Are Barley Dwarfing Genes Important in Tolerance to Abiotic Stress?

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Abstract

DELLA proteins are a highly conserved group of growth inhibitors, mutants of which were integral to the semi-dwarf, high yielding wheat lines of the Green Revolution. In addition to reducing plant growth, the gain of function (GoF) mutants in which DELLA protein is stabilised were shown to confer resistance to salt stress in the model species *Arabidopsis*.

In order to determine whether these findings could be translated from *Arabidopsis* to monocot crop species, GoF and loss of function (LoF) mutants of the barley DELLA orthologue, *Sln1*, were characterised and growth and development assessed. By subjecting DELLA wild-type and mutant barley plants to abiotic stress conditions (salt stress and heat shock) it was established that the increased survival conferred by stabilised DELLA that was reported in *Arabidopsis* was also applicable to barley, and that survival of the LoF barley mutants was decreased. Further evidence for the importance of stabilised DELLA was obtained when additional mutants in the GA signalling pathway (*gse1a,j,n*; *Gse1,* GA receptor mutants) in which DELLA protein is predicted to accumulate, also showed increased tolerance to abiotic stress. These data suggest DELLA protein function is conserved between monocot (cereal) and dicot plants. Attempts to produce transgenic barley plants in which *Sln1* was silenced were inconclusive, likely underlining the essential nature of the gene in growth, development and regeneration. The studies provide a basis for further work to investigate the mechanisms underlying DELLA function in cereals.

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Contents

Abbreviations

µL	microlitre
µM	micromolar
aa	amino acid(s)
ABA	abscisic acid
APX	ascorbate peroxidase
AsA	ascorbic acid
BCI	barley callus induction
BLAST	Basic Local Alignment Search Tool
bp	base pair
BR	barley regeneration
BT	barley transition
cc	cubic centimetre
cDNA	complementary DNA
CER	controlled environment room
cm	centimetre
C_t	threshold cycle
CTAB	cetyl trimethylammonium bromide
cv	cultivar
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
GA	gibberellin(s)
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GID1	GA insensitive dwarf1
GoF	gain of function
GP	Golden Promise
GSH	glutathione
h	hour(s)
HQI	hydrargyrum quartz iodide
HSP	heat shock protein(s)
L	litre
LEA	late embryogenesis abundant
LiCl	lithium chloride
LoF	loss of function

LZ	leucine zipper(s)
M	molar
min	minute(s)
mL	millilitre
mM	millimolar
mRNA	messenger RNA
NCBI	National Centre for Biotechnology Information
NLS	nuclear localisation signals
PCD	programmed cell death
PCR	polymerase chain reaction
PEG	polyethylene glycol
PIF	phytochrome interacting factor
PIL	phytochrome interacting factor 3-like
ppm	parts per million
qRT-PCR	quantitative reverse transcription PCR
RCD	radical-induced cell death
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	reverse transcription
rt	room temperature
s	second(s)
SC1	silencing construct 1
SLR1	slender-like rice1
SNP(s)	single nucleotide polymorphism(s)
SOD	superoxide dismutase
SOS	salt overly sensitive
SPT	spatula
TGW	thousand grain weight
TSS	transcription start site
UV	ultraviolet
V	volts
wk(s)	week(s)
WT	wild-type
Y2H	yeast two-hybrid

Chapter 1: General Introduction

1.1 Dwarfing genes and the 'Green Revolution'

The integration of dwarfing genes into agricultural populations was integral to achieving the increase in crop yields witnessed during the 'Green Revolution' of the 1960's and 70's (Hedden, 2003; Peng *et al.*, 1999), with wheat and rice production doubling since the introduction of the first high yield lines in 1961 and 1966 respectively (Gollin, 2006; Khush, 1999; Khush & Virk, 2002). High yields of these staple crops had previously been restricted by the breakage of stems under the weight of grain, an effect termed 'lodging'. Lodging is particularly prevalent during periods of strong wind or heavy precipitation, which place considerable physical stress upon the stem (Baker *et al.*, 1998). Dwarf and semi-dwarf cultivars reduce lodging, as the shorted stems are more robust than those of taller varieties. Additionally, dwarfed cultivars have an increased harvest index, as assimilate, (production of which is commonly encouraged by addition of nitrogen fertiliser), is invested in the grain rather than the stem (Hedden, 2003). Characterisation of the Green Revolution dwarfing alleles revealed dwarfing is due to mutations within the gibberellin (GA) signalling pathway, with the rice dwarfing allele *sd1* inhibiting GA biosynthesis, and wheat dwarf alleles *Rht-B1b* and *Rht-D1b*, inhibiting GA signal transduction (Hedden, 2003). Thus, the identification and characterisation of the GA signalling pathway has provided the basis for explaining dwarfing phenotypes in wheat and rice, and established GA function as being integral to growth and development (Hooley, 1994). The proliferation of dwarfed wheat and rice varieties has been widespread. High yielding wheat dwarfing alleles *Rht-B1b* and *Rht-D1b* (also termed *Rht1* and *Rht2*, Börner *et al.*, 1996), derived from Norin 10 are present in over 70% of commercial wheat cultivars worldwide (Hedden, 2003), and mutant dwarfing lines allelic to *sd1* are utilised in *Japonica* and *Indica* commercial varieties (Asano *et al*., 2007, 2011; Rutger, 2008).

1.2 The GA signalling pathway

1.2.1 GA function

GA are a tetracylic diterpenoid class of phytohormone that control growth and development processes throughout the plant life cycle, including cell growth and division, vernalisation, and flower, fruit and seed production (Hooley, 1994; Richards *et al.*, 2001; Eckardt, 2002).

GA was first isolated from *Gibberella fujikuroi*, a causative agent of 'foolish seedling' disease in rice, characterised by rapid growth, hypertrophy, chlorosis, limited grain development (leading to reduced fertility), and susceptibility to lodging. The role of GA was subsequently confirmed by the chemical identification of GA in higher plants, resulting from studies using GA deficient mutants (Hedden & Phillips, 2000; Griffiths *et al.*, 2006). Since its initial isolation, over a hundred GA have been identified, but of these only GA_1, GA_3, GA_4, and GA_7 are biologically active (Richards *et al.*, 2001). Furthermore, different GA isoforms are synthesised as the plant develops, for example, in *Arabidopsis* and pumpkin, GA_1 formation is favoured in growing seedlings, while GA_4 formation is favoured in adult plants (Pimenta Lange & Lange, 2006).

1.3 GA biosynthesis

1.3.1 The GA biosynthesis pathway

The GA biosynthesis pathway has largely been elucidated from studies based on *Arabidopsis* and pumpkin (*Cucurbita maxima*) models (Hedden & Phillips, 2000) with additional information provided from plants such as maize and pea (Spray *et al*., 1996; Davidson *et al.*, 2005). GA biosynthesis in higher plants takes place in the chloroplast and cytoplasm of the plant cell, and involves a large number of enzymes and biosynthetic intermediates (Olszewski *et al.*, 2002). Many of the enzymes are multi-functional and several of the enzymes are encoded by multiple genes, each with different systems of regulation, suggesting the GA biosynthesis pathway is under complex multifactorial control (Hedden & Phillips, 2000). The pathway can be summarised into three key stages involving six core enzymes (Figure 1.1). (1) In the proplastid, geranylgeranyl diphosphate (GGDP) is converted to *ent*-kaurene with *ent*-copalyl diphosphate (CDP) as the intermediate, by the enzymes *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS). (2) *Ent*-kaurene is converted to *ent*-kaurenoic acid via *ent*-kaurene oxidase (KO). *Ent*-kaurenoic acid oxidase (KAO) catalyses the production of GA_{12} from *ent*-kaurenoic acid. GA_{12} can be converted further to form GA_{53} by 13-hydroxylation catalysed by GA 13-oxidases (GA13ox). These reactions reportedly occur within the ER membrane. (3) In the cytoplasm, GA_{12} and GA_{53} are converted by a series of oxidation steps to form both bioactive (GA_1, GA_4), and inactive (GA_8, GA_9, GA_{20}, GA_{34}) GA. The enzymes involved in these oxidation steps are GA 20-oxidases (GA20ox), GA 3-oxidases (GA3ox), and GA 2-oxidases (GA2ox) (Olszewski *et al.*, 2002).

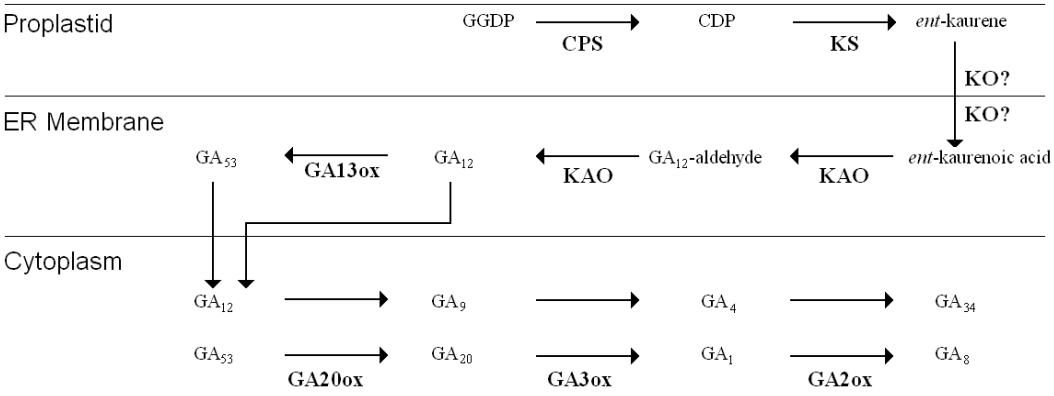


Figure 1.1 Major GA biosynthetic and catabolic pathways in higher plants (modified from Olszewski *et al.*, 2002) The biosynthesis of GA can be separated in three stages 1) biosynthesis of *ent*-kaurene in proplastids; 2) conversion of *ent*-kaurene to GA_{12}; 3) formation of bioactive GA_1 and GA_4 and inactive GA_{34} and GA_8 in the cytoplasm.

1.3.2 Regulation of GA biosynthesis

GA biosynthesis is regulated in response to environmental stimuli (e.g. light and temperature), internal signals, or homeostatic response to changes in GA levels (Olszewski *et al.,* 2002; Hedden & Phillips, 2000). GA are not unique regulators of plant development (Hooley, 1994). The GA signalling pathway closely interacts with other metabolic pathways to regulate plant growth and development, with the degree of interaction likely to be extremely complex (Olszewski *et al.,* 2002; Eckardt, 2007). Auxin, cytokinins, ethylene, brassinosteroid, calcium, and sugars have been shown to affect stem elongation, likely through interaction with the GA signalling pathway (Eckardt, 2002). Auxin levels have been shown to promote biosynthesis of GA_1 in pea plants (*Pisum sativum*) by increasing expression of *PsGA3ox,* encoding GA3-oxidase (Ross *et al.*, 2000). A number of studies have investigated the role of light in GA biosynthesis and downstream plant development. Studies in *Arabidopsis* and other higher plants suggest that expression of genes encoding the GA biosynthesis enzyme GA-20 oxidase is regulated by photoperiod (Hisamatsu *et al.*, 2005; Xu *et al.*, 1995; Carrera *et al.*, 2000; Kim *et al.*, 2006). Furthermore, PHYTOCHROME INTERACTING FACTOR 3-LIKE 5 (PIL5) inhibits seed germination by repressing expression of genes encoding the GA biosynthesis enzyme GA3-oxidase, and activating the expression of the genes encoding the GA catabolic enzyme, GA2-oxidase. PIL5 is degraded in response to light stimulus, allowing GA biosynthesis and subsequent plant growth and development (Oh *et al.*, 2006). Low temperature contributes to *Arabidopsis* seed germination through

expression of the *AtGA3ox1* and *AtGA3ox2* genes encoding GA3-oxidase. Homeostatic regulation of GA has been illustrated in pea where the application of exogenous GA resulted in decreased levels of endogenous GA (Martin *et al.*, 1996), and GA deficiency initiates *AtGA3ox1* expression (Yamauchi *et al*., 2004).

1.3.3 GA biosynthesis mutants

GA biosynthesis mutants have been identified in many plant species including *Arabidopsis*, pea, maize, tomato and rice (Winkler & Helentjaris, 1995; Chasan, 1995). Dwarf mutants with lesions in the GA biosynthesitic pathway are partially or fully recoverable to the wild-type phenotype by the addition of exogenous GA, and addition of GA from cultures of *G. fujikuroi* has been shown to stimulate growth in GA-deficient dwarf mutants of pea and maize (Hedden & Phillips, 2000).

GA biosynthesis in *Arabidopsis* is regulated by the expression of genes at five loci, *GA1*, *GA2*, *GA3*, *GA4* and *GA5*. The enzymes involved in the early stages of GA biosynthesis CPS, KS and KO are encoded by single copy genes *GA1*, *GA2* and *GA3* respectively (Yamaguchi *et al.*, 1998; Hedden & Phillips, 2000). Loss of function (LoF) mutations at these alleles results in a severe dwarf phenotype. In *ga1-3* deletion mutants, the ability to produce a functional enzyme is lost, although small quantities of GA are still produced, most likely by a related diterpene cyclase system that feeds into the biosynthesis pathway. The inability to synthesise and utilise GA effectively affects reproductive viability, leading to sterility in severely GA deficient or insensitive mutants, as with the *ga1-3* mutant which is male sterile due to abortive anther development. The enzymes involved in the later stages of GA biosynthesis are encoded by small multigene families consisting of at least four genes for *GA20ox* and *GA3ox*, and at least six in *GA2ox* with some database searches suggesting additional copies (e.g. Dugardeyn *et al.,* 2008). Plants with null mutations in *AtGA3ox1* (GA_4) and *AtGA20ox1* (GA_5) exhibit a semi-dwarf phenotype due to the functional redundancy of the isozymes (Hedden & Phillips, 2000). The tomato (*Lycopersicon esculentum*) mutant *gib-1* is GA deficient as it has a reduced ability to convert geranylgeranyl pyrophosphate to copalyl pyrophosphate, resulting in a dwarfed plant with limited seed germination and flowering. The wild-type phenotype can be partially restored by application of exogenous GA (Jacobsen & Olszewski, 1991). In maize, five GA biosynthesis mutants have been characterised. The *d5* mutant is defective in the early stages of GA biosynthesis, due to the defective production of

the *ent*-kaurene synthase B enzyme which converts CPP (synonymous with: CDP) to *ent*-kaurene (Hedden & Phinney, 1979). The *d3* mutant is defective in the production of an enzyme early in the GA biosynthesis pathway, most likely *ent*-kaurene oxidase, affecting the conversion of *ent*-kaurene to *ent*-kaurenoic acid (Winkler & Helentjaris, 1995). The *d1* dwarf mutant was initially reported to be defective in the production of the enzyme GA3ox that acts downstream in the GA biosynthesis pathway, and catalyses the conversion of GA_{20} to GA_1 by 3β–hydroxylation (Spray *et al.*, 1984) but was more recently also shown to be defective in steps converting GA_{20} to GA_5, and GA_5 to GA_3 (Spray *et al.*, 1996). The semi-dwarf variety of rice integral to the success of the Green Revolution, IR8 (*sd1*), is unable to efficiently convert GA_{53} to GA_{20}, due to a mutation affecting the GA20ox enzyme. Rice contains at least two *GA20ox* genes; *GA20ox-1* and *GA20ox-2*. Of the two, only *GA20ox-2* is tightly associated with the *SD1* locus. The two genes show tissue-specific expression; *GA20ox-1* is expressed in reproductive organs whilst *GA20ox-2* is strongly expressed in the leaf blade and stem. Thus, the *sd1* mutant exhibits a dwarf plant height, yet is fully fertile (Sasaki *et al*., 2002).

1.3.4 Transgenic alteration of the GA biosynthesis pathway

Alteration of plant growth and development via the GA pathway has focused on modifying the levels of GA synthetic (GA20-oxidase and GA3-oxidase) and catabolic (GA2-oxidase) enzymes that function in the later stages of the GA biosynthesis pathway. This approach has been extensively explored in dicots but less so in the monocots because of their general recalcitrance to transformation and regeneration.

Overexpression of GA20-oxidase

Overexpression of any of the three *Arabidopsis* GA20-oxidase genes (*AtGA20ox1*, *AtGA20ox2*, *AtGA20ox3*) in transgenic *Arabidopsis* resulted in seedlings with elongated hypocotyls, increased height at maturity, early flowering, and a two- to three-fold increase in GA_4 levels in vegetative rosettes compared to wild-type plants (Coles, 1999). A similar study in transgenic potatoes (*Solanum tuberosum*) found overexpression of one of the three potato GA20-oxidase genes (*StGA20ox1*) resulted in taller plants with elongated internodes and decreased tuber dormancy compared to wild-type plants under short day conditions (Carrera *et al.*, 2000). Similarly, transgenic tobacco plants constitutively expressing *AtGA20-ox* from the CaMV 35S promoter had the elongated hypocotyls and early flowering

phenotype observed in *Arabidopsis* overexpressing GA20-oxidase as well as paler leaves, and increased biomass, thereby showing that the *Arabidopsis* gene could function similarly in an unrelated plant species (Biemelt *et al*., 2004). These authors found an increased number of lignified vessels in the transformed plants, results that were consistent with overexpression of the same gene in hybrid aspen (Eriksson *et al*., 2000).

Silencing of GA20-oxidase

The effect of GA20-oxidase gene silencing has been investigated in *Arabidopsis* and potato plants. *Arabidopsis* plants expressing antisense transcripts of *AtGA20ox1* showed decreased growth, shortened hypocotyls, late flowering and reduced rates of stem elongation. Furthermore, GA_4 levels in rosettes and shoot tips were lower than those in wild-type plants (Coles, 1999). In potato, expression of antisense *StGA20ox1* resulted in shorter stems with decreased internode length, and early, high yield tuber production compared to control plants. Furthermore, decreased endogenous GA_1 and GA_{20} levels were detected in apex and first leaf material of the *StGA20ox1* silenced potato plants (Carrera *et al.*, 2000).

GA2-oxidase overexpressors

Transgenic tobacco plants constitutively expressing the *Arabidopsis* GA catabolic enzyme *AtGA2-ox* from the CaMV 35S promoter, exhibited reduced biomass, shoot growth and stem height (16% that of wild-type plants) due to shortened internode length. Leaves on the *AtGA2-ox* overexpressing tobacco plants were small and dark green, containing high levels of chlorophyll, and flower development was delayed and seed formation reduced compared to control plants (Biemelt *et al.*, 2004). Unlike the tobacco plants overexpressing *AtGA20-ox*, the *AtGA2-ox* expressing plants were responsive to the addition of exogenous GA_3, which restored stature to that of wild-type plants. Expression of the runner bean *GA2-ox* gene (*PcGA2ox1*) in wheat resulted in plants with decreased level of bioactive GA and a range of dwarfing severity. The dark green leaves and increased tillering seen in these plants was similar to that seen in wild-type plants treated with paclobutrazol, a GA synthesis inhibitor (Appleford *et al*., 2007). Following expression of *OsGA2ox1* from the actin promoter in rice, severely dwarfed plants were obtained that were unable to set grain, although when the gene was expressed from the *OsGA3ox2* promoter the plants were semi-dwarf and exhibited normal flowering and seed development (Sakamoto *et al*., 2003).

Silencing of GA2-oxidase

Suppression of genes encoding GA2-ox enzymes would be expected to decrease GA levels. A study (Gou *et al*., 2010) in which two *GA2-ox* genes predominantly expressed in roots were silenced, showed that GA levels could be manipulated in specific tissues. Both GA_1 and GA_4 levels were decreased in roots of the transgenic poplar plants and these plants showed decreased lateral root formation but no effect on aerial development. GA have previously been implicated in root development, with plants having reduced GA levels exhibiting stulated root development. The authors suggested that GA is important for stress tolerance since smaller plants with lower demands on environmental resources, but with enhanced root systems, are more likely to survive stress conditions.

1.4 GA-DELLA signal transduction pathway

1.4.1 DELLA proteins

GA signal transduction is dependent on GA-mediated degradation of nuclear localised DELLA repressor proteins (Achard *et al.*, 2006). The GA-DELLA signal transduction pathway is a highly conserved mechanism in higher plants (Yasumura *et al.*, 2007), regulating plant growth by restricting cell proliferation and expansion in the absence of GA (Thomas & Sun, 2004; Zentella *et al.*, 2007). DELLAs are a subfamily of GRAS regulatory proteins, with domain analysis and expression studies suggesting DELLAs also control plant growth by functioning as transcriptional regulators (Dill *et al.*, 2004; Zentella *et al.*, 2007).

1.4.2 Relief of DELLA growth restraint

GA is perceived by both soluble and membrane bound receptors (Ueguchi-Tanaka *et al.*, 2005), with the GA Insensitive Dwarf1 (GID1) proteins identified as one such class of soluble GA receptor (Itoh *et al.*, 2002; Achard *et al*., 2006). DELLA growth repression is relieved as proposed by the "relief of restraint" model (Figure 1.2; Harberd, 2003). GA removes growth inhibition by forming a GID1-GA complex that targets DELLA for degradation. It is likely that the phosphorylated form of the DELLA protein is targeted by the 26S proteasome. Once DELLA is present in the GA-GID1 complex it is stabilised, thereby enhancing DELLA degradation (Eckardt, 2007; Ueguchi-Tanaka *et al.*, 2007a,b). Targeted DELLA is recognised by the GID2-SCF complex (GID2 is an F-box protein), leading to ubiquitination of DELLA and

subsequent degradation. The proteasomal targeting of the barley DELLA protein, SLN1, was reported by Fu *et al*. (2002).

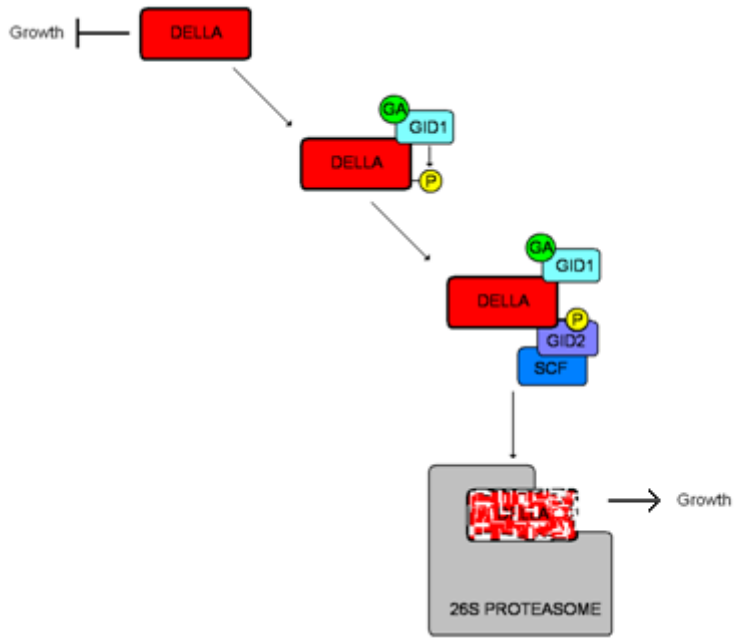


Figure 1.2 The DELLA 'relief of restraint' model (modified from Harberd, 2003) DELLA proteins inhibit growth. GA is perceived by the GA receptor GID1. The GA-GID1 complex leads to the phosphorylation of DELLA. The phosphorylated DELLA protein is recognised by the GID2-SCF complex which targets the DELLA protein for proteosomic degradation.

GID1

Identification of the soluble GA receptors (GID1) in rice and *Arabidopsis* was integral to understanding the GA signal transduction pathway (Ueguchi-Tanaka *et al.*, 2005; Griffiths *et al.*, 2006). The first indication that GID1 is directly involved in GA signalling was the identification of a GA-dependent interaction between GID1 and the rice DELLA protein, slender-like rice1 (SLR1) in a yeast two-hybrid (Y2H) assay, suggesting GID1 is a soluble GA receptor that mediates GA signalling through DELLA interaction (Ueguchi-Tanaka *et al.*, 2005; Willige *et al.*, 2007). Ueguchi-Tanaka *et al.* (2007a, b) tested the dose dependency of GA-mediated GID1-SLR1 interaction in Y2H assays using four bioactive GA: GA_1, GA_2, GA_3, and GA_4. GA_4 had the highest affinity for GID1; suggesting GA_4 is the most effective GA in stimulating DELLA degradation. The GID1-SLR interaction was shown to occur also *in planta* (Ueguchi-Tanaka *et al*., 2007b), with GA_4 also being the most effective isoform. The interaction between GA and the GID1-SLR1 complex appears to be highly isoform and dose specific, with higher levels of GA resulting in increased levels of growth in wild-type plants (Richards *et al.*, 2001; Ueguchi-Tanaka *et al.*, 2007a). GA_4 is less stable than GA_3, being

rapidly degraded in the presence of GA-inactivating enzymes, whilst GA_3 remains active even in their presence. This may explain why levels of GA_3 can be significantly higher than that of GA_4 in growing plants (Ueguchi-Tanaka *et al.*, 2007a). Whilst rice and barley possess only a single *GID1*, *Arabidopsis* contains three homologues (Griffiths *et al.*, 2006). The *GID1* gene is known as *Gse1* in barley (Chandler *et al*., 2008).

GID2

GA-INSENSITIVE DWARF2 (GID2) is a putative F-box protein and is a subunit of the SCF-ubiquitin ligase complex that is essential for GA-mediated DELLA protein degradation (Sasaki *et al*., 2003). In wild-type rice plants, GID2 was found to be preferentially expressed in organs actively synthesising GA (Gomi *et al.*, 2004). However, recent work using *gid2* mutants has shown that derepression of SLR1 activity does not require GID2 function (Ueguchi-Tanaka *et al*., 2008), leading the authors to suggest that other unknown factors might interact with SLR1 to induce its suppressive activity. Further work by Hirano *et al.* (2010) has indicated the complexity of the degradation of SLR1, suggesting the F box protein, rather than recognising a post-translational modification of SLR1, recognises the GA-dependent SLR1-GID1 complex, with GRAS domain binding to GID1 serving as the recognition signal. The GID2 protein is known as SLEEPY (SLY) in *Arabidopsis* (McGinnis *et al*., 2003).

1.4.3 DELLAs as integrators of multiple signalling pathways

DELLAs are nuclear localised growth-repressors that integrate responses to independent hormonal and environmental stimuli (Achard *et al.*, 2003, 2006; Fu & Harberd, 2003; Itoh *et al.*, 2002). DELLA enhances and represses the expression of genes involved in growth and development, whilst DELLA levels are in turn regulated by the actions of other signalling pathways.

Signals affecting DELLA function and stability

DELLA and GA levels are regulated in a homeostatic manner, with high levels of DELLA resulting in increased GA levels through expression of components of the GA signalling pathway including GA biosynthesis enzymes, GA receptors and ubiquitinases. DELLA stability can be regulated through the action of plant growth hormones other than GA. Using an RGA-GFP reporter construct in transgenic plants, ethylene was shown to delay the degradation of DELLA in *Arabidopsis* root cells, even in the presence of bioactive GA (Achard

et al., 2003). The authors found that root growth was inhibited in a DELLA-dependent manner.

