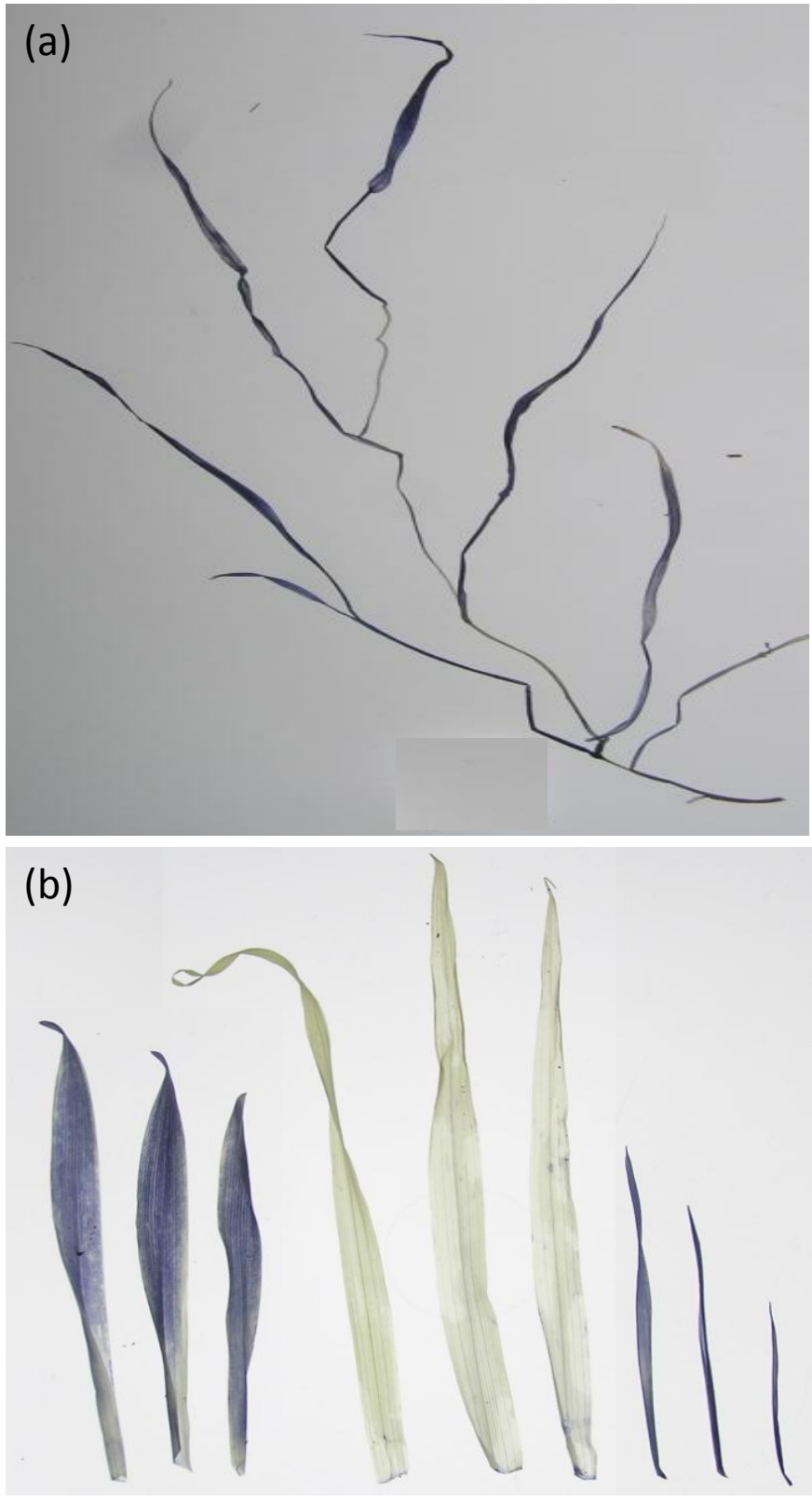


Figure 5.4. Endogenous O_2^- accumulation reported by nitroblue tetrazolium; (a) Representative sample of a Himalaya *sln1c* plant. (b) developmental series of Himalaya *Sln1a* leaves.