Auxin has been shown to promote the accumulation of bioactive GA. The effect of auxin on GA-mediated DELLA degradation was observed by removal of auxin producing shoot apices of pRGA:GFP-RGA seedlings and measurement of response to the application of exogenous GA. Intact pRGA:GFP-RGA seedlings treated with exogenous GA showed rapid degradation of GFP-RGA. Conversely GFP-RGA was still present in pRGA:GFP-RGA seedlings with apices removed after 4 h treatment with GA, with degradation only restored upon addition of exogenous auxin at the site of apex removal (Fu & Harberd, 2003). Inhibition of auxin efflux in *Arabidopsis* by exogenous addition of the 1-N-Naphthylphthalamic acid to *ga1-3* GA biosynthesis mutants inhibited growth even after the addition of exogenous GA. *ga1-3* mutants also lacking GAI and RGA function reverted to near normal growth on addition of exogenous GA, suggesting interaction between auxin growth regulation and the GA-DELLA signal transduction pathway. Ethylene therefore stabilises DELLA and slows growth by inhibiting GA-mediated DELLA degradation, whilst auxin partly promotes growth by enhancing DELLA degradation (Fu & Harberd, 2003). Although it has been generally thought that DELLAs and auxin act together to increase GA levels, more recent work (O'Neill *et al.*, 2010) using a pea double mutant lacking both DELLA proteins, has shed more light on the mechanisms involved. Synthesis of bioactive GA was promoted both by auxin and DELLAs, and both were able to inhibit deactivation of GA. However, it was found that DELLA and auxin independently regulated the GA pathway, although the extent to which DELLA was able to counteract auxin regulation differed depending upon the target genes tested (*GA20-ox*, *GA3-ox* and *GA2-ox*) with effects varying even between *GA2ox* genes. It is clear that further work will be required to fully understand the mechanisms involved.

Downstream effects of DELLA function

DELLAs have been shown to exert their function through protein-protein interaction. For example, DELLAs inhibit the action of phytochrome interacting factors PIF3 and PIF4. PIF3 and PIF4 are transcription factors involved in phytochrome-mediated signalling in response to light. GA-mediated degradation of DELLA releases PIF3 and PIF4 inhibition, allowing the promotion of expression of the PIF3 and PIF4 target genes as well as yet uncharacterised growth promoting genes (Feng *et al.*, 2008; De Lucus *et al.*, 2008). A further three transcription factors have been shown to interact with DELLA in Y2H assays: PIF1, SPATULA

(SPT), and phytochrome-interacting factor 3-like 2 (PIL2, Gallego-Bartolome *et al.*, 2010). PIF1, PIF3, SPT, and PIL2 contain basic helix loop helix (bHLH) structures, suggesting this structure may be important for DELLA interaction.

DELLAs lack recognised DNA binding domains, suggesting they are unlikely to interact directly with genomic DNA to elicit expression responses. Domain analysis, chromatin immunoprecipitation (ChiP) and expression studies have shown DELLAs act as transcriptional regulators, controlling plant development through repression of transcription factor action and function (Dill *et al.*, 2004; Zentella *et al.*, 2007). It is suggested that DELLA promotes the expression of genes encoding ubiquitin enzymes and abscisic acid (ABA). As ABA is antagonistic to GA function, DELLA proteins are able to control GA-mediated growth via manipulation of GA and ABA pathways (Zentella *et al.*, 2007). DELLA mediates between GA and ABA pathways via the XERICO gene, which upregulates ABA expression in response to stress. Furthermore, using ChiP analysis *Arabidopsis* RGA has been shown to bind to the promoters of eight GA response genes either individually or as part of a complex (Zentella *et al.*, 2007). Despite several approaches taken by several groups, the number of genes targeted by DELLA remains unclear, and differential results obtained may reflect the fact that DELLAS are likely to regulate different genes both temporally and spatially (Hartweck, 2008).

1.4.4 GA-DELLA signal transduction pathway mutants

Mutations in genes involved in the GA signalling pathway produce dwarf, semi-dwarf and slender phenotypes (Hooley, 1994; Hedden, 2003). Dwarf varieties are classified as mutants with a plant height less than 50% of that of the wild-type plant, and semi-dwarf as having a height between 50% and 100% that of the wild-type (Hedden, 2003). The dwarf phenotype is characterised by short stature, reduced internode length and short broad leaves (Harberd & Freeling, 1989; Falk, 1994). Slender mutants have a phenotype similar to a wild-type plant that has been treated repeatedly with exogenous GA (Hooley, 1994). Slender plants are infertile, and have elongated epidermal cells, resulting in a tall, narrow whole plant phenotype with elongated internodes (Schünmann *et al.*, 1994). In cases where the slender phenotype is not caused by the overproduction of GA, the plant is a GA constitutive response mutant. The occurrence of these mutants is rare by comparison with dwarf and semi-dwarf mutants (Hooley, 1994).

1.4.5 DELLA mutants

GA-DELLA signalling transduction mutants have been extensively characterised in crop and model plants, with dominant or semi-dominant GA insensitive DELLA dwarf mutants found in *Arabidopsis*, wheat, maize, rice and barley, as well as a smaller number of recessive LoF mutants, discussed more fully in Chapter 3.

***Arabidopsis* DELLA mutants**

Arabidopsis has five DELLA genes: *RGA* (REPRESSOR OF ga1-3), *GAI* (GA-INSENSITIVE), *RGL1, RGL2* and *RGL3* (RGA-LIKE1, 2 and 3, respectively), (Wen & Chang, 2002; Zentella *et al.*, 2007). *Arabidopsis* DELLA mutants exhibit a cumulative effect on plant phenotype, although the redundancy associated with the five homologous DELLA genes makes the effect of a mutation at a single locus difficult to determine. This is illustrated by GAI and RGA, which are highly homologous, and appear to have partially redundant or overlapping functions, with plants containing single null mutations at these loci exhibiting a wild-type phenotype (Dill & Sun, 2001; Peng *et al.*, 1997; Silverstone *et al.*, 1998; Willige *et al*., 2007). The *gai-1* gain of function (GoF) mutant results from a deletion within the conserved DELLA domain of the GAI DELLA protein (Peng *et al.*, 1997). *gai-1* is unresponsive to the application of exogenous GA, and exhibits phenotypic characteristics typical of dwarfed growth mutants, including reduced height, dark green colour and late flowering (Willige *et al*., 2007). The *Arabidopsis* "quadruple-DELLA mutant" lacks GAI, RGA, RGL1 and RGL2, four of the five *Arabidopsis* DELLAs (Achard *et al*., 2006). These mutants bolt and flower earlier than wild-types, and are significantly taller in comparison. The quadruple-DELLA mutant exhibits full petal and stamen growth, and produces fertile flowers and seeds. The "global DELLA mutant" plants in which all five genes are disrupted (Koini *et al*., 2009) have a similar phenotype but also exhibit parthenocarpic fruit development.

Wheat DELLA mutants

The alleles that produced the increased yield and semi-dwarfing trait characterised by the Green Revolution are the DELLA orthologues *Rht-B1b* (formerly *Rht1*) and *Rht-D1b* (formerly *Rht2*) located on the 4B and 4D genome chromosomes respectively (Peng *et al.*, 1999, Muangprom *et al.*, 2005). Wheat is hexaploid, with three homeologous sets of chromosomes, referred to as the A, B and D genomes. The hexaploid nature of wheat makes the study of GA signalling more problematic than in model diploid organisms such as barley or rice. Both the *Rht-B1b* and *Rht-D1b* mutants have a mutation within the DELLA domain

(Peng *et al*., 1999) resulting in a GoF mutant that exhibits a semi-dwarf phenotype due to reduced GA sensitivity, similar to that seen in the *gai* mutant. Each mutant allele produces a similar effect on plant height, and their effect is additive (Hedden, 2003). Further *Rht* mutant alleles have been identified. The *Rht3* allele (*RhtB1c*, Pearce *et al.*, 2011; Wu *et al*., 2011), contains a 30 amino acid insertion within the DELLA domain causing a severe dwarf phenotype. The extreme dwarfing seen in *Rht10* (*RhtD1c*) plants is a result of overexpression of the *D1b* allele caused by an increase in gene copy number (Pearce *et al*., 2011).

Rice DELLA mutants

Rice shares many similarities in the GA signalling pathway with *Arabidopsis*, however rice contains a single DELLA gene, *SLENDER RICE-1* (*SLR1*), in contrast to *Arabidopsis* which contains five. The rice genome is small and has been entirely sequenced. These factors make it a good candidate to link model plant work with crop applications. With its single DELLA gene, rice produces either a strong or weak growth phenotype rather than a cumulative one, making the effect of any mutation simpler to determine. The *slr1* mutant protein was the first mutant protein to be characterised that produced a slender, LoF phenotype (Ikeda *et al.*, 2001). The *slr1-1* to *slr-1-4* mutants result from mutations in the GRAS domain of the DELLA gene, resulting in a slender phenotype similar to that of a constitutive expresser of GA, whereas truncation of the DELLA motif in *SLR1* (*pSLRtr*) produced a dwarf phenotype (Ikeda *et al.*, 2001).

Barley DELLA mutants

As with rice, both GoF and LoF DELLA mutants exist in barley. The mutant *sln1d* is a GoF dwarf mutant orthologous to *Rht-B1b* and *Rht-D1b* mutants in wheat (Peng *et al*., 1999) and *gai1* and *gai2* in *Arabidopsis* (Peng *et al*., 1997; Koorneef *et al*., 1985; Chandler *et al.*, 2002). The *sln1d* dominant dwarf phenotype results from a mutation in the DELLA domain, encoding a DELLA protein which is stable even in the presence of GA. Conversely, the *sln1c* recessive slender phenotype results from a mutation in the GRAS domain (Chandler *et al.*, 2002). Plants homozygous for the *sln1c* mutant allele have increased leaf extension rate and long, attenuated light green leaves. Epidermal leaf cells are narrow and elongated compared with those of the wild-type phenotype (Foster, 1977; Schünmann *et al.*, 1994). Anthocyanin pigmentation of the leaf sheaths and stem nodes is much more pronounced in the mutant compared with that of the wild-type (Foster, 1977). In terms of aspects of growth, barley *slender* plants exhibit many phenotypes similar to those of wild-type plants exposed to

exogenous (applied) GA, however, the endogenous concentrations of bioactive GA_1 and GA_3 in the mutant plants is much lower than those of wild-type varieties. This suggests that GA overproduction is not linked to slender phenotype; rather the mutant is a GA response mutant (Schünmann *et al.*, 1994). The production of fertile seed by *slender* barley is impaired due to male sterility (Schünmann *et al.*, 1994). Barley mutants are described further in Chapter 3.

1.4.6 GID1 and GID2 mutants

GID1 mutants

Arabidopsis contains three homologous GID1 genes: *AtGID1a*, *AtGID1b* and *AtGID1c*. Loss of gene function of the three genes in a single plant produces an extreme GoF mutant, characterised by an extreme dwarf phenotype and insensitivity to the addition of exogenous GA (Griffiths *et al*., 2006; Willige *et al*., 2007).

GID1-GA recognition and GID1 function is integral to DELLA degradation via the GA-DELLA signal transduction pathway. The GID1 receptor was first identified in rice along with the mutant allele *gid1* (Ueguchi-Tanaka *et al.*, 2005), which exhibited an extreme dwarf phenotype with wide dark-green leaves, and insensitivity to the addition of exogenous GA. In wild-type plants, GA production is inhibited and catabolism promoted when bioactive GA levels exceed a homeostatic threshold, however, this mechanism is absent in rice *gid1* mutants (Ueguchi-Tanaka *et al.*, 2005). Of eight *gid1* mutants characterised, six were severe dwarfs, one, a moderate dwarf and one had a mild dwarf phenotype with only the last producing fertile seed (Ueguchi-Tanaka *et al*. 2007).

In barley, sixteen *gse1* (orthologous to *GID1*) mutants (*gse1a* to *gse1p*) were generated from a sodium azide treated population. Each mutant carried a unique single nucleotide substitution resulting in a single amino acid (aa) change in each of the gse1 mutant proteins, with the exception of one mutant that contained a substitution in the 5' untranslated region (UTR) close to the translation initiation site. All of the *gse1* mutants exhibited reduced sensitivity to exogenous addition of GA_3, and had phenotypes ranging from mild to severe dwarf. Although the severe dwarfs had reduced grain set all were fertile (Chandler *et al.*, 2008). Currently characterised GID1 alleles are shown in Table 1.1.

Table 1.1 GID1 genes of the GA-DELLA signalling pathway involved in growth regulation.

Species	Allele	Identified mutants	Reference
Barley	*Gse1*	*gse1a* to *gse1p*	Chandler *et al.*, 2008
Rice	*GID1*	*gid1-1* to *gid1-4*	Ueguchi-Tanaka *et al.*, 2005
Arabidopsis	*AtGID1a to AtGID1c*	*Atgid1a to Atgidc*	Griffiths *et al.*, 2006

GID2 mutants

The rice *gid2* mutants exhibit a similar phenotype to that of the *gid1* mutants, being GA-insensitive with wide, dark green leaves, and a severe dwarf phenotype. DELLA accumulates in *gid2* mutants, even after GA treatment, whilst DELLA in wild-type plants is rapidly degraded (Sasaki *et al.*, 2003). Similarly, the *Arabidopsis sly1* mutant is a GA-insensitive dwarf mutant, with a phenotype similar to other GA insensitive mutants, including increased seed dormancy, dark green tissue colour, delayed flowering and reduced fertility (McGinnis *et al*., 2003). Currently characterised GID2 alleles are shown in Table 1.2.

Table 1.2 GID2 genes of the GA-DELLA signalling pathway involved in growth regulation.

Species	Allele	Identified mutants	Reference
Rice	*GID2*	*gid2*	Sasaki *et al.*, 2003
Arabidopsis	*SLY1*	*sly1*	McGinnis *et al.*, 2003

1.5 Abiotic stress tolerance

1.5.1 Pressures on agriculture

The increase in crop production seen during the Green Revolution is being matched by the needs of an increasing world population (Khush & Virk, 2002). Global climate change is an increasing threat to crop production, reducing yields through plant damage and reducing the availability of agricultural land due to soil erosion, urbanisation and industrialisation (Khush, 1999; Vinocur & Altman, 2005; Ericsson & Nilsson, 2006). Environmental stress, especially salinity and drought, are the primary cause of crop losses worldwide (Vinocur & Altman, 2005). A common feature of abiotic stress is limitation of water availability. Water has an

essential biological role as a transport medium for nutrients and plant metabolites, as an electron donor in the Hill reaction of photosynthesis, and as an evaporative coolant (Bohnert *et al*., 1995). Plants live in fixed locations and must survive adversity by responding to diverse environmental signals (Achard *et al.*, 2006). Understanding the mechanisms that allow plants to survive such stresses whilst identifying key yield and quality genes and assimilating them into agricultural populations is integral to meeting the needs of a growing world population.

1.5.2 Reactive oxygen species (ROS)

ROS are produced by plants as a consequence of environmental stress, and at high levels, their accumulation leads to plant damage. Initially believed to be solely deleterious to plant health, reactive oxygen species (ROS) have latterly been identified as integral to plant development, acting as signal transduction molecules controlling processes including growth, development, response to biotic and abiotic environmental stimuli, and programmed cell death (PCD) (Mittler, 2002; Bailey-Serres & Mittler, 2006). ROS are generated as a consequence of aerobic metabolic processes such as respiration and photosynthesis (Apel & Hirt, 2004). Under normal growth conditions ROS homeostasis is maintained by the interplay between ROS producing and ROS scavenging mechanisms (Mittler, 2002), however under stress conditions levels of ROS may increase to the point whereby homeostasis cannot be maintained, resulting in membrane lipid peroxidation, enzyme inhibition, and DNA and RNA damage (Mittler, 2002; Fridovich, 1986). Cell death occurs through extensive oxidative damage, or through ROS-mediated activation of PCD pathways (Mittler, 2002). Plants minimise oxidative stress by producing antioxidants and ROS detoxifying enzymes, which interrupt cascades of uncontrolled oxidation (Noctor & Foyer, 1998). The importance of ROS detoxifying enzyme activity is highlighted by the *Arabidopsis* mutant, *pst1*, which has enhanced superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities, conferring significantly increased tolerance to salt induced oxidative stress (post germination) compared to the wild-type (Tsugane *et al.*, 1999).

1.5.3 Salt stress

Salt imposes a major constraint on global crop production (Botella *et al.*, 2005), affecting an estimated 20% of agricultural land and 40% of irrigated land worldwide, with a further 1000

km^2 and 150 km^2, respectively lost annually as a result of human activity and environmental processes (World Health Organisation, 2005; Khan *et al.*, 2006). Irrigation is the primary manmade cause of salinisation, particularly on agricultural land reliant on groundwater based irrigation systems, as excessive use causes the water table to rise, drawing contaminated groundwater towards the topsoil (Utset & Borroto, 2001). Areas of high crop productivity are disproportionally affected, as despite optimal day length and temperature, irrigation is frequently used to compensate for limited rainfall (Aus der Beek *et al.*, 2010). Lesser causes of salinisation include the long term addition of livestock manure, run off from neighbouring environments (e.g. road salt), land clearance and waterlogging (Abrol *et al.*, 1988; Chang *et al.*, 1990). Coastal regions are prone to salinisation, due to the potential for seawater contamination of groundwater, and by encroachment of seawater upon agricultural land (Milnes & Renard, 2004). Salinisation also occurs through gradual natural processes such as salt transportation and deposition by wind movement (Aeolian processes), and soil erosion (Rengasamy, 2006). The dissociation of sodium chloride in aqueous soil environments produces sodium ions (Na^+), the accumulation of which leads to the alkalinisation and sodification of soils which is problematic to agriculture (Van Breemen *et al.*, 1983).

The majority of modern grain crops are derived from plants lacking the genetic basis for salt tolerance (Glenn & Brown, 1999; Munns *et al.*, 2006). These plants, termed glycophytes, comprise 99% of the world's flora and are susceptible to even low levels of salinity (ECe <4 dS m^{-1}) (Flowers & Colmer, 2008; Chinnusamy, *et al.* 2005). Glycophytes are able to adapt to higher levels of salinity, provided the salinity is increased in moderate increments (Botella *et al.*, 2005). A high priority for plant biologists is the identification of naturally-occurring salt tolerant varieties through laboratory and field assessment, coupled with genomic analysis and the use of genetic modification to confer increased resistance (Gale, 2003).

1.5.4 Heat stress

Heat stress as a result of increases in ambient temperature threatens global crop production (Hall, 2001). Although predictions as to the degree of climate change vary due to differences in the climate models used, the Intergovernmental Panel on Climate Change (IPCC) predicts an increase of ~0.2 °C per decade over the next two decades (Christensen *et al.*, 2007). Increases in climate change are widely believed to be the result of manmade activity, namely

the atmospheric release of the greenhouse gases: methane, carbon monoxide, nitrous oxide, CFCs and carbon dioxide (Lashof & Ahuja, 1990). Heat stress commonly refers to high temperature for sufficient time to cause irreversible damage to plant growth and development, however lesser heat stress levels may also cause damage by increasing the rate of reproductive development resulting in a reduction of the photosynthetic contribution to seed production, thereby decreasing fruit or grain yields. Although the duration of the heat stress is important, transitory high temperatures, especially at developmentally-susceptible stages, can cause an array of morphological and physiological changes that frequently result in decreased crop yields. One example of this is the sterility in wheat caused by high temperature at anthesis (Ferris *et al.*, 1998). Plant responses to heat stress have been investigated extensively, with induction of heat shock proteins (HSP) and other stress-related proteins, as well as the causes of ROS production receiving the most attention. Plant response to heat stress has been reviewed by Wahid *et al*. (2007). High temperature can also indirectly affect plants by reducing water content of soils through evaporation, which increases solute concentration and soil salinity thereby exposing plants to drought and salinity stress, which reduces seed germination. (Hillel, 1998; Lee & Zhu, 2010; Khodarahmpour & Motamedi, 2011; Sharma *et al.*, 2004)

1.5.5 Abiotic stress perception

Salt stress perception

Plants sense saline conditions primarily through ion specific signals (in the form of Na^+ and ROS), and signals resulting from changes in osmolarity (Chinnusamy *et al.*, 2005; Mittler, 2002; Zhu, 2003). Na^+ is sensed by external membrane receptors, internal membrane proteins, or by cytosolic Na^+ sensitive enzymes, e.g. SOS1 (Salt Overly Sensitive 1), (Zhu, 2003).

Changes in osmotic pressure affecting plasma membrane fluidity trigger cell stress responses. Under hyperosmotic stress conditions, the cell membrane contracts, eventually retracting from the cell wall (Lang-Pauluzzi, 2000). This retraction is sensed by transmembrane protein kinases and stretch activated channels (Cosgrove & Hedrich, 1991; Urao *et al*., 1999). Stretch activated channels have been identified which allow Cl^-, K^+, and Ca^{2+} to permeate the cell (Cosgrove & Hedrich, 1991), allowing specific ion uptake to be regulated under osmotic stress conditions.

Calcium functions as a major signalling molecule, mobilising plant response to generic abiotic stress responses via the mitogen-activated protein kinases (MAPK), and Ca^{2+}-dependent protein kinase (CDPK) signalling cascades (Kader & Lindberg, 2010; Sung *et al.*, 2003). Increased calcium supply has been shown to reduce the effect of salt stress by alleviating sodium toxicity via increased K^+ and Ca^{2+} assimilation and reduction of cellular levels of Na^+ (Zhu, 2000). Cytosolic Ca^{2+} levels increase in response to salt stress as a result of extracellular or intracellular perception by the cell membrane (Knight *et al.*, 1999). Membrane depolarisation and cytosolic Ca^{2+} accumulation signal downstream salt stress responses, with Ca^{2+} likely to activate signalling pathways for K^+ and Na^+ transport, allowing homeostatic maintenance of K^+ and Na^+ through influx, efflux and compartmentalisation (Xiong & Zhu, 2002; Zhu, 2002).

Heat stress perception

Despite the characterisation of multiple plant responses to increases in temperature, the actual mechanisms of heat stress perception and the identity of the early components of the temperature signal transduction pathway are largely unknown (Penfield, 2008; Murata & Los, 1997). Observations of temperature perception in microbial organisms form the basis of understanding temperature perception in higher plants. It is proposed that membrane fluidity is the primary mechanism of temperature perception (both high and low temperature), with sensors capable of detecting physical phase transition located in the microdomains of membranes, leading to conformational changes and/or phosphorylation/dephosphorylation cycles (Plieth, 1999). The role of membrane fluidity in temperature tolerance has been investigated using mutation analysis, and transgenic and physiological studies (Sung *et al.*, 2003). Mutants of *Arabidopsis* and soybean deficient in fatty acid unsaturation exhibit increased tolerance to high temperature, (Alfonso *et al.*, 2001; Hugly *et al.*, 1989), and increases in lipid saturation in tobacco caused by the silencing of a *ω-3* desaturase gene also conferred high temperature tolerance (Murakami *et al.*, 2000). Based on the expression profile of heat-shock genes, rigidification of the thylakoid membrane and not the plasma membrane appears to trigger temperature stress response (Horváth *et al.*, 1998). The thylakoid membrane would be an appropriate sensor for temperature change, as it is both temperature sensitive due to its unsaturated structure, and is close to photosystems, which are particularly prone to damage by temperature change (Sung *et al.*, 2003).

As with salt tolerance, calcium plays a key role in temperature tolerance, Ca^{2+} influx rapidly increases during the plant recovery phase after heat shock. The importance of high levels of cytosolic Ca^{2+} is illustrated in *Arabidopsis*, where treatment with calcium channel blockers and calmodulin inhibitors increased the degree of heat induced oxidative damage (Larkindale & Knight, 2002). As with salt response, calcium activates the MAPK and CDPK signalling pathways, activating generic abiotic stress response (Sung *et al.*, 2003). The expression of the Heat-Shock Factor (HSF) class of transcription factors increases in response to heat shock stimulus. HSFs bind to conserved Heat-Shock Elements (HSEs) in the promoters of genes which mitigate the effects of high temperature induced damage (Wu, 1995).

1.5.6 Abiotic stress tolerance

1.5.6.1 General mechanisms of abiotic stress tolerance

Mechanisms of plant defence against abiotic stress

Prolonged exposure to adverse environmental conditions prevents plants from attaining their full potential (Boyer, 1982). For example, reduced access to water can lead to a decline in plant function, and will ultimately result in plant death. Similarities in response shown by both vascular and non vascular plants, and the conservation of common sets of genes and proteins associated with stress tolerance, suggest plants have an encoded capability for stress perception and response (Bohnert *et al*., 1995). Plant response to environmental stress is coordinated by a complex series of cascade reactions that regulate molecular networks (Cushman & Bohnert, 2000). These reactions activate stress response mechanisms that re-establish homeostasis preventing further damage to the plant, and instigate mechanisms to repair damage to proteins and membranes (Vinocur & Altman, 2005).

Antioxidant compounds

Antioxidants act both non-enzymatically and as substrates in enzyme catalysed ROS detoxification reactions (Grene, 2002). Ascorbic acid (AsA), glutathione (GSH), and α-tocopherol are well characterised antioxidants. Evidence for the role of antioxidants in protecting plants from oxidative stress has emerged from observations of stress response and localisation of antioxidants. Antioxidants have been found in high concentrations in highly metabolically active organelles. AsA and GSH levels were found in high concentrations

in the chloroplasts of pea, spinach and barley species (5-20 mM AsA and 1-5 mM GSH), suggesting a crucial role in preventing ROS and free radical accumulation (Noctor & Foyer, 1998). Antioxidant expression levels are closely related to the metabolic state of the cell and changes in environmental conditions (Stöhr & Stremlau 2006; Mullineaux *et al.*, 2006; Ahmad *et al.*, 2008). AsA directly scavenges $^1O_2$, $O_2^{\cdot-}$ and $^{\cdot}OH$, regenerates tocopheroxyl from the tocopheroxyl radical, and acts as a co-factor in enzymatic ROS detoxification reactions (Smirnoff, 2000; Ahmad *et al.*, 2008). Oxidative stress activates the expression of genes responsible for the synthesis of tocopherols in higher plants, preventing lipid auto-oxidation by free radicals (Wu *et al.*, 2007; Ahmad *et al.*, 2008). Anthocyanins accumulate in the *Arabidopsis* leaves in response to salt stress, suggesting a role in mediation of abiotic stress (Xiong & Zhu, 2002).

ROS detoxifying enzymes

ROS detoxifying enzymes are fundamental to the prevention of oxidative damage caused by the build up of ROS. SOD, APX and catalase (CAT) activity maintain a homeostatic balance of superoxide radicals and hydrogen peroxide. The difference in affinities of APX (μM range) and CAT (mM range) for H_2O_2 suggest APX may control the fine modulation of ROS for signalling, whilst CAT is responsible for the removal of ROS during periods of high stress. ROS scavenging pathways are present in almost all cellular compartments, with the APX-mediated ascorbate-glutathione cycle being the most ubiquitous, suggesting ROS is finely controlled in most cellular compartments (Mittler, 2002). CAT is solely present in the peroxisomes, which proliferate during periods of high stress (Lopez-Huertas *et al.*, 2000). SOD converts O_2^- to H_2O_2, preventing immediate oxidative damage, and allowing further conversion by APX, glutathione peroxidise (GPX) and CAT to water and diatomic oxygen (Mittler, 2002).