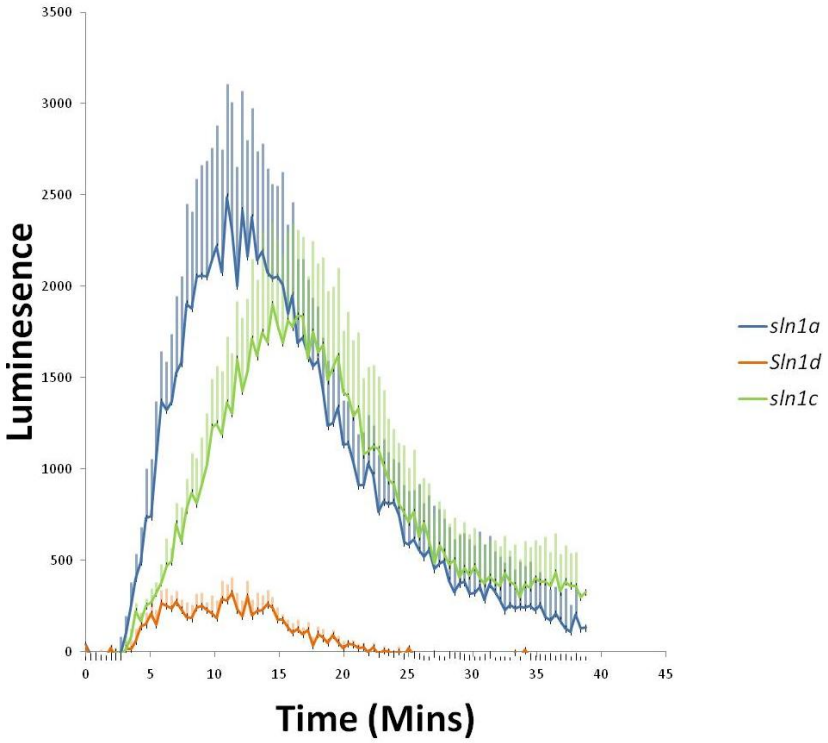


Figure 5.5. flg22-elicited oxidative bursts of *Sln1* allelic series. Luminescence, reporting H_2O_2 accumulation, was measured over time following flg22 treatment. Results presented are from a single representative experiment, n = 32 leaf disks, Bars = SEM.

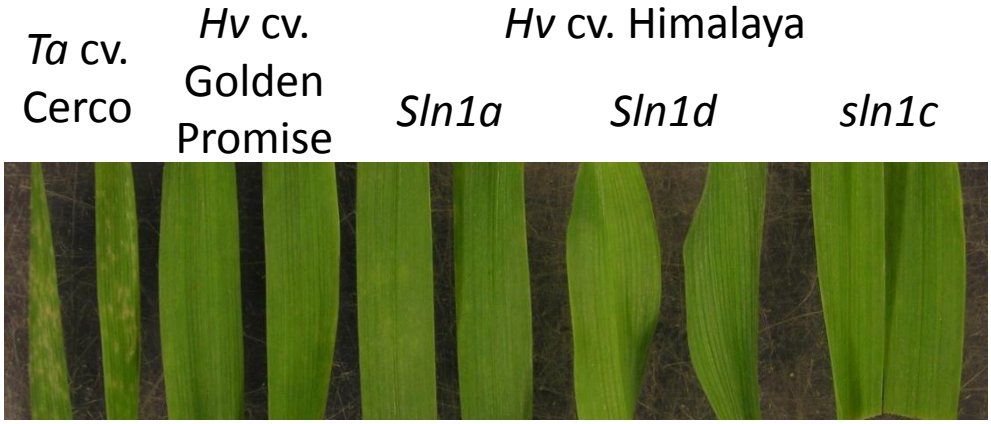


Figure 5.6. Barley lines allelic at the *Sln1* locus challenged with an isolate of an inappropriate formae speciales; *B. graminis f. sp. tritici.* Representative disease phenotype 8 days after inoculation. Wheat (*Ta*) cv. Cerco, the susceptible control, is infected, none of the barley (*Hv*) lines displayed macroscopic symptoms of infection.