Physiological adaptations to counter abiotic stress

Anatomical adaptations such as leaf movement and curling, development of a refracting epidermis and hiding of stomata in specialised structures allow plants to adjust to abiotic stress and thereby avoid ROS production (Mittler, 2002).

Enhancement of abiotic stress resistance

Efforts to improve abiotic stress tolerance have had limited success due to the physiological and genetic complexity of the trait (Flowers, 2004; Sung *et al.*, 2003; Vij & Tyagi, 2007), with

the molecular networks involved in stress perception, signal transduction, and expression of stress related genes and metabolites being both complex and interdependent (Vinocur & Altman, 2005). Attempts to integrate stress tolerance into agricultural lines via traditional breeding methods has had limited success due to the negative linkages that exist between stress tolerance and yield (Flowers, 2004). Several genes encoding plant antioxidant enzymes have been characterised and used in the construction of transgenic lines (Sarowar *et al.*, 2005). Plants with high levels of antioxidants, either through induction or constitutive expression of these genes, have generally exhibited greater resistance to oxidative damage than plants with lower levels (Bailey-Serres & Mittler, 2006). *Arabidopsis* genetically engineered to over-produce reactive oxygen-scavenging osmolytes showed enhanced tolerance to salt, cold and heat stresses (Hayashi *et al.*, 1997), whilst the ozone-sensitive *Arabidopsis* mutant, *soz1* (now known as mutant vitamin c1; *vtc1*), exhibiting decreased levels of AsA, was more sensitive to oxidative stress than the wild-type (Conklin *et al.*, 1996). Overexpression of the *Arabidopsis* gene nucleoside diphosphate kinase 2 (*AtNDPK2*), encoding a protein that has ROS detoxifying function, enhanced tolerance to saline and oxidative stress in transgenic *Arabidopsis* (Moon *et al.*, 2003), whilst overexpression of the antioxidant β-carotene hydroxylase produced increased tolerance to oxidative stress induced by high light conditions (Davidson *et al.*, 2002). Other studies found no benefit or even a cost in over-expressing antioxidant compounds and enzymes. Over-expression of SOD in tobacco chloroplasts provided no additional tolerance to oxidative stress, suggesting other antioxidant mechanisms may be limiting (Allen, 1995), whilst enhanced GSH biosynthesis in tobacco chloroplasts resulted in increased oxidative damage to cells, likely due to alteration of the overall redox state of chloroplasts (Creissen *et al.*, 1999). These studies suggest that, if a genetic manipulation approach is taken, it may be important not to search for the “best” gene, but to optimise the timing or strength of expression, for example by using inducible promoters. Many target genes have been identified by changes in expression under abiotic stress and one of the most studied group is the DREBs/CEBs transcription factors (dehydration responsive element binding proteins/C-repeat binding proteins). DREB expression is upregulated by water, cold and salt stress and the proteins bind to drought, cold and salt stress responsive promoter elements, therefore playing a central role in abiotic stress response (Liu *et al.*, 1998). Constitutive over-expression of these genes resulted in reduced barley yield in the absence of water stress (Morran *et al.*, 2011) However, when *TaDREB2* or *TaDREB3* were expressed from the maize *Rab17* promoter in wheat, there was little or no expression in well watered conditions, but high expression

when drought stress was applied. Expression levels rapidly returned to normal on watering, with the result that plants grew normally when well watered. Trials of these plants are underway in Australia to determine whether these lines will respond well under less defined conditions (Lopato & Langridge, 2011).

1.5.6.2 Salt stress tolerance

Maintenance of osmotic homeostasis

Plant roots absorb water by exploiting the difference in osmotic potential between root epidermal cells and the surrounding soil environment. Uptake of water from saline soils is maintained through the controlled uptake of Na^+ ions counterbalanced with negatively charged ions, usually Cl^-. Under normal or low salinity soil conditions, the solute concentration within the root is higher than that of the surrounding soil, and water is drawn into the root. In saline soils, this osmotic balance is disturbed, resulting in a decrease in water intake into the roots. In extreme saline conditions, hyperosmolarity may occur, whereby water is drawn out of the roots into the soil resulting in dehydration of the roots and physiological drought effects (Botella *et al.*, 2005; Xiong & Zhu, 2002). Drought increases the salinity of the soil solution, as lower water availability increases salt concentration (Moore, 2008). Root cells mediate changes in water potential by facilitating water movement, controlling ion entry and efflux from the cytosol, sequestering ions once in the cytosol, and by producing osmoprotectants to counteract osmotic imbalance. When water availability is limited, plants restrict growth through hormone signals generated by the roots (Munns, 2002), thereby increasing the likelihood of plant survival.

Root cells respond to external increases in solute concentration by synthesising osomoregulatory compounds such as osmolytes (organic solutes) and late embryogenesis abundant (LEA) proteins. Osmolytes, which include proline, glycine-betaine, trehalose, and sugar alcohols such as mannitol and sorbitol, are abundantly produced in cells undergoing salt stress and have a key role in reducing the water potential of the cytosol (Sahi *et al*., 2006). Osmolytes may act as inert osmoprotectants which decrease osmotic potential without disturbing metabolic functions, or may act as free radical scavengers and, in concert with HSP, as stabilisers of membranes and proteins (Vinocur & Altman, 2005). *Arabidopsis* transformed to express the bacterial *codA* gene encoding choline oxidase, which converts choline to the osmolyte glycinebetaine, showed increased tolerance to salt stress during

germination and seedling growth phases (Hayashi *et al.*, 1997). However, further attempts to genetically engineer glycophytes to increase osmolyte expression resulted in only a marginal increase in salt tolerance, suggesting osmolyte production is not a limiting factor for salt tolerance. In contrast, Garg *et al*. (2002) reported that T_4 generation rice plants transformed with the *E. coli* trehalose biosynthetic genes *otsA* and *otsB* survived and grew better under low temperature, salt and drought stress. These lines also maintained a higher level of selectivity for K^+ over Na^+ uptake in the roots and Na^+ exclusion in the shoots than non-transformed controls and showed improved photosystem II function.

Sudden changes in soil salinity can cause osmotic shock due the sudden influx or efflux of water across the cell membrane. Severe osmotic shock can disrupt substrate transport and result in damage to the membrane. Aquaporins facilitate the rapid transport of water between the cytosol and the external environment allowing water uptake or relieving osmotic pressure (Tyerman *et al.*, 1999). Aquaporins may aid the uptake of water by root cells under drought or unfavourable osmotic conditions. Aquaporins may be gated through interaction with Ca^{2+} and gating proteins, providing a further level of osmotic control (Azad *et al.*, 2004).

Detection of salinity by root cells upregulates the biosynthesis of the plant stress hormone ABA. ABA is then translocated to the leaves where it regulates the osmotic potential of stomatal guard cells, rapidly closing stomata to prevent water loss and the transpirational pull of water from roots to the leaves, thus maintaining a favourable osmotic potential in root cells. Furthermore, control of transpiration limits salt ions to the vacuoles in shoots, minimising damage to areas of high metabolic activity (Apse *et al.*, 1999). ABA responds to salt stress by restricting growth and germination, maintaining ion and osmotic homeostasis, and regulating stress damage control and repair (Koornneef *et al*., 1984; Zhu, 2002, Chinnusamy *et al.*, 2005). ABA contributes to salt stress induced growth inhibition by enhancing DELLA restraint (Achard *et al.*, 2006).

The Salt Overly Sensitive (SOS) signalling pathway has a key role in exporting Na^+ through the Na^+/H^+ antiporter system. Cytosolic Ca^{2+} accumulation results in increased SOS3 accumulation. SOS3 binds to Ca^{2+} activating the protein kinase SOS2. The SOS3-SOS2 complex increases *SOS1* expression and activates the SOS1 protein, a Na^+/H^+ antiporter that exports Na^+ from the cell. The SOS pathway may also regulate other transporters at a post-

translational level to maintain ion homeostasis (Zhu, 2000). *SOS1* activity is ubiquitous to virtually all plant tissue, but activity is greatest in root epidermal cells, particularly at the root tip and the cells surrounding the xylem, suggesting Na^+ is loaded into the xylem for transport away from the root cells (Shi *et al.*, 2002b). Over-expression of *SOS1* improved salt tolerance in *Arabidopsis* (Shi *et al*., 2002a, b), likely due to interaction with RCD1 (Radical-Induced Cell Death1), which protects cells against oxidative damage caused by the ROS molecule hydrogen peroxide (Katiyar-Agarwal *et al.*, 2006). An *Arabidopsis* mutant of *AtSOS1* exhibited salt sensitivity coupled with the death of root cells under salt stress conditions (Oh *et al*., 2010). It has also been proposed that SOS1 may act as a plasma membrane Na^+ sensor, based on the presence of 10 – 12 transmembrane tails and a long cytoplasmic tail (Zhu, 2003). A further Na^+/H^+ antiporter system, AtNHX, has been characterised in *Arabidopsis*, controlling Na^+ sequestration to the vacuole via an ABA-dependent mechanism (Yokoi *et al.*, 2002). Overexpression of the *AtNHX5* gene in paper mulberry plants resulted in increased salt tolerance (Li *et al*., 2011) and expression of the yeast Na^+/H^+ membrane antiporter (*SOD2*) gene in rice also conveyed increased salt tolerance, likely through increased root proton export capacity, increased photosynthetic capability, and reduced ROS generation (Zhao *et al*., 2006). The SOS pathway may have a further role in salt stress tolerance through interaction with pathways controlling cell division and expansion, and crosstalk with other stress signalling pathways (Zhu, 2000).

Competition for ion uptake

Root cells must absorb nutrient ions despite the prevalence of toxic ions in the surrounding environment. For most plants, sodium is not a limiting factor in plant growth; however, potassium is an essential nutrient (Blumwald *et al.*, 2000, Mäser *et al.*, 2002). Na^+ and K^+ ions have a similar radius and ion hydration energy, leading to competition for cellular uptake, particularly where the external concentration of Na^+ is higher than K^+ (Niu *et al.*, 1995; Rodríguez-Navarro, 2000). Na^+ may enter root cells through the high-affinity K+ transporter HKT1, non-selective cation channels, and Na^+ leakage into the transpiration stream via the apoplast (Zhu, 2003). High K^+:Na^+ ratio is maintained by primary active transport mediated by H^+-ATPases, and secondary transport mediated by channels and co-transporters (Zhu, 2003).

1.5.7 DELLA and abiotic stress tolerance

DELLAs and response to abiotic stress

DELLA mediates response to environmental stress by limiting growth and up-regulating expression of ROS detoxifying enzymes in environmentally unfavourable conditions, allowing plants to respond to environmental changes through regulation of DELLA activity (Achard *et al*., 2006). Wild-type plants show decreased levels of GA and increased levels of DELLA in response to salt stress, resulting in inhibited growth. Growth inhibition is beneficial to survival as smaller plants have a lower requirement for resources, and are thereby able to survive in environments where resources are scarce. The effect of environmental stress on DELLA-mediated growth is illustrated by the DELLA GoF and LoF *Arabidopsis* mutants subjected to salt stress. The growth and development of the tall "quadruple-DELLA mutant" of *Arabidopsis* (lacking GAI, RGA, RGL1 and RGL2) is less inhibited by saline conditions when compared with the wild-type, yet has a lower rate of survival to salt toxicity (Achard *et al*., 2008a). In contrast, the *gai* dwarf mutant is able to survive saline conditions better than the wild-type. DELLAs are also important in freezing tolerance. Using wild-type and DELLA mutant lines Achard *et al*. (2008b) showed that the *gai* mutant was better able to withstand freezing than the wild-type controls which, in turn, survived better than the double mutant *gai-t6/rga-24*. Furthermore, this mutant was less tolerant even following cold acclimation. The authors hypothesised that this tolerance may also be mediated through DELLA-mediated suppression of ROS signalling.

DELLAs regulate root hair growth in *Arabidopsis* via a ROS dependent mechanism, with root hair growth inhibited under salt stress conditions. Elongation of roots in the "quadruple-DELLA" mutant in salt conditions suggests that RGL3 may play a role in growth inhibition under stress conditions (Achard *et al*., 2006). Indeed, Achard *et al*. (2008b) provided additional evidence for the involvement of *RGL3* in cold stress response, with increased expression of the gene reported in response to cold treatment. Furthermore, specific enhancement of *RGL3* expression in transgenic *Arabidopsis* expressing the *CBF1* (C=repeat drought responsive element binding factor) gene, underlined the importance of this protein in stress tolerance, but also showed that the freezing tolerance mechanism was not through the CBF regulon. Cold treatment increased the level of *GA2ox1*, *GA2ox3 and GA2ox6* transcripts which led to a decrease in bioactive GA and an increase in DELLA protein which in turn led to increased transcript levels for *Ga3ox* and *Ga20ox* through a feedback mechanism. Treatment of barley with growth inhibitors which reduce GA biosynthesis and therefore

likely stabilise DELLA have been shown to confer tolerance to abiotic stress in barley (Sarkar *et al.*, 2004). It has long been reported that GA play a role in abiotic stress response and recently Alonso-Ramirez *et al*. (2009) suggested that this was likely to be as a result of their ability to increase salicylic acid (SA) biosynthesis. Since there is increasing evidence that SA elicits plant defence reactions in several abiotic stress conditions (Horvath *et al*., 2007), the authors hypothesized that DELLA proteins (and/or the absence of GA) restrain growth by repressing SA biosynthesis. DELLAs have been shown to delay flowering in *Arabidopsis* by means of two distinct mechanisms: 1) DELLA inhibits plant development, increasing the duration of the vegetative phase 2) DELLA inhibits expression of the *LEAFY* (*LFY*) gene, a proponent of floral development (Achard *et al.*, 2004). Wild-type *Arabidopsis* plants growing in saline conditions exhibit delayed flowering, through activation of both ABA and ethylene signalling pathways whose effects are integrated at the level of DELLA response (Achard *et al.*, 2006). In contrast, "quadruple-DELLA" mutants growing in saline conditions flowered earlier than wild-type plants under the same growth conditions. Saline conditions produced total inhibition of flowering in GA deficient and insensitive mutants (*ga1-3* and *gai* mutants; Achard *et al.*, 2006). The delay in flowering under stress conditions may negatively affect the yield potential of plants and the link of DELLAs with flowering time has been reported in crop plants. The semi-dwarfing wheat mutant alleles *rhtB1b* and *rhtD1b* have pleiotropic effects on flowering time (Silverstone & Sun, 2000; Khush, 2001) with flowering delayed by drought (Dr. M. Boulton, personal communication) and severely dwarfed mutants (*rhtB1c* and *rht*D1c) in which the DELLA proteins are highly stable, have markedly delayed flowering (Dr. M. Boulton, personal communication). It is therefore clear that the use of DELLA mutants in agriculture requires careful consideration to identify the alleles most suited to the changing environment.

Chapter 2: Materials and Methods

2.1 Plant materials and plant culture

2.1.1 Seed origin

Wild-type and mutant barley (*Hordeum vulgare*) lines were used in this study. Wild-type lines for cultivars Bowman, Golden Promise, Herta, Himalaya and Triumph, as well as the mutant lines 380, 382 (cv Bowman), *sln1-1* (cv Herta), *gse1a*, *sln1c* and *sln1d* (cv Himalaya) were acquired from the John Innes Centre germplasm collection or from seed packets available in the Boulton laboratory. Seeds of cv H930-36 wild-type and *dwf2* mutant were originally provided by Dr. D.E. Falk (University of Guelph, Ontario), but were available as bulked seed in packets in the Boulton laboratory. During this study, further seed was collected from dwarf, medium and wild-type size tillers from a single revertant chimeric plant (termed *dwf2-1*) that spontaneously resulted from *dwf2* seed. The cv Himalaya *gse1j* and *gse1n* mutant seed was obtained from Dr. P. Chandler (CSIRO, Canberra), and had been backcrossed eight times. The cv Triumph mutant seed was collected from 4 plants (*γ-1*, *-2*, *-3*, *-5*). These plants were produced from seed collected from a plant that had shown a late flowering phenotype identified during analysis of a population derived from γ irradiated seed grown in field plots at the JIC (unpublished data, Dr. D. Laurie). The cv Bowman 827 and 885 mutant lines were obtained from Dr. A. Druka (Scottish Crops Research Institute, Dundee).

To produce bulk seed stocks, seedlings were transferred to 2 L pots containing barley mix compost (see Section 2.1.3.2) and grown in a glasshouse (see Section 2.1.2). Developing plant heads (pre-anthesis) were contained in polyethylene bags to prevent cross-pollination. Mature seeds were collected from desiccated plants.

2.1.2 Plant growth conditions

Growth cabinets (Sanyo MLR Plant Growth Chamber, Sanyo, Leicestershire, UK) were used for the growth of seedlings used in genotype characterisation and heat shock experiments. Plants were grown at 20 °C, 16 h light period; 8 h dark period, and relative humidity of 60%. Irradiance was approximately 150 µmol m^{-2} s^{-1}.

A Controlled Environment Room (CER) was used to germinate and grow seedlings preceding and post heat shock treatment, and preceding and during hydroponics experiments. Seedlings grown for characterisation of mutant phenotype were also grown in the CER. Plants were grown at 20 °C, 16 h light period; 8 h dark period and relative humidity of 60%. Irradiance was approximately 150 µmol m^{-2} s^{-1}.

For transformation experiments, donor plants for embryo isolation and post-tissue culture T_0 seedlings were grown as described by Harwood *et al.* (2008) in a CER with a 16 h light period (500 µmol m^{-2} s^{-1} at mature canopy level provided by metal halide lamps (HQI) supplemented with tungsten bulbs) and 80% humidity, with watering as required. Transformed callus was regenerated in a tissue culture room. Plants were grown at 23 °C, 16 h light period; 8 h dark period and 60% humidity. Irradiance was approximately 150 µmol m^{-2} s^{-1}.

2.1.3 Plant culture

2.1.3.1 Seed stratification

Seeds were stratified at 4 °C on a double layer of 90 mm filter paper (Sartorius Stedim Biotech, Aubagne, France) in a standard Petri dish (Sterilin, South Wales, UK) with approximately 8 ml sterile distilled water (SDW). After five days seed were sown in barley mix compost and transferred to a controlled environment room to germinate (16 h photoperiod, 20 °C day, 15 °C night), or for hydroponics based experiments, transferred to a controlled environment room and germinated in the Petri dish.

2.1.3.2 Growth in soil

Barley was grown in 70 – 2000 cc plastic containers (Desch Plantpak, Waalwijk, The Netherlands) in Barley Mix compost (JIC Horticultural Services, Appendix 1.1). Seeds were stratified as described in Section 2.1.3.1, then germinated in 70 or 100 cc containers and transferred to larger containers as they developed before root growth was constrained. Barley mix compost was prepared as described in Appendix 1.1.

2.1.3.3 Growth in hydroponic culture

Plants were grown in hydroponic culture in modified Hoagland's solution (Hoagland & Arnon, 1950; Appendix 1.2). Seeds were stratified as described in Section 2.1.3.1 before transfer to CER or growth cabinet conditions (see Section 2.1.2). Seedlings were gently removed from the filter paper when roots were sufficiently developed (minimum length 2 cm) and held at the base of the stem by foam bungs, which were secured in 50 ml polypropylene centrifuge tubes (Corning Incorporated, Corning, NY, USA) with the bases and caps removed. These tubes were placed in custom steel racks, which in turn were placed in 10 L plastic containers (Tontarelli, Castelfidardo, Italy) containing Hoagland's solution (x 0.5). Seedlings were positioned randomly in the racks according to randomisation patterns generated using Genstat^{TM} ver. 10 (VSN International, UK), and grown for two days before transfer to respective treatment solutions. Plants designated for salt treatment were transferred to Hoagland's solution (x 0.5) containing 100 - 500 mM NaCl dependent on the treatment group, whilst control lines were transferred to fresh Hoagland's solution (x 0.5). Seedlings of a similar developmental stage were selected for treatment. Solutions were aerated by daily transference of solutions between two containers, or by constant aeration using air pumps (Rena Air 100 & 200, Rena Aquarium Equipment, Charlotte, NC, USA; Tetratec APS 100, Spectrum Brands, Madison, WI, USA).

2.1.3.4 Growth media for transgenic plants

Media used for the production of transgenic barley plants (barley callus induction (BCI) medium, barley transition (BT) medium, and barley regeneration (BR) medium), were prepared as described in Appendix 1.3.

2.1.4 Isolation of plant nucleic acid

2.1.4.1 Isolation of plant genomic DNA

Young leaf material was collected from seedling and mature plants for DNA extraction. DNA was extracted immediately, or leaf material was immediately flash frozen in liquid nitrogen and stored at -80 °C for subsequent extraction. DNA was isolated using the modified cetyl trimethylammonium bromide (CTAB) method described by Sambrook & Russell (2001) or

using the DNeasy Plant Mini Kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions.

A modified version of the Edwards *et al*. (1991) protocol was used to extract DNA from transgenic barley lines. The following amendments were made: upon harvesting, tissue was macerated in liquid nitrogen rather than at room temperature (rt). All centrifugation steps were carried out at 4 °C. After treatment with extraction buffer, samples were centrifuged for 3 min instead of 1 min. After addition of isopropanol, samples were incubated on ice rather than at room temperature. The DNA pellet was air dried and was dissolved in 0.1 x TE instead of 1 x TE buffer.

2.1.4.2 Isolation of plant RNA

Plant tissue samples were flash frozen in liquid nitrogen immediately after harvesting and subsequently stored at -80 °C prior to RNA isolation. Frozen tissue samples were ground to powder in liquid nitrogen in autoclaved pestle and mortars. RNA was extracted from a maximum of 100 mg of plant tissue using the RNeasy Mini Kit (Qiagen) or RNeasy Plant Mini Kit (Qiagen), according to manufacturer's instructions, with the exception that β-mercaptoethanol was not added to RLT and RLC buffers. RNA was re-suspended in 'Super' water produced using Super-Q Plus Water Purification Systems (Millipore, Bedford, MA, USA) or UltraPure™ DEPC-Treated Water (Invitrogen). RNA quantity and quality was assessed using the Picodrop™ spectrophotometer and agarose gel electrophoresis.

2.2 Bacteria and bacterial culture

2.2.1 Bacterial strains

One Shot® TOP10 Chemically Competent *Escherichia coli* (*E. coli*, Invitrogen, Carlsbad, CA, USA), and homemade *E. coli* DH5α cells were used in routine cloning for insert amplification and sequencing. Homemade chemically competent and electrocompetent DH5α cells, derived from DH1 *E. coli* (Hannahan, 1983) were provided by Dr. Nadia Al-Kaff and Dr. Christine Faulkner (JIC, Norwich, UK) respectively. Library Efficiency® DH5α™ cells (Invitrogen) were used in both routine and Gateway® cloning.

Agrobacterium tumefaciens (*Agrobacterium*) strain AGL1 was used for barley transformation and was provided by Dr. W. Harwood (JIC).

2.2.2 Restriction enzymes and antibiotics

Restriction enzymes and associated incubation buffers were obtained from New England BioLabs (Ipswich, MA, USA) or Roche (Basel, Switzerland). Chemicals were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. The following antibiotics were used for *E. coli* and *Agrobacterium* selection: ampicillin (Sigma-Aldrich), carbenicillin (Formedium, Hunstanton, UK), hygromycin (Duchefa, Haarlem, The Netherlands), kanamycin (Formedium).

2.2.3 Bacterial transformation

Unless otherwise stated, bacterial transformation of electrocompetent and chemically competent cells was conducted according to the manufacturer's instructions. Chemically competent cells were heat shocked using a water bath (JB1, Grant Instruments, Cambridgeshire, UK) or heating block (Thermomixer 5436, Eppendorf, Hamburg, Germany). DNA (2 – 10 ng) was added to 50 µL electrocompetent cells which had been thawed on ice immediately before use. The mixture was then transferred into a 2 mm electroporation curvette pre-chilled on ice. The curvette was placed into an electroporator (BioRad Gene Pulser II (BioRad, Hertfordshire, UK)) and cells pulsed at 2.5 kV (400 ohms, 25 µFd). Cells were then added to 0.5 mL SOC medium (Super Optimal Broth with Catabolite Repression, Hanahan, 1983) and shaken at 37 °C for 2 h. An aliquot (100 µL) of the suspension was then spread on LB-G (LB: Luria-Bertani without glucose, Maniatis *et al.*, 1982) agar plates containing the appropriate selection, and incubated overnight at 37 °C.

Transformation of *Agrobacterium* was performed as for electroporation of *E. coli*, with the exception that cells were transferred to 2 mL L broth and shaken at room temperature for 2 - 3 h after pulsing. Aliquots (10 – 100 µL) of this suspension were then spread on L agar (with rifampicin (50 µg mL^{-1}) for *Agrobacterium* selection and the appropriate selection for the transforming plasmid). Plates were incubated for 2 days at 28 °C.

2.2.4 Bacterial culture

Unless otherwise stated, *E. coli* was cultured at 37 °C overnight on LB–G agar plates or with shaking in LB–G broth, with appropriate selection: X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) or ampicillin. SOC medium was used as a bacterial growth medium when required according to the manufacturer's instructions. Growth media were obtained from the JIC media supply and produced to the formulations described in Appendix 1.4.

2.2.5 Colony PCR

White colonies growing on selective media were identified as putative transformants. The presence of an insert was confirmed by colony PCR using insert and vector specific primers. Colonies were sampled using sterile wooden toothpicks, added to 50 µL SDW, heated to 95 °C for 15 min then cooled and used in colony PCR. Each colony PCR reaction consisted of 2 µL colony preparation, 10 µL GoTaq® DNA Polymerase (Promega), 1.5 µL forward primer, 1.5 µL reverse primer, 0.6 µL Dimethylsuphoxide (DMSO), 4.4 µL SDW. Colony PCR cycling conditions were dependent on the annealing temperature of the primer set and insert size. Appropriate positive and negative controls (null transformant and SDW) were used. Transformed cells were preserved as glycerol stocks, produced by the addition of 50 µL of bacterial culture (grown overnight) to 50 µL glycerol, followed by mixing and storage at -80 °C.

2.2.6 Isolation of plasmids from *E. coli*

Plasmids were isolated from *E. coli* using QIAprep Spin Miniprep Kit (Qiagen) from a maximum of 5 ml bacterial culture in LB-G medium, according to the manufacturer's instructions, however to increase plasmid concentration, 30 µL EB buffer was used in the elution step instead of the recommended 50 µL.

2.3 Molecular biology materials and methods

2.3.1 Precipitation and purification of nucleic acids

2.3.1.1 Precipitation and purification of DNA

Precipitation and purification of nucleic acids was used to increase DNA concentration and remove contaminants that could inhibit subsequent PCR, cloning and sequencing steps. Isopropanol and ethanol precipitation was used to precipitate and purify genomic DNA before PCR amplification, whilst polyethylene glycol (PEG) precipitation and agarose gel purification was used to precipitate and purify PCR product and plasmid DNA before cloning and sequencing. Protocols used were as follows:

Isopropanol and ethanol precipitation was used preceding PCR amplification and were carried out as described by Maniatis *et al*. (1982), with the amendment that precipitated DNA was in some cases resuspended in SDW instead of TE buffer.