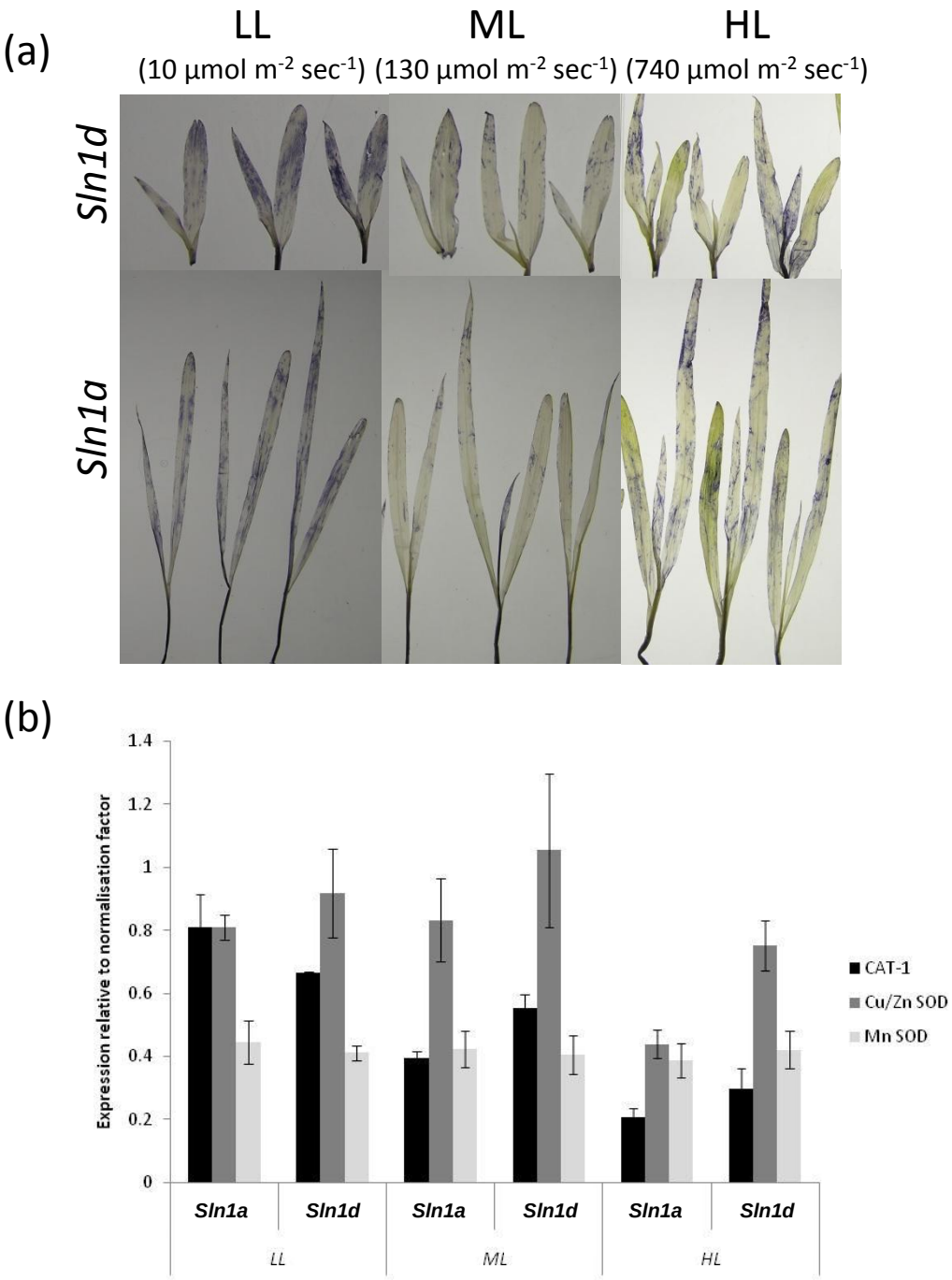
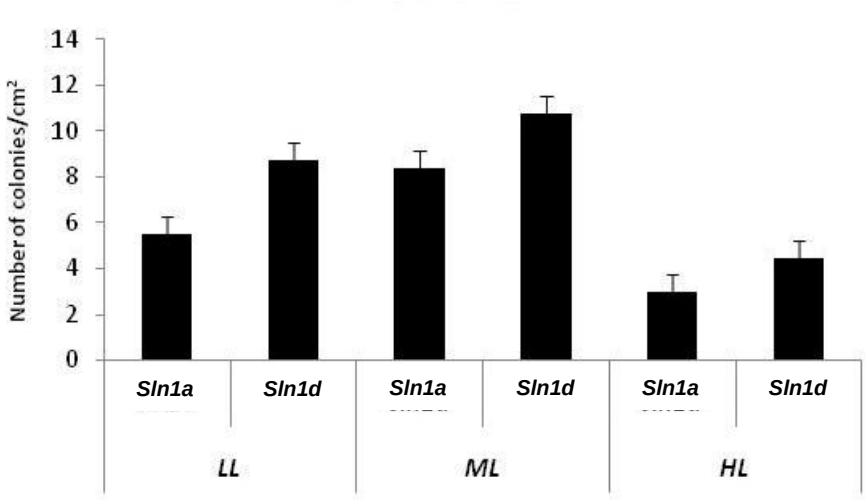


Figure 5.7. An indication of ROS homeostasis influenced by DELLA and light level. (a) *Sln1a* and *Sln1d* lines grown at three light levels have been stained with NBT to report O_2^- accumulation. (b) The expression of genes encoding the antioxidant enzymes catalase (*CAT-1*), Cu/Zn superoxide dismutase (*Cu/ZnSOD*) and Mn superoxide dismutase (*MnSOD*) was quantified relative to normalisation factor in *Sln1a* and *Sln1d* lines grown at three light levels. Normalisation factor is based on *GAPDH* and *EF1*α expression. Bars: 1 SEM. LL; low light, ML; medium light , HL; high light.



Term	d.f.	m.s.	v.r.	F pr.
Replicate	5	34.619	4.89	<.001
Light_level	2	205.951	29.06	<.001
Genotype	1	100.236	14.14	<.001
Light_level.Genotype	2	4.73	0.67	0.517
Residual	61	7.087		
Total	71	15.873		

d.f., refers to degrees of freedom; m.s., refers to mean square; v.r., refers to variance ratio; F. pr. F-test probability.

Figure 5.8. The effect of fluence and DELLA on infection of barley with the biotrophic pathogen; *B. graminis*. *Sln1a* and *Sln1d* barley lines grown at three light levels were challenged with *B. graminis* and scored for susceptibility (measured as number of colonies per cm^2) 8 days after inoculation. Bars; 1 SEM. Analysis of variance, using general linear modelling is presented in table.