Polyethylene glycol precipitation was used preceding PCR amplification, and to increase the concentration of insert DNA before the ligation step of bacterial cloning. Precipitation was conducted as described by Sambrook & Russell, (2001).

QIAquick Extraction Kit (Qiagen) was used to isolate DNA bands from agarose gels. DNA bands were excised from low concentration agarose gels (0.4 – 0.8%, 1 x TAE or TBE buffer) and purified according to the manufacturer's instructions.

2.3.1.2 Precipitation and purification of RNA

Lithium chloride precipitation was performed on DNase treated RNA samples before cDNA synthesis, according to the procedure described by Maniatis *et al*. (1982) with the following modifications: 1 volume of 8 M LiCl was added to a maximum volume of 30 µL of RNA extract, and the mix was stored at -80 °C for 2 h or overnight. Samples were centrifuged at 21 000 x g for 30 min at room temperature and the supernatant carefully removed. Resulting pellets were washed with 200 – 500 µL of 70% ethanol before centrifugation at 21

000 x g for 5 min. Supernatant was removed and the pellet air dried for 10 min, before resuspension in 'Super' or DEPC-treated nuclease free water by incubation at 4 °C for 1 h.

2.3.2 PCR amplification

Full and partial amplification of the *Sln1* gene was used for sequencing and for transgenic construct production. Primers were designed using the criteria described by Dieffenbach *et al.* (1993), or by using the primer design software Primer3 v.4.0 (http://frodo.wi.mit.edu; Rozen & Skaletsky, 2000), or Primerfox (http://www.primerfox.com). Primer specificity was checked against the National Centre for Biotechnology Information (NCBI) *Hordeum vulgare* non-redundant database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using BLASTn (Altschul *et al.*, 1990). PCR efficiency was optimised by selecting the most efficient temperature from a gradient. Primers were ordered form Eurofins MWG Operon (Ebersberg, Germany) and Sigma-Aldrich (Gillingham, UK).

PCR amplification of target sequence was carried out using the following DNA polymerases, as appropriate: Platinum® *Taq* DNA Polymerase High Fidelity, Platinum® *Taq* PCRx DNA Polymerase, Platinum® *Pfx* DNA Polymerase (Invitrogen), Taq DNA Polymerase (Roche), HotStar Taq DNA Polymerase (Qiagen), Phusion® High Fidelity DNA Polymerase (New England BioLabs), GoTaq® DNA Polymerase (Promega). PCR was carried out using PTC-200 DNA Engine (MJ Research Inc., Alameda, CA, USA), Tetrad PTC-225 Thermo cycler (MJ Research Inc.) and GS1 (G-Storm, Surrey, UK) thermal cyclers.

2.3.3 Qualitative and quantitative analysis of nucleic acids

Nucleic acid purity and quantity was assessed by gel electrophoresis and UV spectrophotometry.

Gel electrophoresis was used to analyse precipitated or unprecipitated nucleic acid extracts, PCR products and DNA digests. Clear band formation on an electrophoresis gel (0.6 – 0.8% agarose, 1 x TAE or TBE buffer) was used as an indicator of nucleic acid quality, and the use of molecular weight markers allowed assessment of band sizes. Loading buffers (5 x DNA loading buffer blue (Bioline, London, UK) and 5 x Orange G loading buffer) provided by Dr.

Andrey Korolev (JIC) were added to the nucleic acid before loading on to the agarose gel. Ethidium bromide stained nucleic acid was visualised and recorded using Gel Doc™ XR System and Quantity One® ver. 4.6 software (Bio-Rad, Hertfordshire, UK) or AutoChemi™ System (UVP, Upland, CA, USA) and LabWorks™ ver. 4.6 (Media Cybernetics, Bethesda, MD, USA). The following molecular markers were used, as appropriate: HyperLadder™ I (Bioline), HyperLadder™ IV (Bioline), 1 kb DNA ladder (New England BioLabs), Quick-Load® 1 kb DNA ladder (New England BioLabs), Quick-Load® 100 bp DNA ladder (New England BioLabs), 1 kb DNA Ladder (Invitrogen), BenchTop 1 kb DNA Ladder (Promega, Madison, WI, USA).

UV spectrophotometry was used to analyse purified nucleic acid extracts. Spectrophotometric quantification of RNA or DNA was carried out using a NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, USA) or Picodrop (Picodrop, Cambridge, UK). Contamination and quality of nucleic acids was estimated by the OD_{260}/OD_{280} ratio, with 1.8 considered ideal for DNA and 2.0 considered ideal for RNA (Sambrook & Russell, 2001).

2.3.4 DNA sequencing

Purified PCR products and plasmids were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions and specific primers. Sequencing was performed by The Genome Analysis Centre (TGAC, Norwich, UK).

2.4 Quantification of *Sln1* expression

2.4.1 DNase treatment

To remove DNA from RNA extracts prior to cDNA synthesis, samples were treated with TURBO DNA-*free*™ DNase I (Ambion, Carlsbad, CA, USA) or DNase I recombinant, RNase-free (Roche) according to the manufacturer's instructions. RNA quantity and quality was assessed using the Picodrop™ spectrophotometer and agarose gel electrophoresis. Where RNA concentration or purity was insufficient for cDNA synthesis (<20 ng μL^{-1}) or OD_{260}/OD_{280} ratio was less than 1.8 or more than 2.2, samples were purified and concentrated by lithium chloride precipitation. RNA quantity and quality was re-assessed after precipitation.

A QPCR reaction was performed on a 1:10 or 1:20 dilution of the RNA samples to determine whether genomic DNA was present after DNase treatment or LiCl precipitation. QPCR was performed using a CFX 96™ Real-Time System (Bio-Rad) attached to a C1000™ Thermal Cycler (Bio-Rad). Reactions were prepared in 96 well plates (Thermo-Fast® 96, Thermo Scientific) to a final volume of 20 µL; with 4 µL diluted RNA, 10 µL SYBR® Green JumpStart™ *Taq* ReadyMix™ (Sigma-Aldrich); 2 µL forward primer (see Table 2.1); 2 µL reverse primer (see Table 2.1); 2 µL nuclease free water. Cycling conditions were: 95 °C for 10 min; then 40 cycles of 94 °C for 20 s, 62 °C for 20 s (anneal temp), 72 °C for 30 s (plate read); followed by a final extension of 72 °C for 10 min. Melt curve analysis (50-95 °C) was performed for each sample at the end of the PCR reaction to distinguish double stranded amplicons from PCR artefacts. A control qRT-PCR was performed on the same dilution of RNA used for cDNA synthesis to reveal any genomic DNA remaining after DNase treatment. Only samples without DNA contamination were used for cDNA synthesis.

Table 2.1 Primers used for amplification of the [(a)]target and [(b)]normalisation genes in the qRT-PCR assay. [(c)]Glyceraldehyde 3-phosphate dehydrogenase; [(d)]The primers used for amplification of *Sln1* were designed for amplification of the wheat DELLA gene (*Rht*) but had been shown to amplify *Sln1* (R. Saville, 2011).

Gene	Forward	Reverse	Reference
Sln1[a]	CTACGAGTCCTGCCCCTACC	CCCTGCTTGATGCCGAAGTC	Dr. R. Saville (JIC) [d]
α-tubulin[b]	AGTGTCCTGTCCACCCACTC	AGCATGAAGTGGATCCTTGG	Burton *et al*. (2004)
GAPDH[b,c]	CAGAAACCCCGAGGAGATT CCAT	TGGCTGGCTTGGCAAGTCTAA CAGTCAG	Dunford *et al.* (2005)
Ubiquitin[b]	GCCGCACCCTCGCCGACTAC	CGGCGTTGGGGCACTCCTTC	Rostocks *et al*. (2003)

2.4.2 cDNA synthesis

cDNA was synthesised from a minimum of 80 ng and maximum of 5 µg RNA using the Superscript™ III first strand synthesis kit (Invitrogen) according to the manufacturer's instructions. The reaction was primed with the addition of random hexamers (2.5 ng μL^{-1}, Invitrogen). RNA was digested using RNase-H (Invitrogen) by incubation at 37 °C for 20 min, followed by cooling to 4 °C according to the manufacturer's instructions.

2.4.3 Quantitative RT-PCR (qRT-PCR)

cDNA was diluted to 1:10 or 1:20 in nuclease free water and amplified using the protocol described in Section 2.4.1. Data were analysed using Bio-Rad CFX Manager™ software ver. 1.6 (Bio-Rad). Each reaction was carried out using a minimum of two replicates, and the mean C_t value for each reaction was calculated. The stability and primer efficiency for the three reference genes were tested in all samples as described by Vandesompele *et al*. (2002) using geNorm software ver. 3.5 (http://medgen.ugent.be/~jvdesomp/genorm/). The two reference genes with the most stable expression under experimental conditions were used to calculate a normalisation factor, which was used to normalise the target gene expression data according to Pfaffl *et al*. (2001).

2.5 Bioinformatic and statistical analysis

Sequence alignment

DNA sequences were aligned using ContigExpress, from Vector NTI Suite 10 (Invitrogen).

Statistical analysis

Data were analysed using Excel (Microsoft Office 2007; Microsoft, Redmond, WA, USA), or Genstat™ ver. 10 (VSN International).

Chapter 3: Characterisation of GA Signal Transduction Mutants

3.1 Aims

The aim of the work described in this chapter was to identify and characterise novel and archived barley DELLA mutants that have dwarf or slender-like phenotypes by sequencing the *Sln1* genes of these plants. Novel characterised *Sln1* mutants could then be used in subsequent studies of the role of DELLA in abiotic stress tolerance. The *Sln1* sequences obtained were compared to wild-type and existing *Sln1* mutant sequence to determine whether the abnormal phenotypes were likely to be the result of mutations to *Sln1*, and if so, whether the characterised polymorphisms were present in conserved domains and motifs. The growth and developmental phenotypes of confirmed *Sln1* mutants were also assessed.

The following hypothesise were formulated. H_1: The mutant phenotypes observed in putative DELLA mutants result from differences in *Sln1* nucleotide sequence or expression; H_0: The mutant phenotypes observed in putative DELLA mutants result from mutation to a gene or genes other than *Sln1*, or through post transcriptional regulation of *Sln1* gene transcripts or the SLN1 protein.

3.2 Introduction

3.2.1 GRAS protein family

DELLAs are members of the GRAS family of regulatory proteins. GRAS proteins, named after the first three member proteins to be identified, GAI, RGA and SCR, are a large family of plant specific transcription factors that function in a diverse set of physiological and developmental processes (Engstrom, 2011). The GRAS protein family is comprised of subfamilies, each named after a member protein or common function. In addition to DELLA proteins, other GRAS protein subfamilies include SCARECROW (SCR), LATERAL SUPPRESSOR (Ls), HAIRY MERISTEM (HAM), PHYTOCHROME A SIGNALLING TRANSDUCTION (PAT1), and SHORT-ROOT (SHR) (Table 3.1).

Table 3.1 GRAS protein subfamilies and their regulatory functions. Regulatory functions presented here are described in: [(a)]Peng *et al.*, 1997; [(b)]Di Laurenzio *et al.*, 1996; [(c)]Schumacher *et al.,* 1999; [(d)]Stuurman *et al.*, 2002; [(e)]Bolle *et al.*, 2000; [(f)]Helariutta *et al.*, 2000.

GRAS protein subfamily	Characterised regulatory function
DELLA	Growth inhibition [(a)]
SCARECROW (SCR)	Radial patterning in root and shoot [(b)]
LATERAL SUPPRESSOR (Ls)	Axillary meristem development [(c)]
HAIRY MERISTEM (HAM)	Shoot development [(d)]
PHYTOCHROME A SIGNAL TRANSDUCTION1 (PAT1)	Light stimulated developmental processes including de-etiolation and hypocotyl elongation [(e)]
SHORT-ROOT (SHR)	Root radial patterning and growth [(f)]

3.2.2 Conserved GRAS domains and motifs

GRAS proteins are typically 400 – 700 aa in length, and consist of variable N-terminal and conserved C-terminal domains. The variable N-terminal domain defines individual GRAS protein subfamilies, whilst the C-terminal domain is characterised by conserved GRAS motifs (Figure 3.1). Conserved aa sequence motifs have been identified in the C-terminus: the LXXLL sequence, leucine heptad repeat I (LHR I), VHIID motif, leucine heptad repeat II (LHR II), PFYRE motif, RVER motif, and the SAW motif (Pysh *et al.*, 1999).

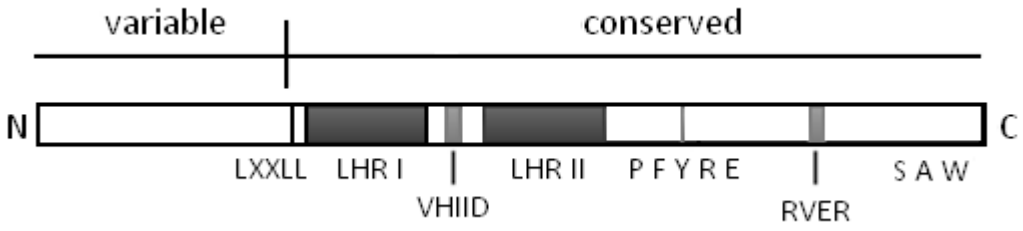


Figure 3.1 Diagrammatic representation of the domains and conserved motifs of GRAS proteins (modified from Bolle, 2004). GRAS proteins are divided into two distinct domains, the C-terminal domain characterised by conserved GRAS motifs, and the variable N-terminal domain that defines individual GRAS protein subfamilies. The motifs are designated after their conserved aa. N and C = amino and carboxyl terminal of the protein, respectively.

An LXXLL sequence at the beginning of the LHR I region marks the beginning of the conserved C-terminal part of several GRAS proteins. LXXLL sequence has been shown to mediate the binding of steroid receptor co-activator complexes to cognate nuclear receptors in mammals (Heery *et al.*, 1997; Bolle, 2004); although an equivalent function is yet to be reported in plant systems.

The LHR regions, LHR I and LHR II, which flank the VHIID motif, are approximately 100 residues in length (Bolle, 2004). The LHR regions in most cases do not consist of leucine heptad repeats, instead they are more commonly regions of leucine richness (Bolle, 2004). Where repeats do occur, the number of repeats is small, normally consisting of two repeats, however, three to five repeats are observed less frequently (Pysh *et al.*, 1999). Leucine rich repeat motifs, 20 – 29 residues long, are associated with protein-protein interactions (Kobe & Kajava, 2001; Itoh *et al.*, 2002), suggesting a role for LHR regions in oligomer formation. The presence of two LHR regions has been identified in several transcription factors, supporting a similar role for GRAS proteins. Furthermore, characterisation of the SCR GRAS protein in *Arabidopsis* identified a stretch of four leucines with spacing consistent with leucine zipper (LZ) formation (Bolle, 2004). LZ are commonly found in DNA-binding proteins (Landschulz *et al.*, 1988) and define the basic-leucine zipper (bZIP) family of transcription factors (Hirsh & Oldroyd, 2009).

The VHIID motif is not absolutely conserved in GRAS proteins, with only the histidine (H) and aspartic acid (D) residues showing absolute conservation (Bolle, 2004). The full significance of this motif is yet to be established, however it has been hypothesised that the LHR I-VHIID-LHRII regions may act as a DNA-binding domain analogous to bZIP protein-DNA interaction (Ellenberger *et al.*, 1992), with the LHRs enabling protein-protein interaction and the VHIID motif enabling protein-DNA interaction (Pysh, 1999).

After the LHRII domain, a putative tyrosine phosphorylation site is present in many members of the GRAS family, overlapping with the tyrosine (Y) in the PFYRE motif (Bolle, 2004). Site directed mutagenesis of the tyrosine in the *Arabidopsis* RGL GRAS protein to mimic constitutive phosphorylation, resulted in reduced GA-mediated degradation of the protein (Hussain *et al.*, 2005). The PFYRE motif is less conserved than the VHIID and SAW motifs, with only the proline (P) residue being absolutely conserved, although there is a high degree of sequence similarity at this region between GRAS proteins (Pysh *et al.*, 1999).

The SAW region consists of three pairs of absolutely conserved residues: R-E, W-G, and W-W. The spacing between R-E and W-G pairs is absolutely conserved; however the spacing between W-G and W-W pairs differs between GRAS proteins. A further motif, RVER, is present between the PFYRE and SAW domains. Although the functions of the RVER and SAW motifs have not currently been identified (Bolle, 2004), the SAW motif at least appears to be essential for DELLA-mediated growth repression, in that a single amino acid substitution at position 606 (T → P) in the SAW motif of the rice SLR1 protein results in a slender phenotype (Itoh *et al.*, 2002).

In addition to the motifs and regions described above, nuclear localisation signals (NLS) have been identified in a number of GRAS proteins (Hirsch & Oldroyd, 2009), with several proteins exhibiting nuclear localisation (Tian *et al.*, 2004). Sequence analyses together with evidence for the nuclear localisation of GRAS proteins is consistent with the proposed role of the GRAS family proteins as transcriptional regulators (Lee *et al.*, 2008).

3.2.3 Motifs conserved in the DELLA proteins

The DELLA and GRAS domains

Sequence and expression analysis of mutant DELLA genes and analysis of mutant protein function (primarily in *Arabidopsis* and rice) has identified conserved motifs and functional regions in DELLA proteins (Ikeda *et al.*, 2001; Pysh *et al.*, 1999). Characteristically for GRAS proteins, DELLA proteins comprise two domains. The N-terminal is highly specific to DELLA, providing the basis for the DELLA subfamily, and is required for interaction with the GA-GID1 complex. Mutations that result in alteration to the DELLA or TVHYNP motifs that define DELLA proteins, or the region between these motifs, commonly result in the development of a GA insensitive, Gain of Function (GoF), dwarf mutant phenotype (Pysh *et al.*, 1999; Itoh *et al.*, 2002). DELLA domain mutants are typically dominant, and exhibit reduced height, dark green colour and late flowering (Chandler *et al.*, 2002; Ikeda *et al.*, 2001; Peng *et al.*, 1997, 1999; Cassani *et al.*, 2009; Liu *et al.*, 2010). In addition to the DELLA and TVHYNP motifs, a polyS/T/V region connects the N-and C-terminal domains of the DELLA proteins. This domain shows marked sequence variation between species but has been characterised most extensively in the SLR1 protein. Itoh *et al.* (2002) found that the polyS/T/V region enhanced the growth suppression imposed by the SLR1 protein, and served as a putative target site for O-linked N-acetylglucosamine (O-GlcNAc) modification by O-GlcNAc transferase encoded by

the *SPY* gene, thereby enhancing the suppressive activity of the DELLA proteins (Itoh *et al.*, 2005; Silverstone *et al.*, 1998). Alanine substitution of residues in the polyS/T/V region did not affect interaction of SLR with either GID1 or GID2 (Hirano *et al*., 2010). The C-terminus of the DELLA proteins (the GRAS domain) is integral to growth repression, as exhibited with the expression of a truncated form of the rice DELLA, SLR1 (ΔC-Ter), which lacks the VHIID, PFYRE and SAW motifs. The highly truncated SLR1 lacked the DELLA growth repression associated with the wild-type form of SLR1 (Itoh *et al.*, 2002), with plants exhibiting a tall (LoF) phenotype. GRAS domain mutants are typically recessive, and in plants containing a single DELLA gene they result in tall, slender phenotypes similar to those of plants saturated with GA, or having a constitutive GA response. Chlorosis and reduced seed fertility are commonly associated with these mutations (Chandler *et al.*, 2002; Ikeda *et al.*, 2001; Bassel *et al.*, 2008; Weston *et al.*, 2008). A summary of selected DELLA ORF mutants characterised to date is shown in Table 3.2.

Table 3.2 Characterised mutations within the DELLA ORF. [(a)]Chandler *et al.*, 2002; [(b)]Ikeda *et al.*, 2001; [(c)]Peng *et al.*, 1999; [(d)]Pearce *et al*., 2011 [(e)]Wu *et al*., 2011 [(f)]Cassani *et al.*, 2009; [(g)]Bassel *et al.*, 2008; [(h)]Weston *et al.*, 2008; [(i)]Peng *et al.*, 1997; [(j)]Liu *et al.*, 2010; [(k)]Muangprom *et al*., 2005. Gene knockouts (e.g. transposon insertions in *Arabidopsis* DELLAs) are not included in this table.

Species	**Locus**	**Identified mutants**
Barley (*Hordeum vulgare*)	*Sln1*	*sln1a* to *sln1d* [(a)]
Rice (*Oryza sativa*)	*SLR1*	*slr1*; *slr1-1* to *slr1-4* [(b)]
Wheat (*Triticum aestivum*)	*RHT-1*	*Rht-B1b* [(c)]; *Rht-B1c* [(d, e)]; *Rht-D1b* [(c)]; *Rht-D1c* [(d)]
Maize (*Zea mays*)	*Dwarf8*	*d8* [(f)]
Tomato (*Solanum lycopersicum*)	*LeGAI*	*pro* [(g)]
Pea (*Pisum sativum*)	*LA*; *CRY*	*la*; *cry-s; cry-c* [(h)]
Arabidopsis (Arabidopsis thaliana)	*GAI*	*gai* [(i)]
Brassica *(Brassica napus/rapa)*	BnRGA	*Bnrga-ds* [(j)]; *Brrga1-d* [(k)]

DELLA domain mutants

DELLA domain mutants have been characterised in both dicot and cereal species. The dwarf *Arabidopsis* mutant named *ga insensitive* (gai) by Koorneef *et al.* (1985) contains a 51 bp deletion in the 5' region of the *GAI* ORF, resulting in a protein lacking 17 aa including the

DELLA motif. As the mutant lacks the N-terminal region required for GA-GID1 recognition, GA-insensitivity is conferred (Peng *et al.*, 1997; Willige *et al.*, 2007). Similar GA insensitivity has been observed in the agronomically important wheat *Rht* mutants, *Rht-B1b* and *Rht-D1b* (also known as Rht1 and Rht2, respectively). These alleles contain substitution mutations that produce premature stop codons within the DELLA domain (Peng *et al.*, 1999). The severely dwarfed mutant (*RhtB1c*, or Rht3*)* contains an insertion which disrupts the DELLA domain (Pearce *et al*., 2011; Wu *et al*., 2011), and severe dwarf Rht10 (*RhtD1c*) results from over-expression of the Rht-D1b protein (Pearce *et al*., 2011). In barley the dwarf *sln1b* allele results from a frameshift mutation at position 278, affecting the protein from amino residue 93 (Thr, ACC to A-C) and producing an early stop codon at aa position 252. The dwarf *sln1d* allele has a G to A substitution at position 137 (residue 46, Gly, GGG to GAG) causing a Gly to Glu change in the DELLA region (^{39}DELLAALG^{46} → ^{39}DELLAALE^{46}; Figure 3.2).

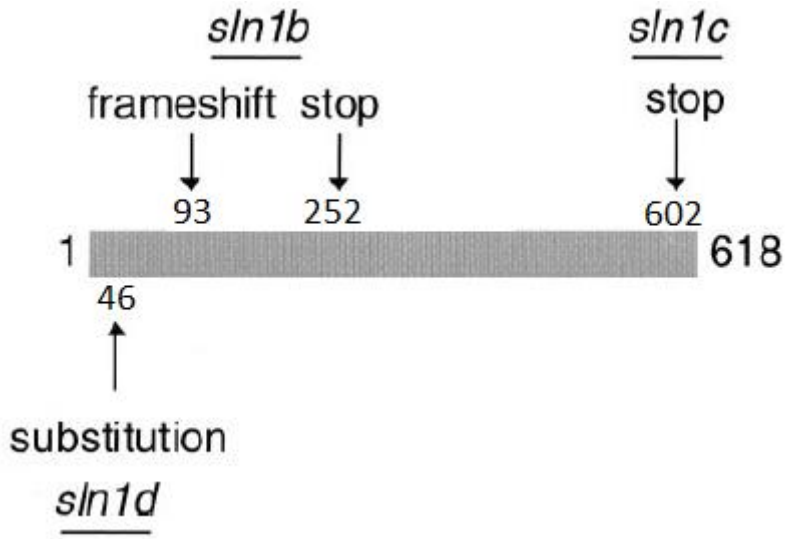


Figure 3.2 Mutants in SLN1 (adapted from Chandler *et al*., 2002). The ORF of cv Himalaya wild-type is 618 aa in length. Slender mutants: *sln1b* has a frameshift mutation in aa residue 93 (Thr, ACC to A-C), resulting in an early stop codon at residue 252, and *sln1c* has a G to A substitution in aa residue 602 (Trp, TGG to TGA), resulting in an early termination codon. Dominant dwarf: *sln1d* has a G to A substitution in aa residue 46 (Gly, GGG to GAG), causing a Gly to Glu change in the DELLA region, ^{39}DELLAALG^{46} → ^{39}DELLAALE^{46}.

Further mutant alleles have been characterised in maize (*Zea mays*) and *Brassica* (*Brassica napus*/*rapa*) species. The *d8* mutant of maize possesses a dominant dwarf phenotype due to a single aa insertion in the TVHYNP motif (Cassani *et al.*, 2009). Previously characterised *d8* mutants with mutations in the DELLA motif exhibited a more severe dwarf phenotype than that of the TVHYNP motif mutant (Cassani *et al.*, 2009). In *Brassica napus* the semi-dwarf

mutant, *ds-1*, results from the *Bnrga-ds* mutant allele that contains a missense mutation in the TVHYNP motif (TVHYNP → TVHYNL; Liu *et al*., 2010). The authors suggested that the conserved proline participates in the DELLA-GID1 interaction. Itoh *et al.* (2002) produced a series of transgenic DELLA domain mutants in rice through the deletions to the DELLA motif, DELLA-TVHYNP inter-motif space, TVHYNP motif, polyS/T/V region, and the LZ region, with deletion in each region resulting in a dwarf phenotype mutant.