(a)

Term	d.f.	m.s.	v.r.	F pr.
Treatment	3	477.64	29.87	<.001
Treatment/Box	4	16.34	1.02	0.4
Side	1	5.42	0.34	0.562
Genotype	3	778.23	48.67	<.001
Treatment.genotype	9	175.2	10.96	<.001
Residual	107	15.99		
Total	127	56.11		

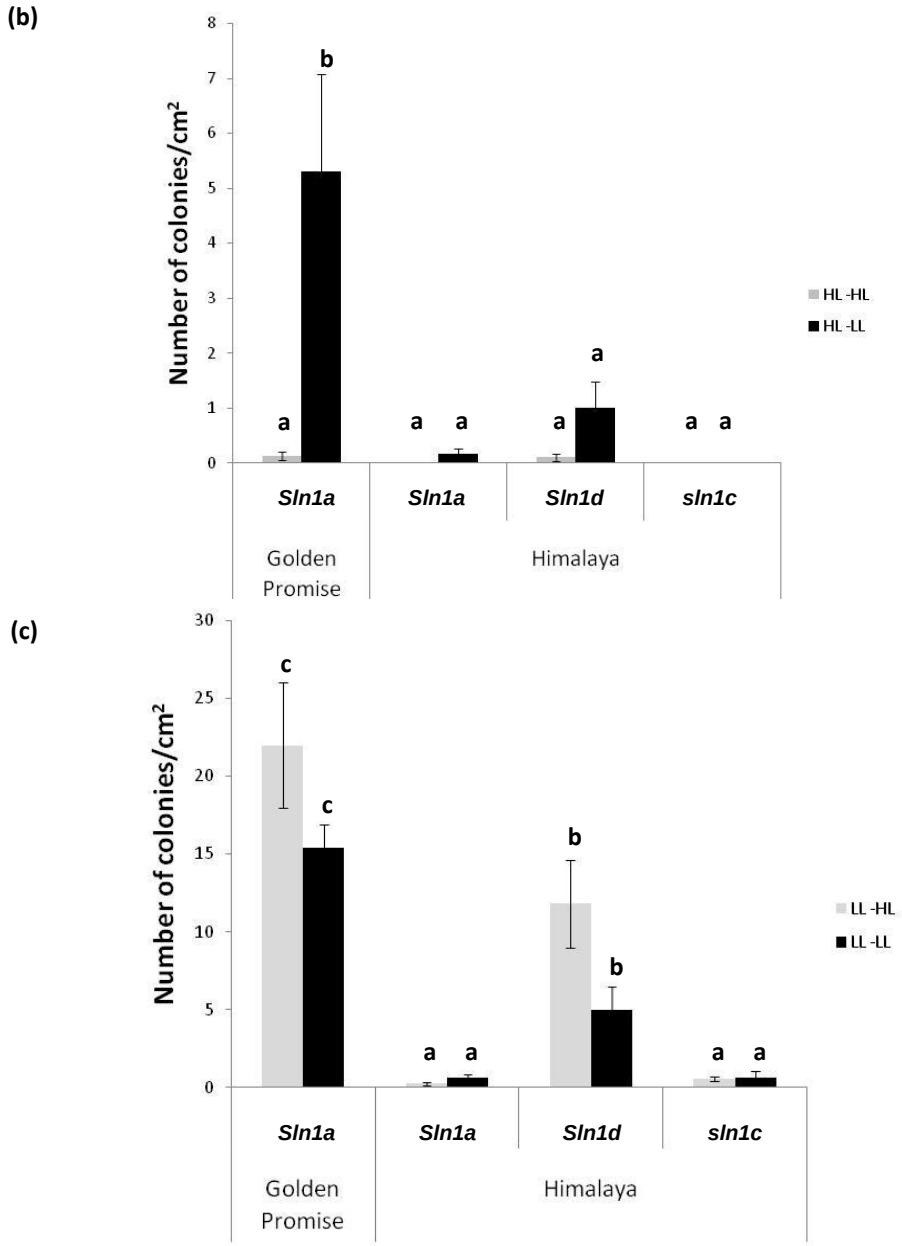
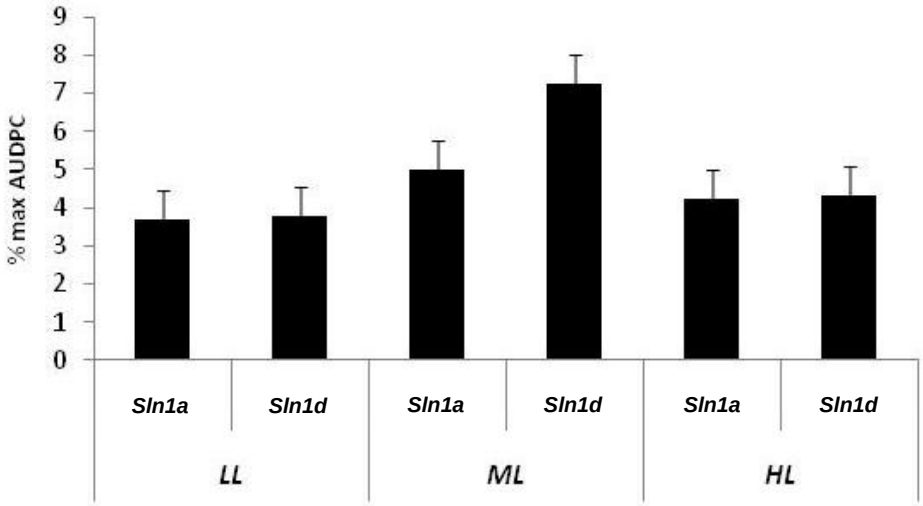