GRAS domain mutants

Fewer GRAS domain mutants have been characterised compared with DELLA domain mutants due to the recessive nature of GRAS mutants. The *slr1-1* allele of rice contains a single nt deletion at position 867 (Leu, CTC to –TC), affecting the protein from aa 289 (within the putative NLS region), while the proteins of the *slr1-2*, *slr1-3* and *slr1-4* mutants contain premature stop codons at aa positions 561, 609 and 620 respectively, close to the C-terminus of the protein. The nucleotide substitution in *slr1-4* occurs just 16 nucleotides upstream of the stop codon (Ikeda *et al.*, 2001). The mutations in *slr1-4* result in slender tall mutant phenotypes, indicating that several aa at the C-terminal are essential for DELLA-mediated growth repression (Ikeda *et al.*, 2001; Itoh *et al.*, 2002). A similar mutation has been characterised in barley (*sln1c*) that has a substitution at nt position 1806, affecting aa 602 (Trp, TGG to TGA), producing an early stop codon resulting in the loss of 17 C-terminal amino acids resulting in a slender phenotype (Chandler *et al.*, 2002; Figure 3.2). The *procera* (*pro*) mutant of tomato has a slender phenotype resulting from a point mutation in the VHIID motif (VHVID to VHEID; Bassel *et al.*, 2008). Pea (*Pisum sativum*) has two DELLA proteins (LA and CRY) and mutations have been identified in each; the *la* mutant allele arises from an insertion of 190 bp at aa position 85, downstream of the TVHYNP motif. Two *cry* mutant alleles (*cry-s* and *cry-c)* have also been characterised, *cry-s* arises from a frameshift deletion at aa position 152, whilst the *cry-c* mutant arises from a nt substitution (G→A) at nt 583 causing a change from Gly to Gln at aa position 163 (Weston *et al.*, 2008). Thus, the *la* and *cry-s* alleles encode non-functional proteins and cause plants to have a slender phenotype whereas the ability of the protein encoded by *cry-c* to inhibit growth is not abolished and the plants are therefore less tall. As described, mutations in the GRAS domain normally result in slender phenotype mutants. A notable exception has been observed in the dwarfed *Brassica rapa* mutant *Brrga1-d*, in which the protein contains a mutation of the conserved aa residue (Q→R) at position 328 in the VHIID motif. The repressor function of the Brrga1-d is maintained, however, Brrga1-d does not interact with the *Arabidopsis* SLY1 (F

box) protein that is required for DELLA degradation, suggesting the mutated protein maintains dwarfism by preventing the interaction needed for DELLA degradation (Muangprom *et al*., 2005).

3.3 Materials and methods

3.3.1 Selection of barley mutant varieties

Mutants of three distinct barley cultivars (Herta, H930-36, Triumph) were selected based on phenotype observations suggesting the presence of mutations in genes involved in the GA-DELLA signal transduction pathway. Unfortunately labelling of the seed was unclear, requiring the molecular characterisation of the DELLA genes in these lines prior to use in further studies. Preliminary molecular analysis at the JIC suggested that the mutation in cv H930-36 was present in *Sln1* (Dr. X. Fu, personal communication), and the Herta seed (labelled only as "Herta sln1") was likely to be the *sln1-1* allele described in Fu *et al*. (2002), derived by diethyl sulphate treatment of cv Herta seed (Foster, 1977). For clarity it has therefore been designated as such in the current study. The three mutants selected for characterisation were therefore *dwf2* (cv H930-36), *sln1-1* (cv Herta), and Triumph *γ-1* (cv Triumph). An additional mutant, designated *dwf2-1*, was observed during bulking of cv H930-36 seed (See Section 2.1.1), and was subsequently investigated.

The *dwf2* mutant (cv H930-36) originates from seed collected from a plant grown by Dr. D.E. Falk (University of Guelph, Ontario), which spontaneously exhibited dwarf characteristics following tissue culture of the wild-type parent (Dwf2; Falk, 1994). In the current study, of over 100 seeds labelled as homozygous for the dwarf mutation that successfully germinated, all but one produced dwarf plants. The single non-dwarf plant that exhibited chimeric characteristics was termed *dwf2-1*, and had dwarf and wild-type height tillers. Plants were grown from seed taken from the dwarf tillers and wild-type tillers in order to determine the stability of, and the molecular basis for, this phenotype.

The Triumph *γ*-1 mutant used in this study was derived from a *γ*-irradiated population of cv Triumph barley (Laurie, D.A. and Byrne, M., JIC, unpublished data). The plant was initially selected (in the field, by Dr. D. Laurie, JIC) based on its late flowering phenotype and subsequently five plants (*γ*-1 - *γ*-5) were grown in the glasshouse from the collected seed. These plants were tall and similar to (but less severe than) the slender tall *Sln1* mutant

phenotype (cv Himalaya) described in the literature (Chandler *et al.*, 2002). The plants were self-pollinated, and the resulting (M_2 generation) seed collected. Insufficient time was available for this seed to be back-crossed before use.

The growth and development of five cv Himalaya mutants was observed to determine how independent mutations in two components of the GA signal transduction pathway, SLN1 and GSE1, affect plant phenotype under the conditions used in the current study. The mutations had previously been molecularly characterised (Chandler *et al.*, 2002, 2008). *Gse1* encodes the GA receptor, GSE1, orthologous to GID1 of *Arabidopsis*. The characterised *DELLA* mutants *sln1c* and *sln1d,* and *Gse1* mutants *gse1a*, *gse1j*, and *gse1n* originated from sodium azide treated seed, and were identified based on response to GA (Chandler *et al.*, 2008). These mutants provided a basis for comparison of phenotypes with the new and uncharacterised mutants under the defined conditions used in the current study. The *Gse1* mutants are further described in Section 1.4.6.

3.3.2 Plant growth and phenotype observation

Seed phenotype

DELLA proteins are reported to be important in regulating seed germination, but their effect on seed morphology has received little attention. In order to assess the effect of mutant SLN1 on seed development the dry seed were assessed visually and seed width, length and thousand grain weight (TGW) measured using the MARVIN seed analyser (GTA Sensorik GmbH, Neubrandenburg, Germany) and Excel (Microsoft) software according to the manufacturer's instructions. The *sln1d* and *gse1a* (cv Himalaya) and Triumph *γ-1* (cv Triumph) mutants and their corresponding wild-types were analysed. MARVIN analyses provided single mean values for seed length and width, as well as range values. TGW analysis provided a single value extrapolated from the mean weight of tested seed.

Plant phenotype

For experiments carried out in soil, seeds were stratified as described in Section 2.1.3.1, and grown in Petri dishes for seven days in CER conditions (20 °C, 16 h light period; 8 h dark period). Seed germination rate was assessed at this point, and germinating seeds were planted and grown in Barley Mix compost (see Section 2.1.3.2). Three days after planting, plants were transferred from the CER to the glasshouse (22 °C, 16 h light period; 15 °C, 8 h

dark period). Seed segregation was assessed based on the phenotype of germinated seed during early plantlet development (2 days to 1 wk after germination). To allow comparison between experiments, plant growth conditions were kept as similar as possible. Mutants were always compared with their respective wild-type genotypes of the same age. Lines under comparison were grown together when possible. Phenotypes were observed on a bi-weekly basis over a four month period. Wild-type to mutant phenotype segregation ratio was calculated during the first month of plant growth, once seedlings were developed enough to clearly differentiate between phenotypes.

For hydroponics based experiments (where root growth could be analysed), seeds were stratified as described in Section 2.1.3.1, and grown in modified 0.5 x Hoagland's solution (Appendix 1.2.), as described in Section 2.1.3.3. Plants were grown for ten days, before root and shoot lengths and dry mass was measured. Mean mass values were calculated for the pooled samples of each genotype.

3.3.3 Bioinformatic analysis of *Sln1* sequence

Identification of the promoter region

The transcription start site (TSS) of the *Sln1* gene has not been identified. Since changes within the promoter region can affect gene expression, *in silico* identification of this region was attempted. The *Sln1* promoter sites were predicted using two independent promoter recognition methods. The Promoter 2.0 software (http://www.cbs.dtu.dk/services/Promoter/) predicts promoter sites based on sequence pattern and motif separation (Knudsen, 1999). The GC-compositional strand bias software (Fujimori *et al.*, 2005) predicts promoter sites based on GC composition, as transcription start sites (TSS) in plant promoters have a CG-compositional strand bias (GC skew), where C is more frequently observed in the transcribed strand, and G more frequently observed in the non-transcribed strand at the TSS (Tatarinova *et al.*, 2003; Fujimori *et al.*, 2005).

***Sln1* gene composition and SLN1 protein structure**

Conserved motifs of *Sln1* were identified by aligning DELLA protein sequence using ClustalW (http://www.ebi.ac.uk). The position of cultivar- and mutant-specific polymorphisms identified in this study could thus be determined relative to the conserved motifs. The GC composition of Morex WT *Sln1* ORF sequence was analysed using Vector NTI Suite 10

(Invitrogen) software and the *Sln1* mRNA secondary structure was predicted using the MFOLD program (http://mfold.ma.albany.edu; Zucker *et al.*, 1999). The Morex wild-type *Sln1* ORF sequence, obtained from the NCBI nucleotide database (AF460219; http://www.ncbi.nim.nih.gov), was used in analyses.

3.3.4 Nucleic acid extraction

DNA extraction

DNA was extracted from young leaf tissue of plants growing in soil, and either extracted immediately or flash-frozen in liquid nitrogen and stored at -80 °C for future extraction. DNA was extracted using the modified CTAB method and agarose gel purified as described in Sections 2.1.4.1 and 2.3.1.1. In addition, young leaf material of Himalaya *sln1d* was provided by Dr. M. Boulton (JIC, Norwich) from a stock stored at -80 °C. Additional Himalaya wild-type DNA was provided by Dr. A. Korolev (JIC, Norwich).

RNA extraction

Plants used for *Sln1* expression analysis were stratified as described in Section 2.1.3.1, then grown in Petri dishes under CER conditions (16 h photoperiod, 20 °C). For RNA extraction, either whole plant or second leaf material was taken from plants at the 2-3 leaf stage. RNA was extracted and purified as described in Section 2.3.1.2. Quantification of *Sln1* mRNA levels using qRT-PCR was conducted as described in Section 2.4.

3.3.5 Amplification and sequencing of *Sln1*

PCR amplification of the *Sln1* gene was conducted on genomic DNA samples using existing primer stocks left by Dr. X. Fu, and primers that were designed based on cv Morex *Sln1* sequence (GenBank accession AF460219). Primer pairs were designed to amplify the *Sln1* ORF, (data are not presented for primer pairs that failed to do this). Initially only partial *Sln1* regions could be amplified, therefore optimisation of the amplification conditions was required, resulting in successful amplification using the primer set shown in Table 3.3 and the following cycling conditions: 94 °C for 3 min; then 10 cycles of 95 °C for 30 s, 57 °C for 1 min (decreasing by 0.5 °C per cycle) and 68 °C for 3 min. This was followed by 30 cycles of 95 °C for 30 s, 52 °C for 15 s, 68 °C for 3 min, followed by a final extension of 68 °C for 30 min. PCR was conducted using proofreading polymerase (Platinum® *Taq* DNA Polymerase High

Fidelity or Platinum® *Pfx* DNA Polymerase High Fidelity (Invitrogen)). The expected product was 2423 bp, consisting of 1857 bp ORF, 274 bp 5' UTR and 292 bp of 3' UTR sequence.

Table 3.3 Primers used to amplify the entire *Sln1* ORF. [(a)]'F' denotes forward, and 'R' reverse primer orientation [(b)]Primer binding site coordinates are presented relative to the first nucleotide of the *Sln1* initiation codon ('A' = co-ordinate 1), '-' refers to the number of bases upstream of this point, '+' refers to the number of bases beyond the final nucleotide of the translational stop codon of *Sln1*. The reference sequence used was *Sln1* of Morex (GenBank accession AF460219).

Orientation [(a)]	Primer binding site [(b)]	Sequence (5'-3')
F	-274 to -255	CACACCACTATGCCCAGATG
R	+273 to +292	ATGGTGAACTGGGAACGAAG

The amplified product was cloned into pCR®4-TOPO® vector using One Shot® TOP10 Chemically Competent *E. coli* kit (Invitrogen, Carlsbad, CA, USA), and homemade DH5α chemically competent cells as described in Section 2.2.3, or sequenced directly after agarose gel purification of the PCR product. Sequencing reactions were performed as described in Section 2.3.4 using the primers shown in Table 3.4, with additional forward and reverse M13 primers, M13F-20 (GTAAAACGACGGCCAGT), and M13R (CAGGAAACAGCTATGACC) being used to sequence the cloned *Sln1* product.

Table 3.4 Primers used in *Sln1* ORF sequencing. [(a)]'F' denotes forward, and 'R' reverse primer orientation; [(b)]Primer binding site coordinates are presented relative to the first nucleotide of the *Sln1* initiation codon ('A' = co-ordinate 1). The reference sequence used was *Sln1* of Morex (GenBank accession AF460219).

Orientation [(a)]	Primer binding site [(b)]	Sequence (5'-3')
F	118 to 137	GAGCTGCTGGCGGCGCTCGG
R	322 to 303	CGTTGAGCTCGGACAGCATG
F	358 to 376	CTCAACGCCTCCACCTCTT
F	803 to 822	GCAAGGTCGCCGCCTACTTC
F	1230 to 1249	CCTGGAGCCGTTCATGCTGC
F	1444 to 1464	TTCACCGAGTCCCTGCACTAC
R	1494 to 1476	GCCCTCGAGAGAATCGAAC
F	1635 to 1655	AGAGCGGCACGAGACACTGGG

3.3.6 Analysis of *Sln1* transcript levels in the *γ-1* (cv Triumph) mutant

To determine whether a difference in *Sln1* expression would account for the slender-like phenotype exhibited by the Triumph γ-1 mutant, qRT-PCR was carried out using second leaf and whole seedling material collected from WT and *γ-1* mutant plants and the methods described in Section 2.4. Plants were grown under CER conditions (16 h photoperiod, 20 °C) and sampled at the three leaf stage. In each case, three biological repeats were used (eight plants per biological repeat) for each genotype, with two technical repeats used for each biological repeat.

3.4 Results

3.4.1 Phenotype analysis

3.4.1.1 Seed size, germination and segregation

Seed size

Visual assessment of seed phenotype suggested the seed length and width of some *Sln1* mutants differed respective to their corresponding wild-types. These mutants (*sln1d*, *gse1a*, γ-1) were analysed further, using a minimum of 580 seeds per genotype. The *sln1c* mutant seed could not be analysed, as this mutant is maintained in a heterozygous population, as homozygous *sln1c* mutants do not produce seed. As no significant differences in seed length and width were observed between genotypes in the Himalaya and Triumph backgrounds (but differences in range were observed), (Figure 3.3), TGW weight anlaysis was used to determine whether differences in seed phenotype were observable in seed weight. The TGW data correlated with decreased seed length and width data in mutant lines, as all three mutants had decreased TGW compared to their respective wild-type seed (Figure 3.4). The calculated value for TGW for the *gse1a* mutant seed was lower than that of the *sln1d* mutant.

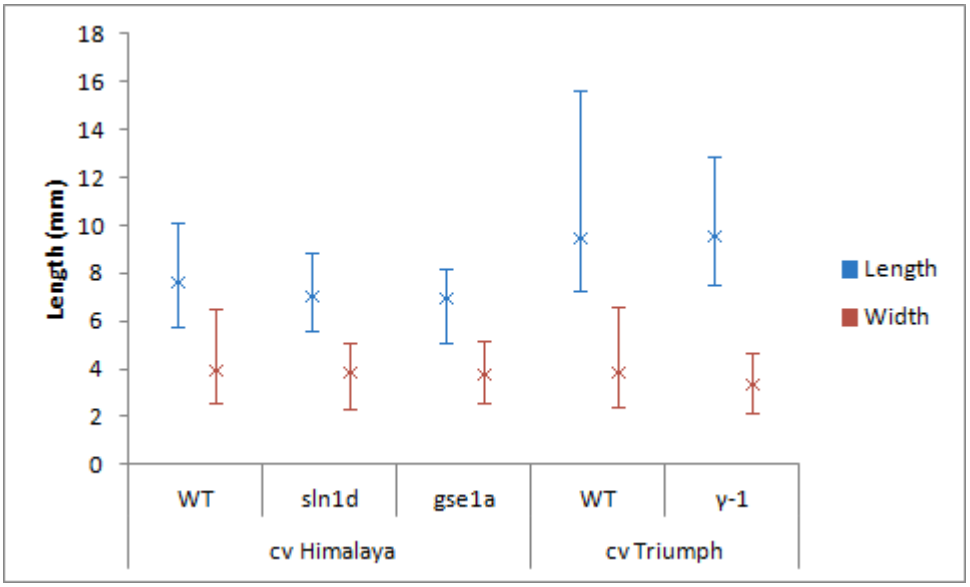


Figure 3.3 Length and width of wild-type and mutant seed of Himalaya and Triumph cultivars. Mean values are shown, with bars denoting range.

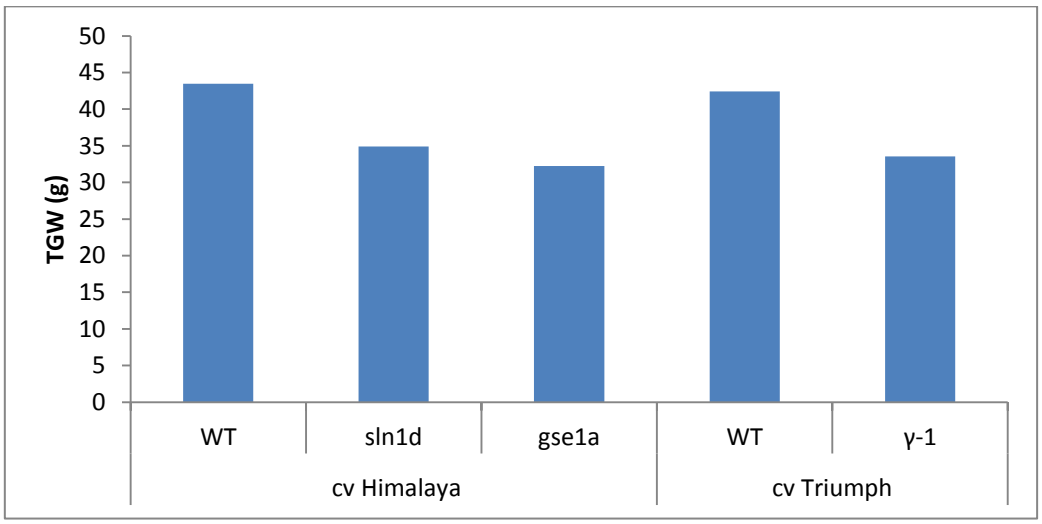


Figure 3.4 Thousand grain weight of wild-type and mutant seed of Himalaya and Triumph cultivars.

Seed germination and segregation

Germination and genetic segregation data were generated based on the observation of over 200 seeds per genotype (Table 3.5). In the Himalaya background, the germination rate of *sln1d* mutant seed (49.4%) was lower than that of the other cv Himalaya genotypes (WT, *gse1a*, *gse1j*, *gse1n*) which varied between 68.7 – 87.5%. Germination rates were similar for wild-type and mutant genotypes in both the H930-36 (86.3 – 92%) and Herta (74.7 – 82.4%) backgrounds. Plants derived from seed of plants containing the putative SLN1 stabilising alleles (*sln1d*, *gse1a*, *gse1j*, *gse1n* and *dwf2*) all showed the expected dwarf phenotypes

confirming the presence of dominant GoF alleles, with the exception of a single plant derived from *dwf2* seed which initially was dwarfed but subsequently gave rise to tillers of height similar to the wild-type plants. Conversely, recessive segregation was observed in *sln1c* (cv Himalaya) and *sln1-1* (cv Herta), as previously reported for *Sln1* LoF mutants. The number of germinated seed in the Triumph background was too low to sufficiently assess germination rate, however all *γ-1* plants exhibited the mutant phenotype. Throughout this study, for all backgrounds only wild-type phenotypes developed from wild-type seed.

Seed was separately collected from each of the wild-type and dwarf height tillers of the *dwf2-1*, putatative chimeric plant. Although the number of germinated seed was too low to accurately assess the germination rate of seed from this plant, seed grown from wild-type height tillers developed into seedlings having a wild-type phenotype, whilst seed from the dwarf tillers produced only dwarf plants (Table 3.5).

Table 3.5 Germination and segregation ratio of mutant and wild-type lines. Homozygous seed was sown for all wild-type and GoF mutant lines, heterozygous seed for *sln1c* and *sln1-1*. [(a)]One *dwf2* plant developed a chimeric phenotype; initially a dwarf phenotype was seen but wild-type height tillers emerged as the plant developed. Segregation was assessed from the seedling phenotype.

Cultivar	Genotype	Germination rate (%)	WT to mutant segregation ratio
Himalaya	WT	68.7	1 : 0
	sln1c	83.2	1 : 0.18
	sln1d	49.4	0 : 1
	gse1a	80.8	0 : 1
	gse1j	84.4	0 : 1
	gse1n	87.5	0 : 1
H930-36	WT	86.3	1 : 0
	dwf2	92	0 : 1 [(a)]
Herta	WT	82.4	1 : 0
	sln1-1	74.7	1 : 0.13

3.4.1.2 Plant phenotype analysis

cv Himalaya mutants

The *sln1c* and *sln1d* mutants of cv Himalaya developed as described by Chandler *et al.* (2002), (Figure 3.5). The dwarf mutant, *sln1d*, showed reduced height and short, wide dark green leaves, whilst homozygous seed of the slender *sln1c* mutant exhibited a tall spindly phenotype resulting from increased internode length, with narrow chlorotic leaves and anthocyanin accumulation at the lower internodes. The *sln1c* mutant exhibited an elongated coleoptilar node phenotype and was unable to maintain an upright stature without staking, and showed reduced tillering compared to the wild-type and *sln1d* mutant, with no secondary tillers emerging at soil level. All *sln1c* plants were sterile. Heterozygous plants were phenotypically indistinguishable from wild-type plants.

Figure 3.5 The phenotype of *Sln1* mutants (cv Himalaya). The *Sln1* dwarf mutant (*sln1d*, centre) and slender mutant (*sln1c*, right), are shown next to a wild-type plant (WT, left). Plants are shown at 9 wks after germination.

cv Herta

The *sln1-1* mutant of cv Herta developed a slender phenotype similar to that of the *sln1c* mutant (Figure 3.6). As with the *sln1c* mutant, *sln1-1* mutant had narrow leaves and

pronounced anthocyanin accumulation at the internodes. The slender phenotype was particularly pronounced at the early stages of plant development (Figure 3.6b); at later stages, the Herta mutant produced tillers from soil level and although it was unable to maintain an upright stature unaided, it was a more robust plant than the *sln1c* mutant (compare Figures 3.5 and 3.6a). As with the cv Himalaya *sln1c* slender mutant, the *sln1-1* mutant was sterile.

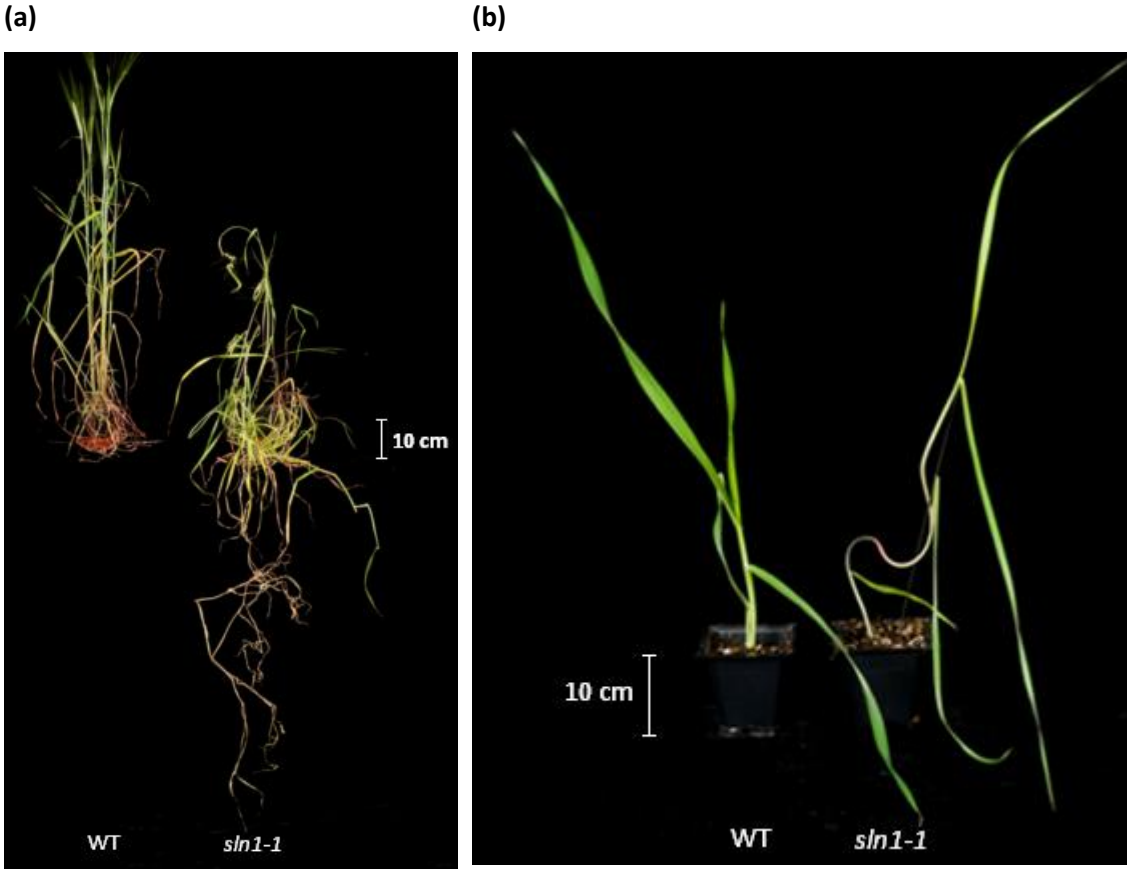


Figure 3.6 The phenotype of cv Herta wild-type and *sln1-1* mutant plants. (a) WT (left) and the *sln1-1* mutant (right) shown at 7 wks after germination, (b) WT (left) and the seedling phenotype of the *sln1-1* slender phenotype mutant (right) shown at 2 wks after germination.

cv H930-36

The *dwf2* mutant exhibited a dwarf phenotype and wide, dark green leaves. The single *dwf2-1* plant initially developed as a dwarf phenotype, however, wild-type height tillers emerged as the plant developed (Figure 3.7).