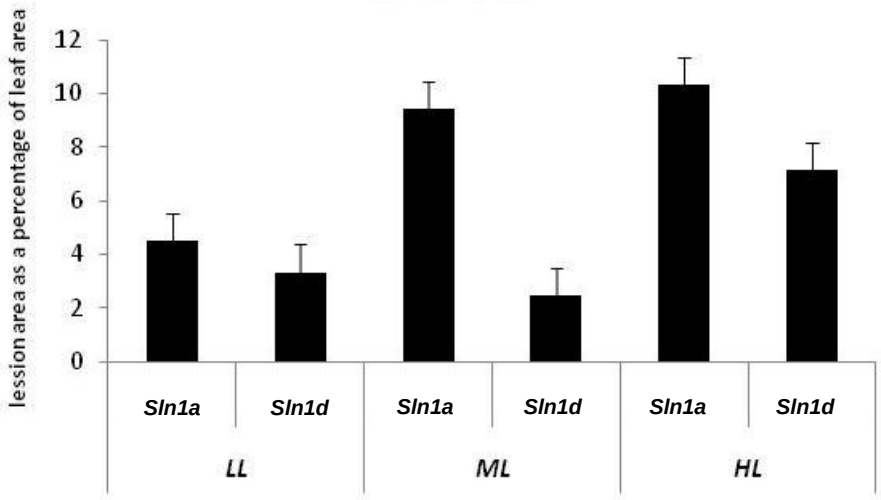
Figure 5.9. The effect of different light regimes on *B. graminis* infection of barley *Sln1* allelic series (a) Analysis of variance, using general linear modelling (b) plants grown in high light preceding inoculation were incubated for 24h either in high light (grey bars) or low light (black bars) after inoculation (c) same as (b) except plants were grown in low light preceding inoculation. Bars; ±1 SEM. Letters above bars represent significant differences (P=<0.05) between disease scores for each line for light treatments separately, using a Tukey's test.



Term	d.f.	m.s.	v.r.	F pr.
Replicate	3	0.873	0.29	0.833
Light_level	2	12.659	4.19	0.036
Genotype	1	4.013	1.33	0.267
Light_level.Genotype	2	3.098	1.03	0.382
Residual	15	3.02		
Total	23	3.628		

d.f., refers to degrees of freedom; m.s., refers to mean square; v.r., refers to variance ratio; F. pr. F-test probability.

Figure 5.10. The effect of fluence and DELLA on infection of barley with the hemibiotrophic pathogen; *R. collo-cygni*. *Sln1a* and *Sln1d* barley lines grown at three light levels were challenged with *R. collo-cygni* and scored for susceptibility (measured as % max AUDPC) from 10 days after inoculation. Bars; 1 SEM. Analysis of variance, using general linear modelling is presented in table.