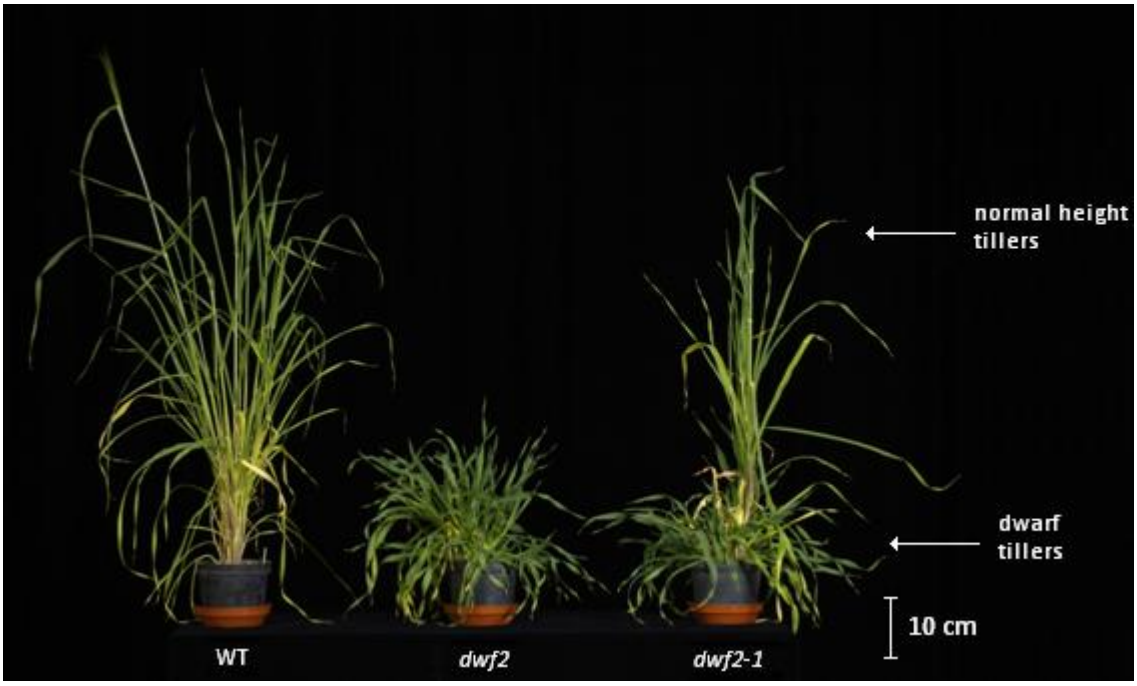


Figure 3.7 Phenotype of wild-type and *dwf2* plants, and the chimeric plant H930-36 *dwf2-1*. The wild-type and *dwf2* mutant (left and centre, respectively are shown for comparison with the chimeric plant H930-36 *dwf2-1* (right) that produced dwarf and wild-type height tillers. Plants are shown at 8 wks after germination.

cv Triumph

Plants grown from M3 seed collected from the four original M2 plants (Triumph *γ-1*, *-2*, *-3*, *-5*) all developed a slender-like phenotype, characterised by rapid growth, tall stature, and pale green leaves. Further slender-like characteristics including anthocyanin accumulation at nodes, and an elongated coleoptilar node similar to that observed in the *sln1c* mutant, were observed in the *γ* mutants (Figure 3.8). In contrast, tiller production was only slightly less than seen for wild-type plants. As all the Triumph *γ* lines had similar phenotype only the *γ-1* line was used for further study.

Figure 3.8 The phenotype of cv Triumph and the Triumph *γ-1* plants. (a) Slender-like phenotype *γ-1* mutant (right), compared to the corresponding wild-type (WT, left). (b) The elongated lower internodes and anthocyanin accumulation typical of the *γ-1* mutant plants. Plants are shown at 9 wks after germination.

3.4.1.3 Root and shoot growth

Analysis of root and shoot growth was conducted on seedlings in the Himalaya background, grown under hydroponic conditions for ten days. Results are based on a minimum of two experiments, each consisting of between 4 - 14 seedlings of each genotype. With the exception of the *sln1d* which had significantly shorter roots than the wild-type, root lengths between the wild-type and mutant genotypes were similar. Differences were more pronounced in regards to shoot length. The mean shoot length of the SLN1 LoF mutant, *sln1c*, was significantly greater than that of the wild-type. Conversely, shoot length in the characterised (*sln1d*) and putative (*gse1a*, *gse1j*, *gse1n*) SLN1 stabilising mutants, was lower than that of the wild-type (Figure 3.9). Root dry mass was lower for the mutant genotypes compared to the wild-type, with the exception of the *gse1n* mutant for which root dry mass

was greater than that of the wild-type. With the exception of *gse1n*, the mutant lines also had decreased shoot mass compared to the wild-type seedlings, although the *sln1c* seedlings had a greater shoot mass than the remaining mutants (Figure 3.10).

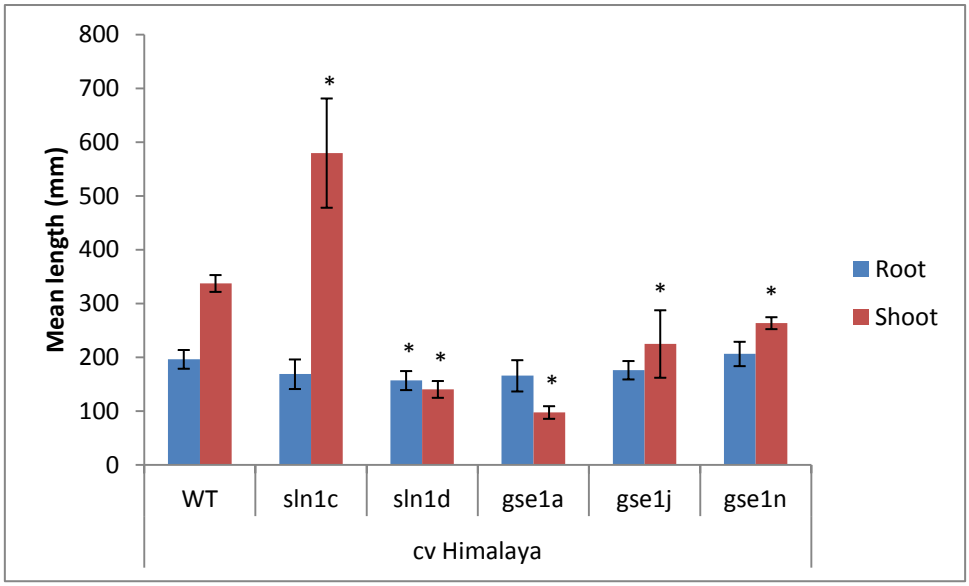


Figure 3.9 Root and shoot lengths of wild-type and mutant seedlings (cv Himalaya). Mean shoot lengths are shown; error bars denote standard deviation, asterisks denote significant difference compared to the WT.

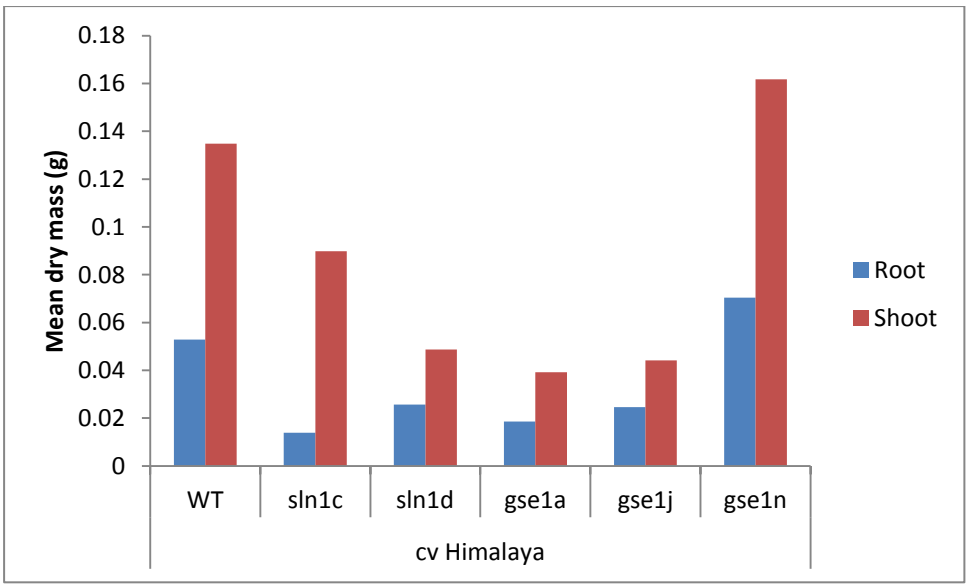


Figure 3.10 Root and shoot dry mass of WT and mutant seedlings (cv Himalaya). Values were calculated from the pooled root and shoot masses for each genotype from each experiment.

3.4.2 Sequence analysis of wild-type and mutant *Sln1* alleles

3.4.2.1 Bioinformatic analysis

Promoter identification

The identification of putative promoter regions was conducted using Promoter 2.0 and CG-compositional strand bias software as described in Section 3.3.3. Promoter 2.0 prediction software identified a transcription start site (TSS) 279 to 249 bp (31 bp in length) upstream, and CG-compositional strand bias software identified a TSS 171 to 151 bp upstream of the ATG start codon of the *Sln1* ORF.

GC composition

In order to determine whether the difficulty in amplifying *Sln1* was because of the primary nucleotide sequence composition and extensive secondary structure, the GC composition of the Morex WT *Sln1* ORF was assessed as described in Section 3.3.3. Analysis showed that the ORF has a GC composition of 70.8 % (Table 3.6).

Table 3.6 Nucleotide composition of the Morex *Sln1* ORF. [(a)]Number of nucleotides in the ORF (ORF = 1857 nt); [(b)]Percentage composition of the ORF per base; [(c)]AT and CG composition of the ORF as a percentage.

Base	No. of nucleotides [(a)]	Base composition of ORF (%) [(b)]	AT/GC composition (%) [(c)]
A	262	14.1	29.2
T	281	15.1	
C	685	36.9	70.8
G	629	33.9	
Total	1857	100	100

***Sln1* mRNA secondary structure**

Sln1 mRNA sequence was analysed using the MFOLD programme as described in Section 3.3.3, in order to identify the extent of secondary structure. The mRNA was predicted to be highly folded with extensive double stranded regions. A number of stem loop structures are also visible (Figure 3.11).

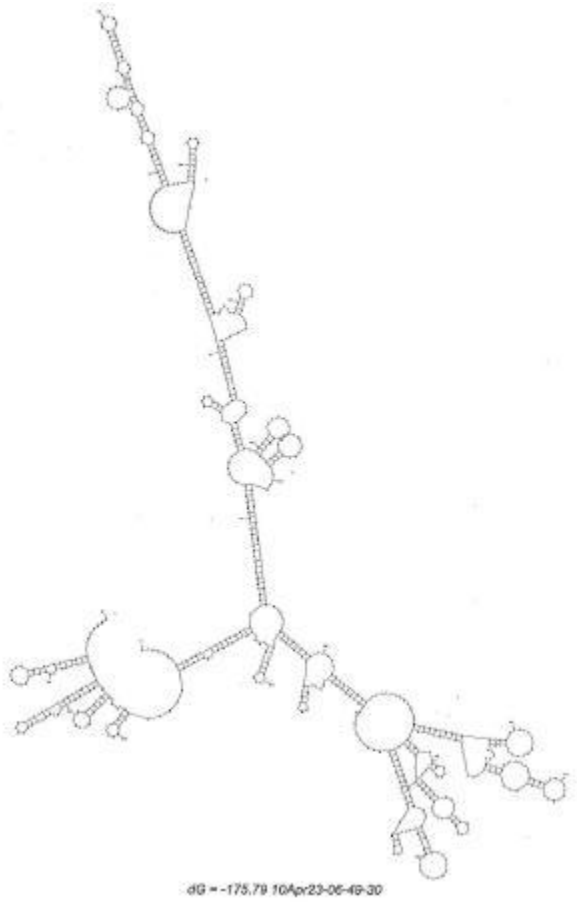


Figure 3.11 The secondary structure of the *Sln1* mRNA.

Identification of conserved motifs

Analysis of the Morex SLN1 sequence using ClustalW software (see Section 3.3.3) identified key motifs common to DELLA proteins, which are shown in Figure 3.12. This analysis was conducted in order to determine if mutations in the characterised mutants were within conserved domains.

[1]MKREYQDGGGSGGGGDEMGSSRDKMMVSSSEAGEGEEV**DELLA**ALGYKVRAS
DMADVAQKLEQLEMAMGMGGPAPDDGFATHLATDTVHYNPTDLSSWVESMLSE
LNAPPPPLPPAPPQLNASTSSTVTGGGGYFDLPPSVDSSSSTYALRPIISPPV
APADLSADSVRDPKRMRTGGSSTSSSSSSSSSLGGGAARSSVVEAAPPVAAAA
AAPALPVVVVDTQEAG***IRLVHALLACAEAVQQENLSAAEALVKQIPLLAAS***QG
GAMRKVAAYFGEALARRVFRFRPQPDSSLLDAAFA***DLLHAHFYESCPYLKFAH***
FTANQAILEAFAGCRRVHVVDFGIKQGMQWPALLQALALRPGGPPSFRLTGVG
PPQPDETDALQQVGWKLAQFAHTIRVDFQYRGLVAATLADLEPFMLQPEGEED
PNEEPE***VIAVNSVFEMHRLLAQP***GALEK***VLGTVRAVRPRIVTVVEQEANHNS***G
S***FLDRFTESLHYYSTMFDSLEGGSSGG***PSEVS***SGGAAPAAAAGTDQVMSEVYL***
GRQICNVVACEGTERTERHETLGQWRNRLGNAGFETVHLGSNAYKQASTLLAL
FAGGDGYKVEEKEG***CLTLGW***HTRPLIATSAWRLAAP[618]

Figure 3.12 Conserved motifs within SLN1. Conserved domains are shown in bold, with the DELLA motif shown in red, LHR I in orange, NLS in light green, VHIID in dark green, LHR II in light blue, PFYRE in purple, and SAW in brown. The DELLA domain is shown in regular font, whilst the GRAS domain is in italics. The 5' terminal amino acid is designated as co-ordinate '1', and the 3' terminal amino acid has a co-ordinate of '618'.

3.4.2.2 Sequencing and analysis

Sequencing the *Sln1* ORF

The *Sln1* ORF of the *dwf2* (cv H930-36) putative SLN1 GoF mutant, and γ-1 (cv Triumph) and *sln1-1* (cv Herta) putative SLN1 LoF mutants and all corresponding wild-types were amplified using the conditions described in Section 3.3.5. The *Sln1* ORF of the wild-type tiller of H930-36 *dwf2-1* was also amplified. Multiple bands were observed when the PCR product was analysed electrophoretically (Figure 3.13), however similar bands were observed for all genotypes. The prominent band of the predicted size (2423 bp) was excised and purified as described in Section 2.3.1.1.

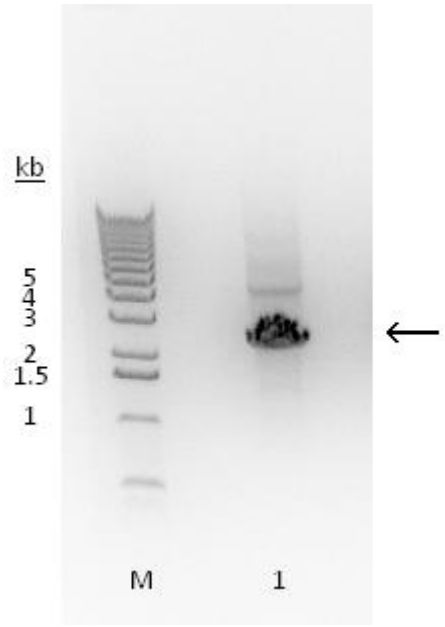


Figure 3.13 Agarose gel electrophoresis of the amplified product of H930-36 *dwf2-1* wild-type tiller *Sln1* ORF. The sample lane (1) was loaded with 2.5 µL of PCR product. Samples were run on a 0.8% agarose gel (TBE buffer) at 100 V. (M) 2 µL Quick-Load® 100 bp DNA ladder (NEB) was used as molecular weight marker.

All *Sln1* ORFs were sequenced (see Section 3.3.5), and nt and aa sequences aligned as described in Section 2.5. Full sequence for each genotype is presented in Appendix 2. Sequencing revealed cultivar specific polymorphisms, and mutations that could account for the altered phenotypes of the cv H930-36 and cv Herta mutants. Comparison of the nt sequence of the *Sln1* ORFs of the wild-type lines showed little cultivar-specific polymorphism. The sequence of H930-36 wild-type was identical to that of the Morex wild-type, whilst the sequence of Himalaya, Triumph and Herta wild-types were identical, with only four single nucleotide polymorphisms (SNPs) identified between their ORFS and that of the Morex wild-type. None of these nt polymorphisms resulted in aa changes, suggesting a low level of diversity between these cultivars (Table 3.7).

Sequencing of the H930-36 *dwf2* mutant revealed a deletion of 9 nt (position 126-134) resulting in the deletion of three amino acids, comprising the final aa (A) of the core DELLA motif, and two aa immediately downstream of the motif (A, L). Four further polymorphisms between the wild-type and the *dwf2* mutant (nt position 90, G→A; nt position 420 and 1074, T→C; nt position 1272, T→G) were identified although all were silent substitutions. Sequencing of clones obtained from the wild-type phenotype tiller of H930-36 *dwf2-1* resulted in heterogeneity at nt position 1155, with two clones containing G (homologous

with wild-type sequence), and two clones containing A at this positions. Direct sequencing of a PCR product was used to clarify the heterogeneity the ORF sequence, and a mixed profile (G or A) was produced at nt position 1155. The G to A substitution at nt position 1155 would result in a premature stop codon at aa position 385 (W → stop) in the LHR II region of the domain, which could result in the protein lacking the 589 C-terminal amino acids. Two further SNPs were identified between H930-36 *dwf2* and H930-36 *dwf2-1* wild-type phenotype tiller, (nt position 90, A→G; nt position 1776, G → A), although these substitutions were silent, producing no change in aa sequence compared to the H930-36 wild-type.

The Herta *sln1-1* mutant contains a SNP at nt position 748 (G → T, in bold Table 3.7), producing a premature stop codon in the LHR I motif of the GRAS domain at aa position 250 (E → stop), resulting in a protein potentially lacking 368 C-terminal amino acids. As the wild-type and *y-1* mutant of cv Triumph exhibited complete homology in *Sln1* sequence, qRT-PCR was used to establish whether altered expression of *Sln1* accounted for differences in phenotype.

Table 3.7 Nucleotide and resulting amino acid polymorphisms in mutant lines relative to wild-type genotypes [(a)]Nucleotide positions are presented relative to the 'A' nucleotide of the *Sln1* initiation codon which is equal to 1; [(b)]amino acid positions are presented relative to the methionine (initiation) amino acid of the SLN1 protein. Nucleotides underlined in bold represent polymorphisms resulting in changes to aa sequence compared to the wild-type of the same background. Full sequence for each genotype is presented in Appendix 2**.**

		nt position [(a)]								**aa position [(b)]**		
Genotype	Phenotype	90	126-134	420	748	1074	1155	1272	1776	43-45	250	385
Morex WT	WT	G	GGCGGCGCT	T	G	T	G	T	G	AAL	E	W
Himalaya WT	WT	G	GGCGGCGCT	C	G	C	G	G	A	AAL	E	W
H930-36 WT	WT	G	GGCGGCGCT	T	G	T	G	T	G	AAL	E	W
H930-36 *dwf2*	Dwarf	A	**---------**	C	G	C	G	G	G	**---**	E	W
H930-36 *dwf2-1*	WT tiller	G	GGCGGCGCT	C	G	C	**G or A**	G	A	AAL	E	**W or Stop**
Triumph WT	WT	G	GGCGGCGCT	C	G	C	G	G	A	AAL	E	W
Triumph *γ-1*	Slender-like	G	GGCGGCGCT	C	G	C	G	G	A	AAL	E	W
Herta WT	WT	G	GGCGGCGCT	C	G	C	G	G	A	AAL	E	W
Herta *sln1-1*	Slender	G	GGCGGCGCT	C	**T**	C	G	G	A	AAL	**Stop**	**NA**

3.4.3 Comparison of *Sln1* transcript levels in Triumph and Triumph γ-1 plants

Sln1 transcript levels were assessed in Triumph WT and *γ-1* whole plant and second leaf tissue as described in Section 3.3.6. *Sln1* transcript levels in WT lines were not significantly different from the *γ-1* mutant in either whole plant or second leaves (Figure 3.14).

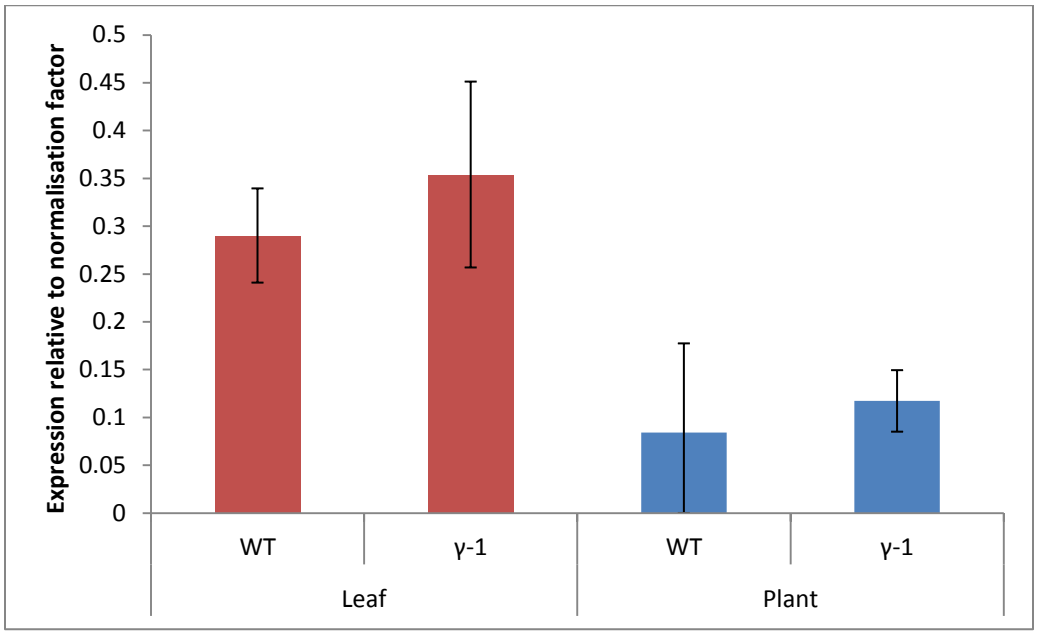


Figure 3.14 *Sln1* expression in second leaf and whole seedlings for wild-type and γ-1 genotypes (cv Triumph). Expression is shown relative to the normalisation factor obtained using two control genes (*α-tubulin*, *GAPDH*). Errors bars show standard error.

3.5 Discussion

Germination rates varied greatly between genotypes, with no apparent link between germination rate and SLN1 function (Table 3.5). The variation between genotypes may result from variability in seed stocks. Seed was germinated from multiple bags of seed, each of which was subjected to differences in harvesting time, seed preparation, and storage time. The seed segregation ratios observed in this study corresponds with those reported for DELLA mutants in other species, with LoF and GoF mutants exhibiting recessive and dominant segregation respectively (see Section 3.2.3). Interestingly, the segregation ratios for the *Sln1* LoF mutants

(*sln1c*, cv Himalaya; *sln1-1*, cv Herta; Table 3.5) was lower than the 1 : 0.25 expected for recessive mutants, suggesting seeds homozygous for the mutant alleles may exhibit reduced germination compared to *WT*/*mutant* heterozygotes or *WT*/*WT* homozygotes. Whilst the germination rate of the *sln1c* mutant was not reported by Chandler *et al*. (2002), it was reported that α-amalase production in endosperm half-grains was greater in *sln1c* mutant seed compared to the wild-type, suggesting differences in seed composition may account for the reduced germination (and subsequent segregation ratios) for LoF mutant homozygotes. Analysis of seed width and length in the *Sln1* GoF and *Gse1* mutants suggests the stabilisation of SLN1 does not affect seed size, although further study is required to determine the effect on seed composition and total yield. In wheat, the dwarf DELLA GoF mutants (*RhtB-B1b*, *Rht-D1b* and *Rht-B1C* (also termed *Rht3*, Börner *et al.*, 1996)) were shown to provide a greater grain yield than taller varieties as a result of the diversion of assimilate from the stem to the developing ear. A higher grain number per ear was also observed in the *Rht* dwarf mutants compared to the wild-type (Flintham *et al.*, 1997), although at the cost of reduced mean weight (Flintham *et al.*, 1997), a trend that the TGW data presented in this study supports (Figure 3.4).

The *sln1-1* (cv Herta) and *dwf2* (cv H930-36) mutants were phenotypically similar to the *sln1c* and *sln1d* mutants (cv Himalaya) first characterised by Chandler *et al.*, 2002, and the observed slender and dwarf phenotypes were consistent with those typical of GRAS and DELLA domain mutants (see Section 3.2.3). Root and shoot dry mass data was only collected from mutants in the Himalaya background, meaning direct numerical comparisons between the novel mutants, *sln1-1* (cv Herta) and *dwf2* (cv H930-36) and their corresponding wild-types could not be made. Observations of root and shoot development suggests the stabilisation of SLN1 can reduce both root and shoot length (*sln1d*; Figure 3.9). Conversely, loss of SLN1 function had little apparent effect on root length, but resulted in increased shoot length compared to the wild-type, *Sln1* GoF and Gse1 mutants (*sln1c*; Figure 3.9). With the exception of the *gse1n* mutant, the stabilisation of SLN1 appeared to reduce overall plant mass, which is consistent with the role of DELLA as an inhibitor of plant growth (Harberd *et al.*, 2003). Reduced overall plant mass was also observed in the *sln1c* mutant (Figure 3.10). Although no notable differences were observed between *sln1c* and wild-type root length, the narrow leaves and slender stems of the *sln1c* plants could account for the reduced shoot biomass compared to the wild-type (Figure 3.10). The growth of the Gse1 mutants in this study was not consistent with the growth observed by

Chandler *et al.*, 2008, who report the *gse1n* mutant had a more dwarfed phenotype than the *gse1a* and *gse1j* mutants. In this study, the *gse1n* mutant showed greater mean shoot length compared to *gse1a* (Figure 3.9), with mean root and shoot mass greater for the *gse1n* mutant than for the *gse1a* and *gse1j* mutants (Figure 3.10). The length and mass results presented in this chapter provided the basis for subsequent studies investigating the effect of salt and heat stress on seedling growth and development.

Bioinformatic analysis was performed on SLN1 sequence to identify conserved regions and motifs so that the impact of mutations affecting these areas could be assessed. Although bioinformatic analysis was conducted on wild-type sequence derived from the Morex background, analysis of the *Sln1* ORF and mRNA secondary structure is widely applicable to all the genotypes sequenced in this study, due to the high level of *Sln1* ORF sequence homology between the genotypes. Despite the presence of several nt differences between the wild-type genotypes of the five analysed cultivars, the high degree of conservation in aa sequence suggests conservation of aa sequence is integral to SLN1 function either through the maintenance of key motifs or regions, or its importance in protein folding. That said, the potential importance of specific nt for maintaining RNA processing stability in RNA cannot be discounted. *Sln1* ORF analysis accounts for the difficulties experienced in this study in characterising *Sln1* at the molecular level. The high GC content of the *Sln1* ORF (approximately 70%; Table 3.6), commonly results in a high level of self-complementary strand binding. Fold analysis predicted an extensively closed structure, with a high degree of folding and stem loop structures; features that impair polymerase binding and activity. Furthermore, PCR amplification of GC rich sequence requires the use of high annealing temperatures, and often restricts the design of primer sequences (to prevent primer secondary structure or primer dimer formation) thereby limiting flexibility in PCR programme design. Although secondary structure can be relaxed using chemical means (e.g. the addition of glycerol or DMSO to the PCR mix; Frackman *et al.,* 1998) this can decrease the fidelity of the PCR. Problems with molecular analysis of *Sln1* were also experienced during sequencing, from which only relatively short reads could be achieved. To overcome these problems, PCR conditions were continuously altered and tested. Changes were made to PCR reagent concentrations, PCR components, primer pairs, and cycling conditions. The key to overcoming the amplification problems came with the implementation of a 'touchdown' PCR programme, designed to amplify with less stringency during early cycles,

followed by high levels of specificity during final cycles. Difficulties with amplification of barley and wheat DELLAs are widely acknowledged (Pearce *et al.,* 2011; personal communications, Dr. A. Phillips, Rothamsted Research, UK., Dr. P. Chandler, CSIRO, Canberra Australia, Dr. R. Saville and Dr. N Al-Kaff, JIC, UK). The extensive secondary structure can also decrease efficiency and fidelity of reverse transcription, to alleviate this, the techniques proven successful for *Rht* mRNA were followed (Saville, 2011).

This study led to the identification of a novel *Sln1* GoF mutant, *dwf2* (cv H930-36), and the characterisation of a stored (but unclearly labelled) seed stock as the *sln1-1* (cv Herta) mutant described in Fu *et al*. (2002), meaning the alternative hypothesis (H_1) was accepted for these two mutant genotypes. The *dwf2* mutant shared phenotypic and *Sln1* ORF sequence similarities with the barley DELLA domain mutant *sln1d* (cv Himalaya), characterised by Chandler *et al.*, 2002. The *sln1d* mutant resulted from a single aa substitution in the DELLA region (DELLAALG → DELLAALE), in the same region deleted in the *dwf2* mutant (DELLAALG → DELL---G). The *dwf2* mutant is consistent with the findings of Itoh *et al.* (2002), in that partial deletion of the DELLA motif results in a GA unresponsive dwarf mutant. The *dwf2* mutant further highlights the importance of the DELLA domain in GA-GID1-mediated degradation of DELLA proteins (Sun *et al*., 2010).

The *sln1-1* (cv Herta) mutant phenotype, although similar to that of the *sln1c* (cv Himalaya) mutant (Chandler *et al.*, 2002), was less severe. While some disparity in phenotype may be accounted for by varietal differences, it might be expected that the *sln1-1* mutant would have a more severe slender phenotype, as the *sln1c* mutant results from a 16 aa deletion at the 3' terminal, just after the SAW domain, and has therefore not lost any conserved motifs, whereas the *sln1-1* mutant lacks most of the LHR II domain, and the entire PFYRE and SAW motifs. A similar effect was reported in rice, where the *slr1-1* to *slr1-4* GRAS domain mutants resulted in slender phenotype mutants (see Section 3.2.3) without a difference in the strength of the slender phenotype being reported (Ikeda *et al.*, 2001). The *sln1c*, *sln1-1*, and *slr1-2* to *slr1-4* mutants result from premature stop codons. Premature stop codons can result in a truncated protein, but may also result in the non-production of the protein because of RNA instability (e.g. nonsense-mediated decay), giving the potential for the production of a dominant negative mutant, a partially functional protein, or no protein at all. The difficulty in determining whether

mutations in the *Sln1* ORF mutations result in LoF DELLA proteins or the non-production of proteins is highlighted by the *sln1b* and *sln1c* mutants in the Himalaya background (Chandler *et al*., 2002). Both mutants result in near identical phenotypes, despite the *sln1c* mutation resulting in a LoF protein, whilst SLN1 is absent in the *sln1b* mutant. It therefore cannot be determined whether the difference in SLN1 protein levels is due to the altered functions of the mutant protein, decreased susceptibility to GA-mediated degradation (e.g. by altered intracellular location), or to general ubiquitin-mediated degradation as a result of protein misfolding.

The nucleotide ORF sequence of the H930-36 *dwf2-1* mutant was unexpected, as it more closely resembled the Himalaya, Triumph or Herta wild-type ORF sequences than the H930-36 wild-type sequence (Table 3.7). The *dwf2-1* phenotype is unlikely to result from two seeds planted in the same pot, as seeds were pre-germinated on filter paper and transferred to soil as young seedlings, one seedling per pot. Great care was taken to ensure that the sequence was correct, with sequence outputs generated from several PCR products checked multiple times. Proof-reading polymerase was consistently used to minimise the possibility of mis-amplification, and laboratory standard operating procedures closely followed to prevent cross-contamination between samples. The *dwf2-1* mutant is likely a chimera, resulting from the simultaneous expression of both wild-type and loss of function or non-functional *Sln1* genes. The emergence of chimeric plants is not uncommon in plants regenerated from tissue culture (Orton, 1980), which was the method used in the generation of the original *dwf2* mutant (Falk, 1994).

The *γ-1* mutant in the Triumph background was initially investigated due to its slender-like phenotype, and for its potential use in stress response experiments. The preliminary stages of analysis did not support the case for the *γ-1* mutant being a *Sln1* LoF mutant. Whilst being phenotypically similar to *Sln1* LoF mutants, the *γ-1* mutant phenotype was less extreme than the characterised *Sln1* LoF mutants. Furthermore segregation ratios did not support the case for the *γ-1* mutant being a *Sln1* LoF mutant, as all germinating *γ-1* mutant seed developed a slender-like phenotype, whereas *Sln1* LoF mutants exhibited recessive segregation for the slender phenotype. Sequencing of the *γ-1* mutant and the corresponding wild-type revealed the ORFs to be completely homologous in terms of both nt and aa sequence, suggesting the *γ-1* mutant phenotype was not the result of differences in SLN1 structure. Finally, the parity in *Sln1* transcript levels between the two genotypes suggest the *γ-1* mutant phenotype is not caused by

differential expression of *Sln1*, rather the *γ-1* mutant results from changes to a gene or genes other than *Sln1*. The alternative hypothesis (H_1) was therefore rejected in favour of the null hypothesis (H_0) for the Triumph *γ-1* mutant genotype. Very few taller than wild-type mutants of cereals have been identified, and the ones that have are characteristically recessive alleles. Recessive mutants such as the *ao-1* and *eui* mutants of rice (Aoki *et al.*, 2002; Zhu *et al.*, 2006) exhibit elongated internode lengths as a result of negative regulation of the GA signalling pathway. Shoot and internode elongation has been observed in rice growing under flood conditions resulting from activation of the *SNORKEL1* and *SNORKEL2* genes that code for transcription factors that regulate ethylene signalling (Hattori *et al.*, 2009). The phenotypic similarities between the *γ-1* mutant and the elongated internode mutants of rice suggest the *γ-1* mutant may result from mutations to the GA or ethylene signalling pathways. Quantification of *Sln1* transcript also inferred information about expression and localisation of SLN1. *Sln1* expression levels were significantly higher in second leaf material than in the whole plant material, suggesting *Sln1* is preferentially expressed in the growing leaf. This is consistent with the findings of Chandler *et al.*, 2002, which found that the SLN1 protein was localized almost exclusively to the leaf elongation zone.

Chapter 4: The Importance of DELLA on Salt Stress Tolerance in Barley

4.1 Aims

The aim of the work described in this chapter was to determine whether data obtained by Achard *et al*. (2006, 2008c) showing that DELLA proteins are important in the survival of salt stress by *Arabidopsis*, could be translated to barley. The approach taken was to identify whether differences in SLN1 function affect the survival of salt stress in barley, by subjecting *Sln1* mutants and their corresponding wild-types to saline conditions. The effect of salt on root and shoot growth of surviving plants was also investigated. To further understand the role of SLN1, preliminary experiments were conducted to determine whether SLN1 status affects accumulation of sodium and uptake of other macroelements, in root and shoot tissues.

The following hypothesise were formulated. H_1: *Sln1* GoF mutants exhibit increased survival and are less susceptible to saline stress compared to the wild-type. Conversely, *Sln1* LoF mutants exhibit decreased survival and increased susceptibility to saline stress compared to the wild-type; H_0: SLN1 has no effect on plant survival or susceptibility to saline stress.

4.2 Introduction

Salinity jeopardises the capacity of agriculture to produce enough food to meet the needs of a burgeoning world population (Flowers, 2004). The amount of land available to agriculture is set to decline due to global warming, which will increase soil degradation, water scarcity, and the unpredictability of weather patterns (Utset & Borroto, 2001; Khush, 1999; Vinocur & Altman, 2005; Ericsson & Nilsson, 2006). Changes in land use through urbanisation and industrialisation will further reduce the availability of land for food production (Khan *et al.*, 2006). Consequently future food production strategies will seek to reduce the rate of land lost through salinisation, whilst maximising output from saline soils through the introduction of novel salt tolerant varieties.

4.2.1 Mechanisms of saline damage

Plants exposed to saline conditions encounter three fundamental problems: 1) a reduction in water availability due to osmotic imbalance between the roots and the surrounding environment, 2) interference of salt ions with metabolic processes within the cell, and the resulting production of ROS, and 3) competition between essential ions and similarly charged toxic ions for uptake by roots (Pasternak, 1987; Apel & Hirt, 2004; Zhu, 2002). Susceptibility to salt depends on plant species, growth stage, and the amount of water passing through the root zone. Plants have evolved mechanisms to cope with saline stress at the cellular and whole plant level, through isolation of ions and ROS in specially adapted morphological structures, and remediation through stress response pathways. Plants most successfully adapted to saline conditions are likely to use more than one of these pathways (El-Sharkawy, 1989). Salt tolerance adaptations are both genetically and physiologically complex, and under polygenic control (Flowers, 2004). Plant response to salt stress is similar to that of drought response, and involves major changes in gene expression.

4.2.2 Salt stress response

Prevention of ion interference and ROS production

Toxic inorganic ions are normally excluded from the cell, however cellular exclusion is used in response to sudden salt shocks, whilst long term adjustment relies on Na^+ compartmentalisation in the vacuoles of shoot and leaf cells (Ellouzi *et al*., 2011). Toxic inorganic ions are removed from the cytoplasm via the Na^+/H^+ antiporter and H^+-ATPase systems on the plasma membrane, or the Na^+/H^+ antiporter on the tonoplast, which sequesters Na^+ to the vacuole. In *Arabidopsis*, the vacuolar Na^+/H^+ exchanger *AtNHX1* (Shi & Zhu, 2002) is expressed in response to increased ABA during osmotic stress. Cytotoxicity is a consequence of the accumulation of ions in the cytosol that result in the substitution of K^+ by Na^+ in biochemical reactions and conformational changes and loss of protein function. The ensuing metabolic imbalances may cause the production of free radical and ROS oxidative agents (Chinnusamy *et al.*, 2005; Zhu, 2002). Concentrations > 0.4 M Na^+ and Cl^- inhibit most enzymes by disturbing the electrostatic bonds required for protein folding and catalysis (Wyn Jones & Pollard, 1983), however concentrations

as low as 0.1 M Na^+ are cytotoxic, suggesting a more direct interference between Na^+ and the substrates of cellular reactions (Serrano, 1996).

DELLA and salt tolerance

The role of DELLAs in resistance to salt stress was first identified in *Arabidopsis* by Achard *et al*. (2006). A strong correlation between the relative growth and developmental effects of DELLA and salt stress tolerance (ρ = -0.96 and 0.94 respectively) was observed in a further study (Achard *et al*., 2008c), suggesting a common regulatory mechanism.

DELLA levels are post transcriptionally regulated through GA-mediated degradation (Achard *et al.*, 2006). Salt treated wild-type *Arabidopsis* plants showed lower levels of bioactive GA (GA_1 and GA_4) compared to untreated plants (Achard *et al*., 2006), whilst GFP tagged DELLA (GFP-RGA) was shown to accumulate to higher levels in the roots of salt treated plants, despite a lack of detectable changes in RGA transcript levels. Salt therefore restricts growth through a DELLA-dependent mechanism associated with reduced accumulation of bioactive GA and subsequent DELLA accumulation (Achard *et al.*, 2006).

Arabidopsis "quadruple-DELLA mutants" lacking four (*GAI*, *RGA*, *RGL1* and *RGL2*), of the five DELLAs encoded by the *Arabidopsis* genome showed reduced growth inhibition under saline conditions compared to the wild-type. Salt slowed the rate of leaf production and expansion, biomass accumulation, and root growth in wild-type plants, but had a less inhibitory effect on the quadruple-DELLA mutant, suggesting DELLA inhibits plant growth and development in response to salt stress (Achard *et al.*, 2006). However, the quadruple-DELLA mutant showed decreased survival in 200 mM NaCl compared to the wild-type (5% and 36% survival, respectively), whilst mutants with increased DELLA accumulation, either through diminished GA biosynthesis (*ga1-3*) or decreased GA-DELLA interaction (*gai*), showed increased survival (93% and 82% respectively) (Achard *et al.*, 2006). Thus, DELLAs provide a mechanism for environmentally-responsive growth regulation in *Arabidopsis*.

4.3 Materials and methods

4.3.1 Plant material

Barley lines were selected based on mutations present in genes involved in the GA signal transduction pathway. Those available contained mutations either within the *Sln1* gene (cv Himalaya: *sln1c*, *sln1d*; cv H930-36: *dwf2*; cv Herta: *sln1-1*), or within the *Gse1* gene (cv Himalaya: *gse1a*, *gse1j*, *gse1n*). The cv Himalaya mutants had been characterised by Chandler *et al*. (2002, 2008), whilst cv H930-36 and cv Herta *Sln1* mutants were characterised as part of this study (see Chapter 3). Investigation of the role of SLN1 in salt stress tolerance initially focused on the Himalaya background in which characterised LoF and GoF *Sln1* mutants and *Gse1* mutants were available (Chandler *et al.*, 2002, 2008). Further mutants (cv Herta: *sln1-1*; cv H930-36: *dwf2*) were included once they had been molecularly characterised. Plants of the *Sln1* LoF mutant genotypes *sln1c* (cv Himalaya) and *sln1-1* (cv Herta) have a slender phenotype, whilst the GoF mutants *sln1d* (cv Himalaya) and *dwf2* (cv H930-36) have a dwarf phenotype. Plants containing the mutant *gse1a*, *gse1j* and *gse1n* alleles exhibit dwarf and semi-dwarf phenotypes (Chandler *et al.*, 2008).

4.3.2 Plant growth and salt stress

Growth conditions

Seeds for the mutant barley genotypes listed in Section 4.3.1, and their corresponding wild-types were stratified as described in Section 2.1.3.1 and then germinated on filter paper in Petri dishes in a CER (16 h photoperiod, 20 °C) for two days. Salt stress response was investigated using hydroponic culture and plants at a similar developmental stage. Seedlings were grown to the 2-3 leaf stage (only those with roots longer than 3 cm were used), then gently removed from the filter paper and supported at the base of the stem by foam bungs, which were secured in 50 ml polypropylene centrifuge tubes (Corning Inc., Corning, NY, USA) which had their bases and caps removed. The tubes were placed in custom steel racks (JIC workshop) in 10 L plastic containers (Tontarelli, Castelfidardo, Italy) containing Hoagland's solution (x 0.5). Seedlings from each line under test were positioned randomly in the racks. The size of the plastic containers and steel racks limited plant numbers to a maximum of 28 seedlings per treatment per experiment. As there was no information available regarding the salt tolerance of the barley

cultivars used, it was necessary to conduct preliminary experiments in order to establish the conditions that would maximise the chance to identify any possible differences between wild-type and mutant lines. Seedlings were acclimatised to hydroponics in Hoagland's solution (x 0.5) for two days before transfer to the respective treatment solutions. Plants were transferred to Hoagland's solution (x 0.5) containing 0, 100, 200 or 300 mM NaCl or 0, 150, 250 or 500 mM NaCl (for preliminary experiments), for a 10 day period. Solutions were aerated by daily transference of solutions between two containers, or for the preliminary experiments, by constant aeration using air pumps (Rena Air 100 & 200, Rena Aquarium Equipment, Charlotte, NC, USA; Tetratec APS 100, Spectrum Brands, Madison, WI, USA). An example of the experimental conditions is shown in Figure 4.1.

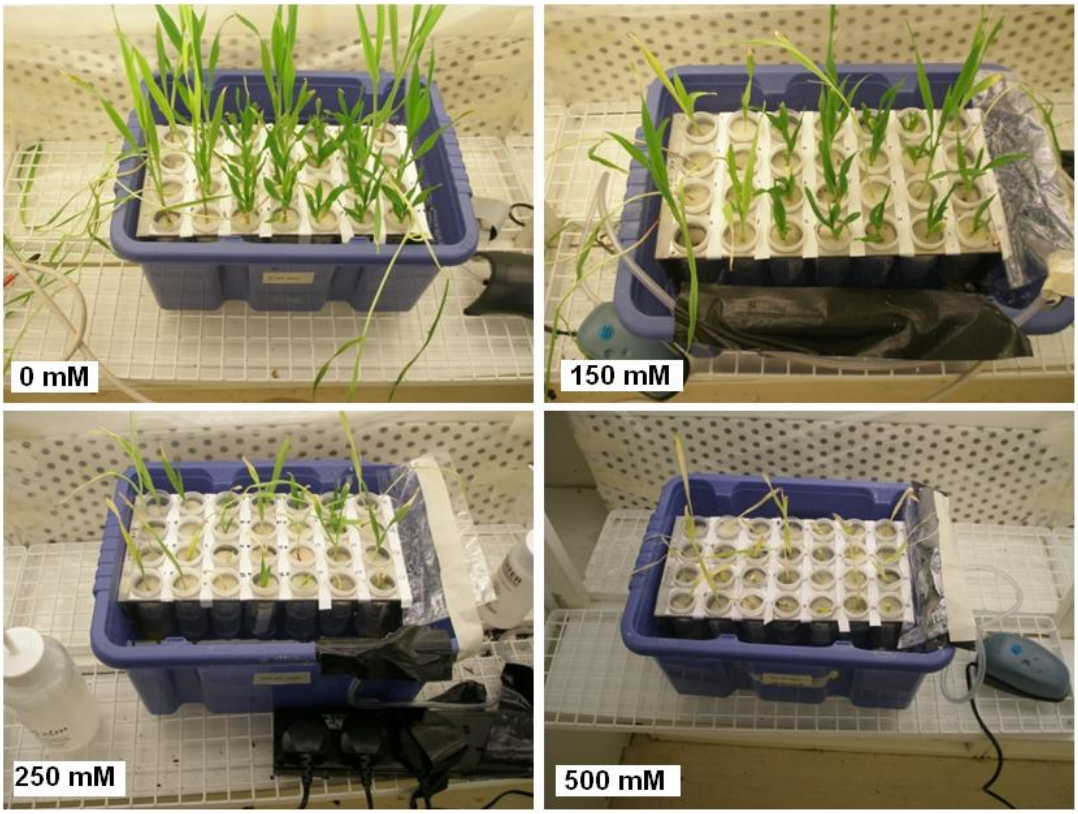


Figure 4.1 Preliminary hydroponics experiment using cv Himalaya seedlings (wild-type, *sln1c*, *sln1d*, *gse1a*, *gse1j*, *gse1n*) showing plant development after 10 days treatment in 0, 150, 250 or 500 mM NaCl. Polythene film was used to prevent splashing of solution on stems and leaves during aeration with an air pump**.**

Sample size

Phenotypic observations and growth measurements were made for each experiment using 4 -14 seedlings of each genotype per treatment. Element accumulation studies were conducted for a single experiment, and pooled samples consisting of 4 – 10 seedlings for each genotype per treatment were collected.

4.3.3 Assessment of plant growth

Survival was assessed visually after 10 days of treatment, with plants defined as dead if leaf and stem necrosis was extensive or total. After 10 days treatment, root and shoot lengths were measured for all seedlings, and roots and shoots separately frozen in liquid nitrogen before being freeze dried (Edwards Modulyo Freeze-Dryer, Edwards Lab, Sandusky, OH, USA) for five days. When recording root and shoot dry mass, samples from dead seedlings were included in the analysis of cv Himalaya, but analysis of cv H930-36 data did not include tissue from dead seedlings. For Himalaya wild-type and mutant plants, mean mass values were calculated for the pooled samples for each genotype for each treatment. For measurements of growth (root and shoot dry mass and length), mean values for salt treated groups were normalised against the control group (0 mM NaCl) for each genotype, by expressing growth in salt as a percentage of growth under control conditions (control group growth being equal to 100%). As root and shoot dry masses in the Himalaya background were calculated from pooled samples, standard deviation could not be calculated. Individual masses were used to obtain standard deviation values for dry mass in the H930-36 background, and root and shoot lengths in Himalaya, H930-36 and Herta backgrounds. Samples were weighed to four decimal places and dry mass recorded. Root length data were collected by measuring the longest root of each seedling. Further observations were made including tiller and leaf number, and number of developing roots, as well as root necrosis and root fine hair formation. As qualitative, non-numerical assessments were made of root necrosis and root fine hair formation; median values are presented in the results.

Chlorosis was observed in seedlings developing during the salt treatment period. As chlorosis is indicative of decreased photosynthetic potential, photosynthetic yield was measured using the MINI-PAM Photosynthesis Analyzer (Walz, Effeltrich, Germany). Initial attempts to measure

chlorophyll levels using a SPAD meter were unsuccessful because seedling leaves were too narrow. Measurements were taken two days into the treatment period. This time point was chosen as leaf chlorosis was observable at this point, but leaf necrosis was not extensive enough to prevent readings from being obtained. Three measurements were taken over the central region of the first and second leaves, and the median values recorded. Median values were used so as to remove extreme values that could result from taking measurements from unrepresentative areas of the leaf.

4.3.4 Analysis of element accumulation

Analyses were carried out to determine whether SLN1 affects the accumulation of elements in roots and shoots in the presence or absence of salt. Wild-type and mutant (*sln1c*, *sln1d*) cv Himalaya seedlings were grown in Hoagland's solution containing 0 mM NaCl or 100 mM NaCl, as described in Section 2.1.3.3. After one week, seedlings were removed from the solutions and excess hydroponics solution removed by dipping the roots several times in a large volume of sterile distilled water followed by gentle blotting on absorbent paper towels. Root and shoot material was separated, and samples from the same genotype and treatment combined, freeze dried, and ground to a fine powder using a 8000 M Mixer/Mill (Glen Creston, Middlesex, UK). Samples were submitted to Mr. Graham Chilvers (University of East Anglia, Norwich, UK) for analysis. For each sample, approximately 3 mg of powdered root or shoot material was incubated at 150 °C for 4 h with 1 ml high purity HNO_3 in sealed boro-silicate tubes. The treated samples were diluted with high purity MQ Water from the Elga water purification system (Elga LabWater, Marlow, Buckinghamshire, UK). Rhodium was added at the dilution stage for use as an internal standard. Spectrophotometric analysis was performed using the Thermo Electron X5 Series IICP-MS (Thermo Scientific, Wilmington, DE, USA). The spectrophotometer was run in standard resolution mode at a nebulising rate of 600 µL min^{-1}. Standard performance checks were run to assure correct performance. Each sample was run in triplicate, with any changes or drift automatically corrected using the internal standard. Levels of sodium (Na), potassium (K), calcium (Ca), magnesium (Mg) and phosphorus (P) were expressed as parts per million (ppm).

4.4 Results

4.4.1 Preliminary experiment

A preliminary experiment was conducted in order to establish the saline conditions needed to produce an approximately 50% survival rate for wild-type seedlings (cv Himalaya). Several mutant lines were also included. Survival results for the preliminary experiment were obtained using 4 - 5 seedlings per genotype per treatment. All wild-type seedlings survived the non-saline control conditions (0 mM NaCl); conversely none survived the highest level of salinity (500 mM NaCl). Intermediate treatments (150 and 250 mM NaCl) produced survival closer to the desired level amongst the wild-type seedlings (60% and 20% respectively; Table 4.1). Although sample numbers were small there was increased survival (100%) of two lines (*sln1c* and *gse1a*) at 150 mM NaCl compared to the wild-type (60% survival). The *Sln1* GoF mutant *sln1d* and the *gse1n* mutants showed slightly increased survival (80% and 75%, respectively) whereas another *Gse1* mutant *gse1j* survived slightly less well (50% survival). A considerable reduction in seedling survival was observed between 150 and 250 mM NaCl treatments for most lines, with only *gse1j* showing little reduction (10%) in survival and *gse1n* showing complete survival at 250 mM NaCl (Table 4.1). None of the alleles were able to confer seedling survival at 500 mM NaCl. Based on the results of this preliminary experiment, treatment at 100, 200, 300 mM NaCl were selected for subsequent experiments.

Table 4.1 Survival (%) of salt stress by cv Himalaya seedlings. The data were obtained from a preliminary experiment designed to identify appropriate NaCl concentrations. Hydroponic treatment was conducted using 0.5 x Hoagland's solution and 0, 150, 250 or 500 mM NaCl. Data was collected 10 days after treatment commenced.

	NaCl concentration			
Genotype	**0 mM**	**150 mM**	**250 mM**	**500 mM**
WT	100	60	20	0
sln1c	100	100	0	0
sln1d	100	80	40	0
gse1a	100	100	40	0
gse1j	100	50	40	0
gse1n	100	75	100	0

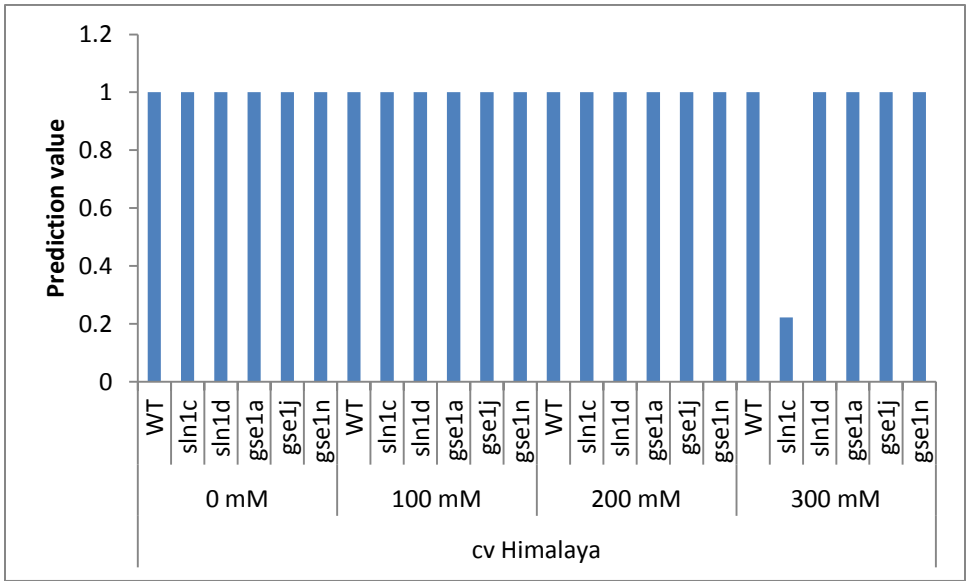
4.4.2 Seedling survival

Using the experimental conditions established by the preliminary experiment, plant survival was measured in further experiments using Himalaya (wild-type, *sln1c*, *sln1d*, *gse1a*, *gse1j*, *gse1n*), H930-36 (wild-type, *dwf2*), and Herta (wild-type, *sln1-1*) backgrounds.