Term	d.f.	m.s.	v.r.	F pr.
Replicate	5	34.05	2.73	0.028
Light_level	2	137.08	10.98	<.001
Genotype	1	258.32	20.69	<.001
Light_level.Genotype	2	50.68	4.06	0.022
Residual	60	12.49		
Total	70	22.19		

d.f., refers to degrees of freedom; m.s., refers to mean square; v.r., refers to variance ratio; F. pr. F-test probability.

Figure 5.11. The effect of fluence and DELLA on infection of barley with the necrotrophic pathogen; *F. graminearum*. Detached leaves of *Sln1a* and *Sln1d* barley plants grown at three light levels were challenged with *F. graminearum* and scored for susceptibility (measured as lesion area as a percentage of leaf area) 4 days after wound inoculation. Bars; 1 SEM. Analysis of variance, using general linear modelling is presented in table.

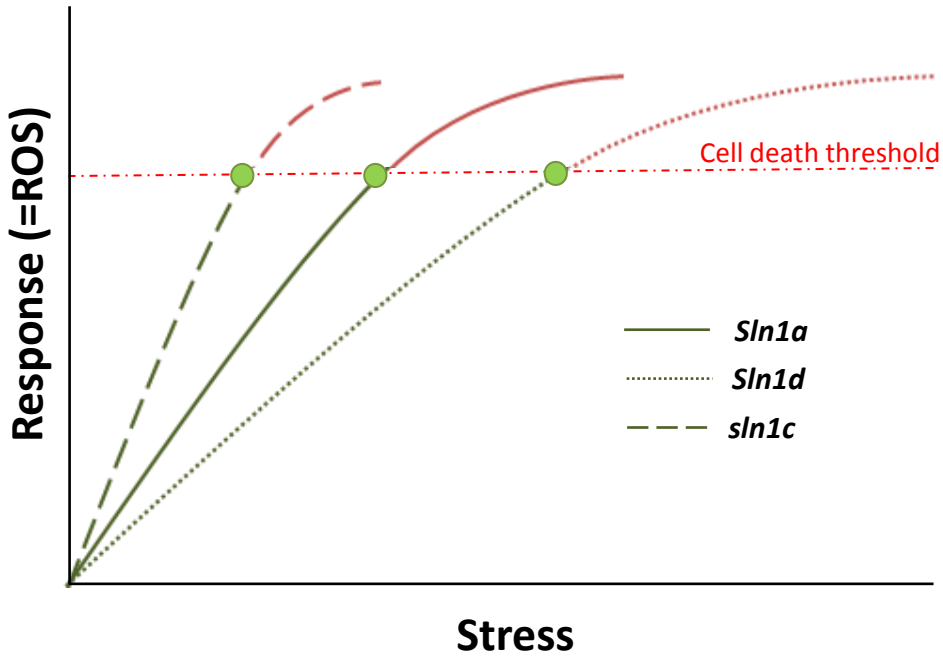


Figure 5.12. A model proposed for altered cell death propensity of barley lines allelic at the *Sln1* locus. A DELLA conferred increase in ROS scavenging means more stress is required before ROS levels accumulate to levels which surpass the cell death threshold in the cells of *Sln1d* carrying lines. Conversely, less stress is required for ROS levels to reach the cell death threshold in the cells of *sln1c* carrying lines.

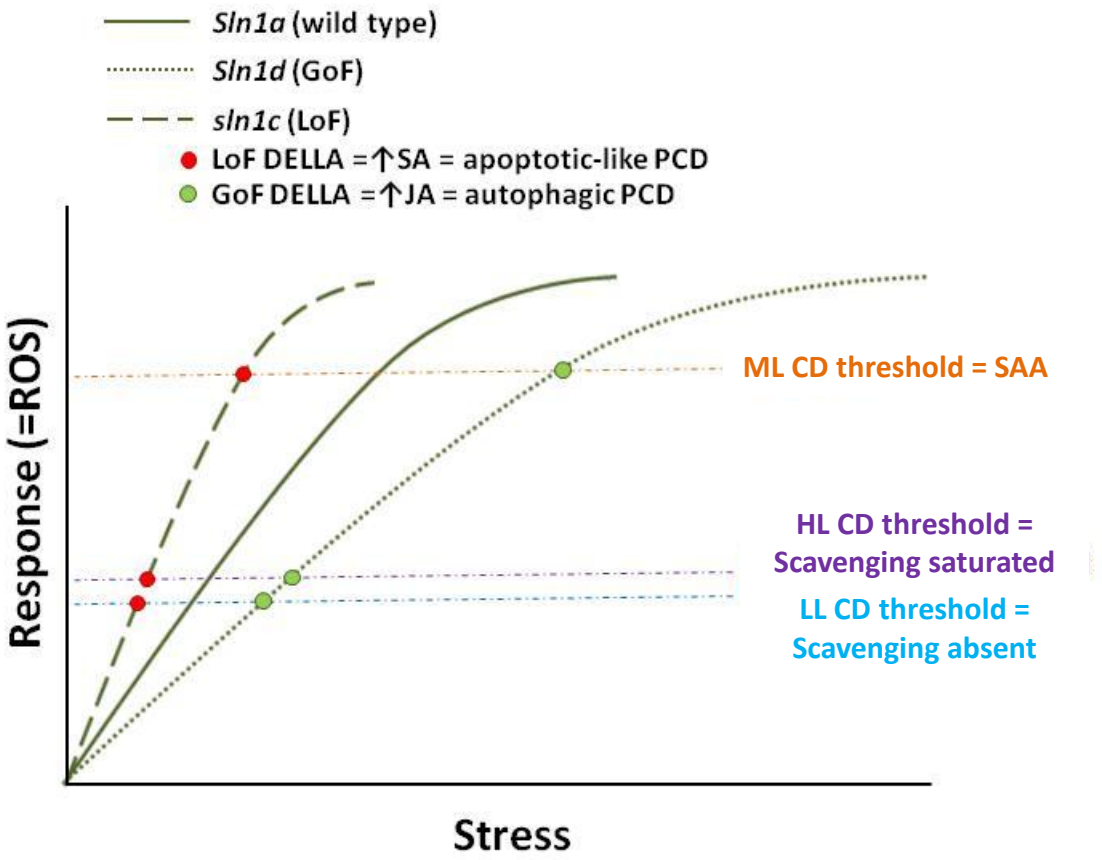


Figure 6.1 A model proposed for the propensity and type of cell death undergone by barley lines allelic at the *Sln1* locus and the influence of environment on the threshold at which cell death occurs. In elaboration of the model proposed previously (Fig. 5.12) data presented in Chapter 5 suggests that the point at which the cell death threshold is set is influenced by the environment. Exposure to abiotic stress induces the plants antioxidant systems for acclimatisation thus raising the cell death (CD) threshold, as postulated for medium light (ML) preconditioned plants. Whilst, severe abiotic stress creates such an intense ROS accumulation that the plant is no longer able to compensate for by detoxification due to the antioxidant system being saturated, thus effectively reducing the CD threshold, as postulated for high light (HL) preconditioned plants. Plants that are not exposed to adverse abiotic factors do not rouse scavenging mechanisms thus effectively reducing the CD threshold, as postulated for low light (LL) preconditioned plants. The altered CD threshold will have differential effects on disease outcome dependent on the nutritional lifestyle of the pathogen. In addition, the model incorporates the yet to be tested hypothesis that DELLA conferred hormone alterations may influence the mode of PCD (apoptotic or autophagic) that cells undergo. SA; Salycilic acid, JA; Jasmonic acid, LL; low light, ML; medium light, HL; high light, SAA; systemic aquired acclimation, CD; cell death.