Survival data was initially assessed using generalised linear models, which generated survival prediction values for each genotype under each test condition (0 mM – 300 mM NaCl). As the data was non-parametric, Mann-Whitney U tests were used to calculate significant differences between the test groups. Survival results for cv Himalaya (Figure 4.2a) were obtained using 4 - 10 seedlings per genotype per treatment. No survival differential was observed between genotypes treated with 0, 100 or 200 mM NaCl, indeed all seedlings survived these concentrations. However at 300 mM NaCl, the LoF mutant *sln1c* showed lower survival (only 2/9 seedlings survived) compared to the wild-type (10/10 seedlings survived), although this difference was not statistically significant (*P*: 0.167). Furthermore, there was no significant difference in survival between *sln1c* and the putative SLN1 stabilising mutants (*sln1d*, *gse1a*,

gse1j, *gse1n*), (*P*: 0.167 – 0.333). The total survival of all wild-type seedlings under the conditions tested meant it was not possible to determine whether the *Sln1* GoF mutant *sln1d*, and the *Gse1* mutants *gse1a*, *gse1j*, *gse1n* were more tolerant to NaCl than the wild-type. Survival results for cv H930-36 (Figure 4.2b) are calculated using 10 - 14 seedlings per genotype per treatment. All cv H930-36 wild-type and *dwf2* seedlings survived treatment in 0, 100 and 200 mM NaCl, however a survival differential between the wild-type and *dwf2* genotypes was observed at 300 mM NaCl, with a lower level of survival observed for wild-type seedlings (23/28 survived) compared to *dwf2* mutant seedlings (24/24). This difference was however, non-significant (*P*: 0.5). Survival of cv Herta wild-type and *sln1-1* (SLN1 LoF) mutant seedlings in saline conditions was observed using 4 - 10 seedlings per genotype per treatment. All the wild-type and *sln1-1* seedlings in the Herta background survived all tested saline conditions (100, 200, 300 mM NaCl), consequently no survival differential was observed between the two cv Herta genotypes.

(a)



(b)

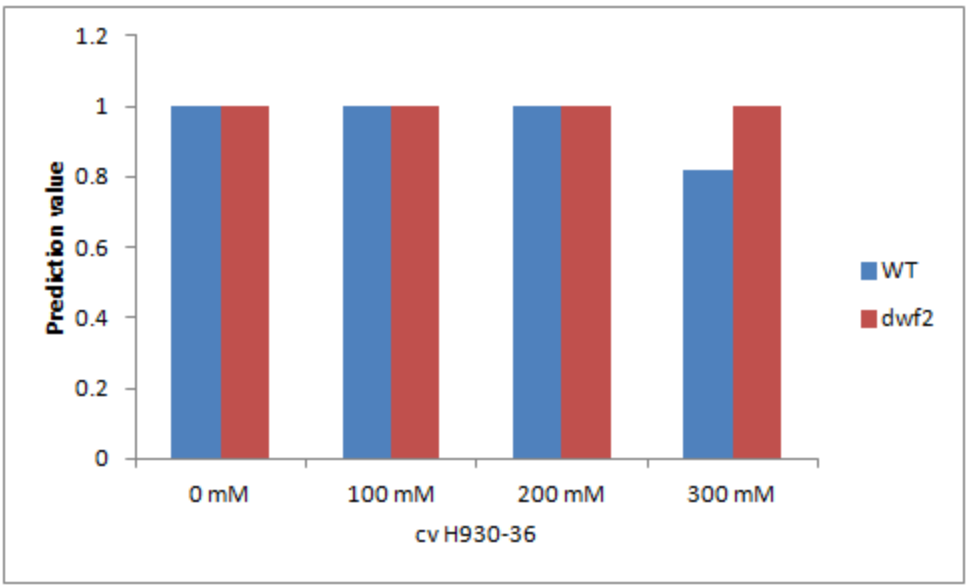


Figure 4.2 Survival values for plants grown for 10 days in 100, 200, 300 mM NaCl or control (0 mM NaCl) conditions. Prediction values were generated from survival data using general linear models. Values of 1 represent the prediction of total survival, and 0 total death of all samples of the genotype under the stated treatment condition. Genotype and NaCl concentrations (mM) are on the x-axis. Results are presented for (a) cv Himalaya, (b) cv H930-36.

4.4.3 Root and shoot mass

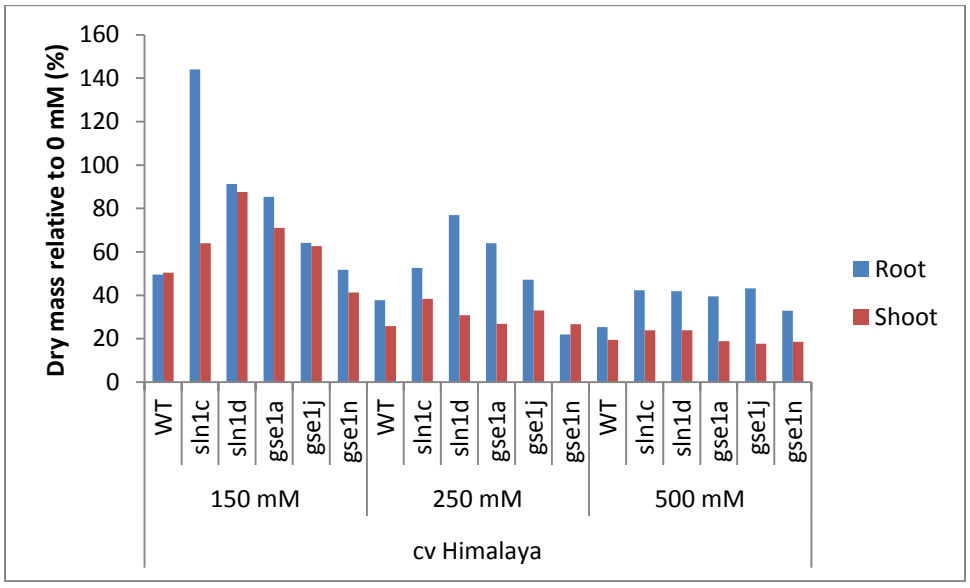
Root and shoot mass were used as an indicator of plant growth. In the preliminary experiment, data were obtained using cv Himalaya and 4 - 5 seedlings per genotype per treatment. All saline conditions (150, 250 and 500 mM NaCl) resulted in reduced mean root and shoot mass (dry weight) for both wild-type and putative SLN1 stabilising mutant seedlings (*sln1d*, *gse1a*, *gse1j*, *gse1n*) compared with seedlings grown in the absence of NaCl (Figure 4.3a). In contrast, the mean root mass of *sln1c* seedlings treated with 150 mM NaCl exceeded that of control (treated in 0 mM NaCl) *sln1c* seedlings. Indeed, the root mass of *sln1c* seedlings decreased less under saline treatment (150, 250 and 500 mM) than for the wild-type. Accumulation of root biomass in three of the four putative SLN1 stabilising mutants, (*sln1d*, *gse1a*, *gse1j*) was less inhibited than (or equal to) that of the wild-type at 150, 250 and 500 mM NaCl conditions. Following treatment with 150 and 250 mM NaCl, root growth inhibition in the putative SLN1 stabilising mutants decreased in the order of *sln1d* > *gse1a* > *gse1j* > *gse1n*, although in 250 mM NaCl the *gse1n* mutant showed decreased mass compared to the wild-type roots (Figure 4.3a). All mutants retained root mass better than the wild-type seedlings after treatment with 500 mM NaCl. Following treatment in 150 mM NaCl, the trend of shoot growth generally corresponded to root growth with the exception of the *sln1c* mutant, for which shoot biomass accumulation was similar to (or only slightly greater than) that of the wild-type (Figure 4.3a, red bars). Differences in shoot dry biomass between the wild-type and mutants at 250 and 500 mM NaCl were relatively small, with all mutants showing only slightly higher levels of growth than the wild-type at 250 mM NaCl. As these observations were based on single combined mass values, only limited conclusions could be drawn.

Further experiments using 4 - 10 seedlings per cv Himalaya genotype per treatment provided additional biomass data (Figure 4.3b). In these experiments saline treatments of 100, 200 and 300 mM NaCl resulted in reduced mean root and shoot mass (dry weight) for both wild-type and mutant genotypes compared to the growth under control (0 mM NaCl) conditions, with the exception of the *Gse1* mutant, *gse1a*, which showed greater biomass accumulation following treatment at 100 mM NaCl compared with 0 mM. As observed in the preliminary experiment, under low salinity (100 mM) the rate of shoot growth was similar to that of root growth. Although following 100 mM NaCl treatment there was no evidence of generally increased salt tolerance of the mutants predicted to have increased DELLA stability compared to the wild-type;

at higher concentrations (200 and 300 mM NaCl), greater growth was observed in mutants *sln1d, and gse1a*, as well as with the LoF mutant *sln1c*. Equivalent results were not seen with mutants *gse1j and gse1n*. As with the preliminary experiment, the observations were based on either one or two experimental repeats, therefore only limited conclusions could be drawn.

To assess the effect of salt on growth of a SLN1 GoF mutant in a different background, an experiment was conducted using the SLN1 GoF mutant (*dwf2*) in the H930-36 background. The use of only one mutant allowed more replicates to be used (14 samples per genotype per treatment), increasing the chances of identifying any significant differences between the two genotypes. As observed with seedlings of the Himalaya background, increasing levels of salinity corresponded with a decrease in both root and shoot biomass for the wild-type and mutant lines (Figure 4.3c). At 300 mM NaCl root biomass accumulation was significantly less inhibited in *dwf2* seedlings than in the wild-type (*P*: 0.004), a trend also seen in seedlings treated with 100 and 200 mM NaCl (*P*: 0.007). A similar trend was seen for shoot biomass, which was maintained significantly better in *dwf2* seedlings compared to the wild-type at 300 mM NaCl (*P*: 0.007).

(a)



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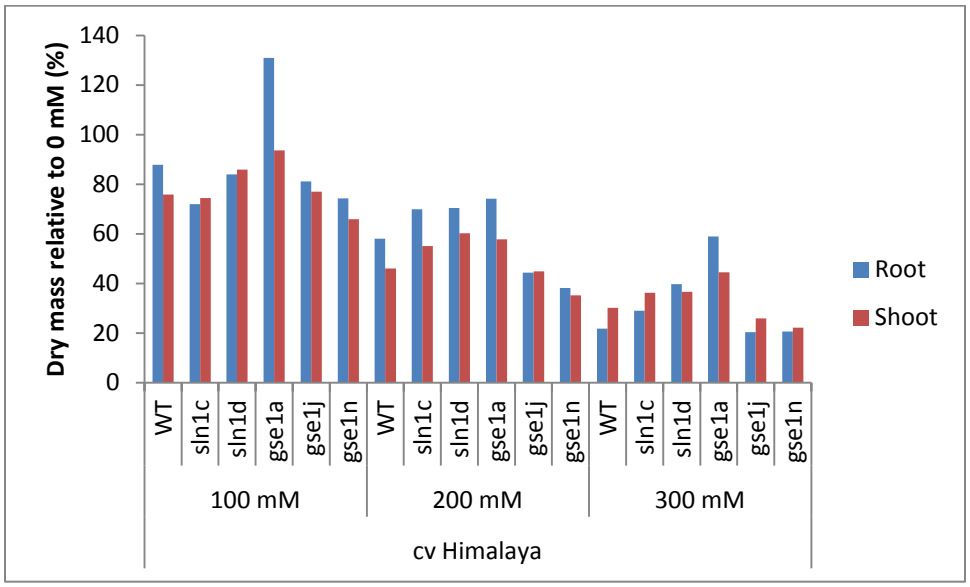

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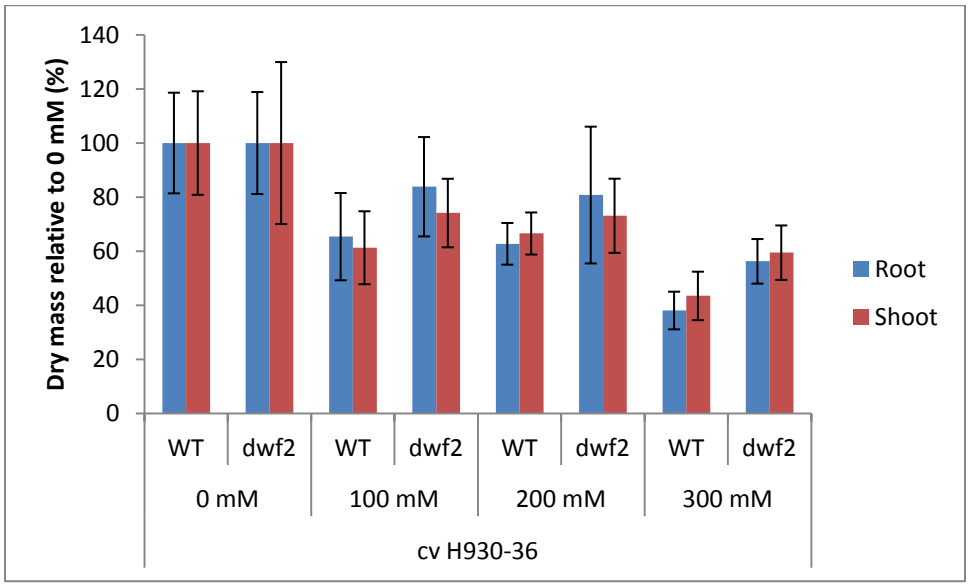


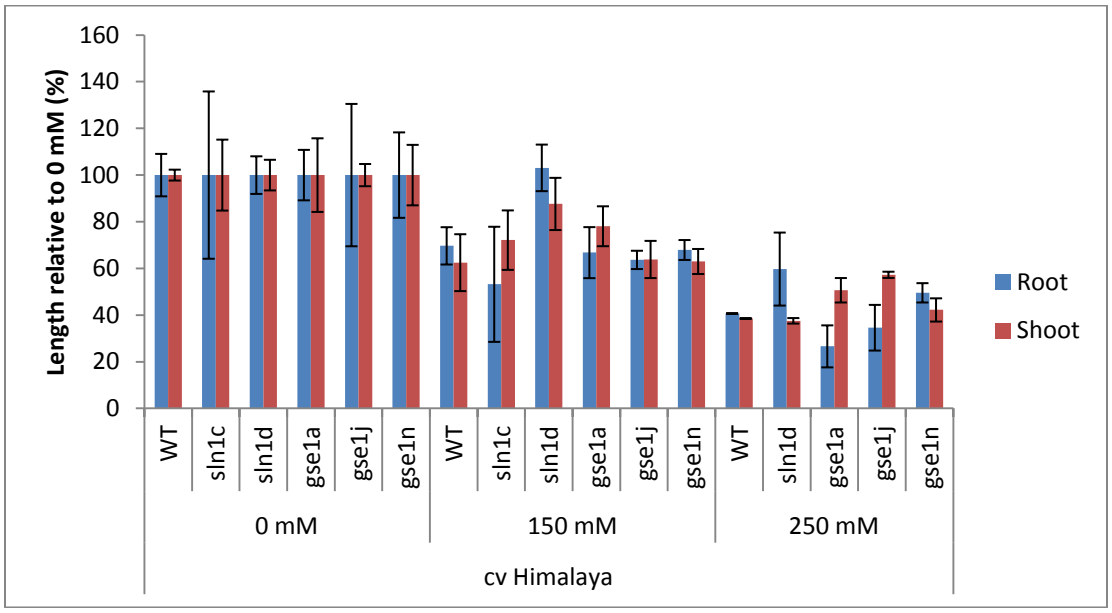
Figure 4.3 Root and shoot dry mass of wild-type and mutant seedlings treated with 0, 100, 200 and 300 mM NaCl. Growth (as represented by biomass) in salt is shown as a percentage of growth under control conditions (0 mM NaCl) for each genotype. Salt concentrations are shown on the x-axis. (a) data from the preliminary cv Himalaya experiment, (b) cv Himalaya, (c) cv H930-36. Blue and red bars indicate root and shoot data, respectively. Bars represent standard deviation.

4.4.4 Root and shoot length

Root and shoot length analysis is shown in Figure 4.4. In the preliminary experiment using cv Himalaya seedlings (Figure 4.4a), growth in saline conditions (150, 250 mM NaCl) resulted in reduced root and shoot length for all genotypes, yet the difference was most acute at 250 mM NaCl. The reduced root growth was, however, clear in wild-type, *gse1a*, *gse1j* and *gse1n* genotypes treated at 150 mM NaCl. In parallel shoot growth was also reduced in wild-type, *gse1j* and *gse1n* seedlings. Two further experiments were carried out in which 4 – 5 cv Himalaya seedlings per genotype per treatment were tested. Again, it was clear that NaCl treatment decreased shoot and root length for all genotypes, with greatest effects being seen at the higher salt concentrations (200 mM and 300 mM NaCl, Figure 4.4b). The large variation in root length of individual seedlings of each genotype in each treatment group made it difficult to identify any differences at 100 mM NaCl, although the root length of the *sln1c* mutant was significantly decreased compared to the control plants (*P:* <0.001). Differences between the root length of the genotypes were more marked at 200 mM NaCl, with the wild-type and *sln1c* (LoF) root growth being markedly reduced (*P*: <0.001), whereas the root length of the severely dwarfed *gse1n* mutant was not significantly different from that at 0 mM NaCl (*P*: 1.0). Plants treated with 300 mM NaCl all had significantly decreased root length compared to plants grown at 0 mM NaCl. Analysis of the shoot length data was again hampered by the seedling variation within a genotype, resulting in large error bars. Nevertheless, the shoot length of the *gse1* mutants was significantly less affected by salt treatment at 100 mM and 200 mM NaCl than the wild-type seedlings (*P*: <0.001 – 0.002), (Figure 4.4b). Following treatment at 300 mM NaCl this difference was not so clearly manifested, with only the *gse1a* mutant showing shoot growth substantially greater than the wild-type plants (*P*: 0.007). To determine whether salt stress responses differed between cultivars (and mutants of that cultivar), the experiment was repeated using cv H930-36 seedlings. Data were combined from three experiments to give sample sizes of 8 - 19 seedlings per genotype, per treatment. Salt treatment decreased shoot and root lengths for both the wild-type and *dwf2* seedlings as was seen with cv Himalaya plants. The decrease was most marked at 300 mM NaCl where final root length was less than half that seen in control (0 mM NaCl) plants (Figure 4.4c).

To further address potential cultivar-specific responses to salt, an additional experiment was conducted using the SLN1 LoF mutant in the Herta background (*sln1-1*, Figure 4.4d). Data (obtained using 4 - 8 seedlings per genotype per treatment) showed that root length was significantly decreased in both the wild-type and *sln1-1* genotypes following treatment in 200 or 300 mM NaCl (*P*: <0.001). At 100 mM NaCl the root length of wild-type seedlings was not significantly decreased (*P*: 0.087), whereas that of the *sln1-1* mutant was (*P*: 0.003). This genotype-dependent difference was not seen at higher NaCl concentrations. As observed with the other cultivars, shoot growth was less affected than root growth by salt, although both wild-type and *sln1-1* seedlings treated at 300 mM had significantly shorter shoot length than the control plants (*P*: <0.001). At 200 mM NaCl the shoot elongation of the *sln1-1* mutant was less affected than the wild-type (*P*: 0.087), (Figure 4.4d).

(a)


Length relative to 0 mM (%)
160
140
120
100
80
60
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WT
sln1c
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gse1a
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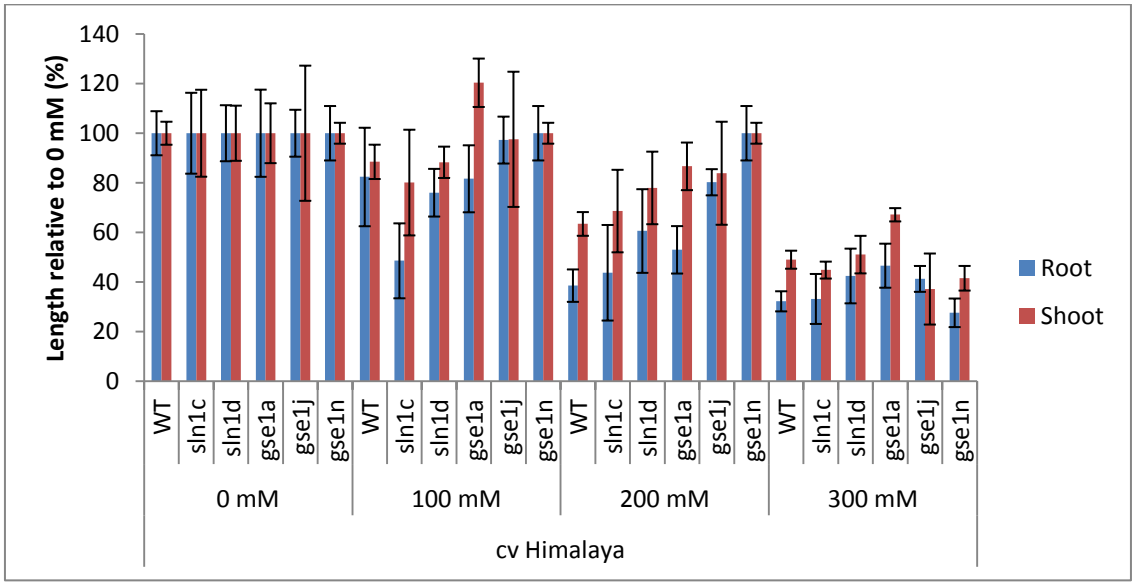
150 mM
250 mM
cv Himalaya
Root
Shoot

(b)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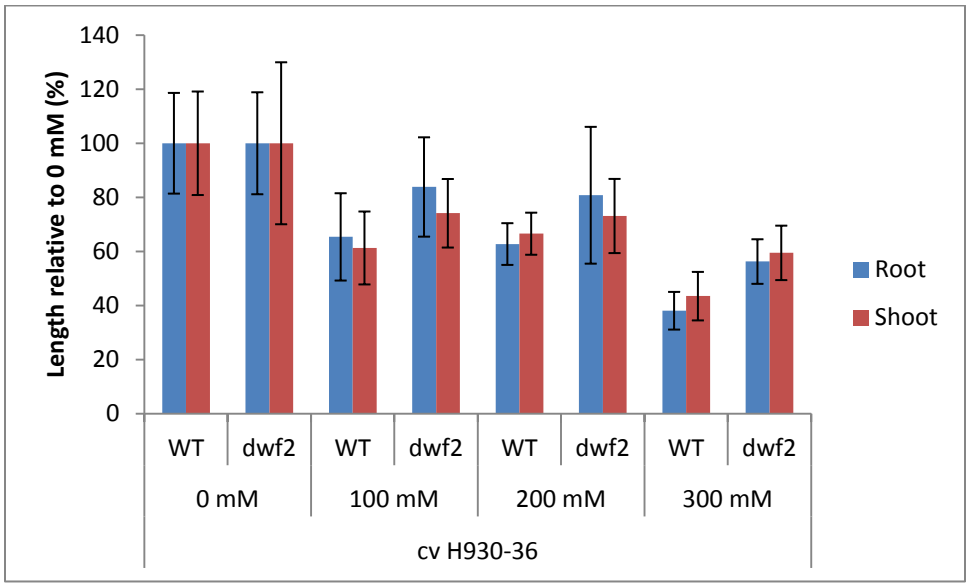
(c)



(d)

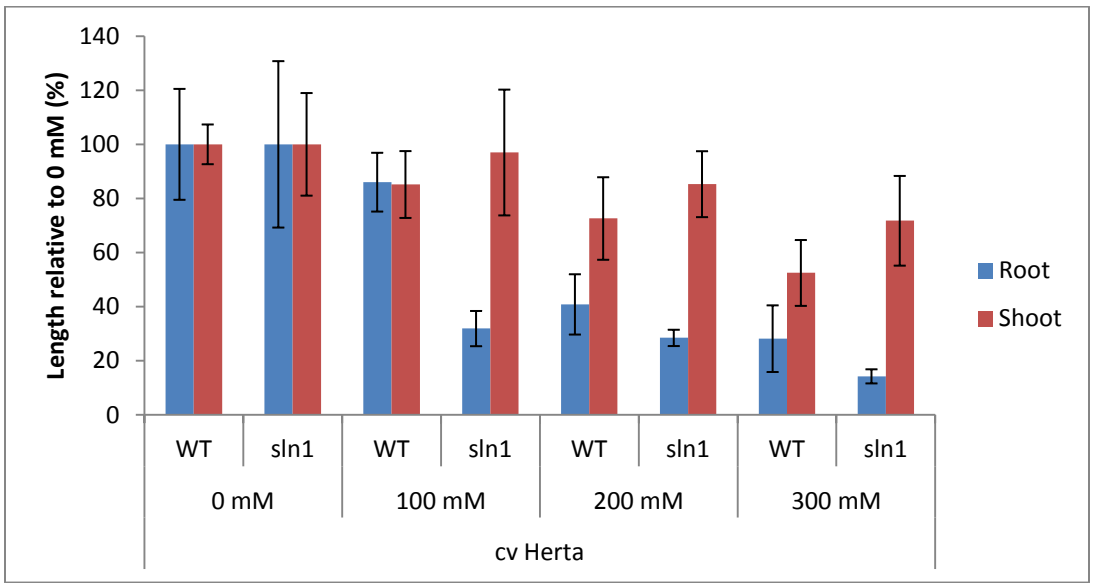


Figure 4.4 Root and shoot length of wild-type and mutant seedlings treated with 0, 100, 150, 200, 250 or 300 mM NaCl. Root and shoot lengths are shown as a percentage of growth under control conditions (0 mM NaCl) for each genotype. Salt concentrations (mM) are shown on the x-axis. Data (blue or red bars indicate data for roots or shoots, respectively) are shown for (a) cv Himalaya preliminary experiment, (b) cv Himalaya, (c) cv H930-36, (d) cv Herta. Bars represent standard deviation.

4.4.5 Leaf, root and tiller number

Leaf, root and tiller numbers were recorded for each plant at the end of the treatment period. Data were not recorded for the preliminary experiment, but were recorded from two further experiments using cv Himalaya seedlings, one using cv H930-36 seedlings, and one using cv Herta seedlings. Data from dead seedlings were not included in the analyses, and mean values were calculated. At the time of transfer to salt treatment, all of the seedlings had only a single main stem, and unless stated were at the two leaf stage.

Leaf number

Increasing salinity caused a reduction in mean leaf number for both wild-type and mutant genotypes in all cultivars (Table 4.2) although in the Himalaya background, leaf number in the *gse1j* and *gse1n* mutants was less affected than the wild-type at 100 mM and 200 mM NaCl (Table 4.2a). Fewer leaves were produced by seedlings of the wild-type, *sln1d*, *gse1a* genotypes after treatment with 100 mM NaCl compared with seedlings grown in the absence of NaCl, whilst mean leaf number of the *gse1j* and *gse1n* mutants remained constant (8 and 7, respectively). Under high salinity (300 mM NaCl), leaf production was decreased for all genotypes (2 – 3 leaves). In the H930-36 background, leaf formation in the *dwf2* (*Sln1* GoF) mutant was marginally less inhibited under low salinity (100 mM NaCl) than leaf formation in the wild-type genotype (Table 4.2b). At higher salinities (200 and 300 mM NaCl), leaf formation was equal (a mean of 2 leaves). In the Herta background, a similar response to that obtained with cv Himalaya seedlings was seen with a decrease in leaf production in both the wild-type and the *sln1-1* (*Sln1* LoF) mutant being observed as salinity increased. Mean leaf number for the wild-type genotype was high compared to the *sln1-1* mutant under control conditions (8 and 5 respectively; 0 mM NaCl, Table 4.2c), yet decreased sharply as salinity increased. Mean leaf number decreased in the wild-type from 8 to 5 leaves as salinity increased from 0 mM to 100 mM NaCl (Table 4.2c).

Table 4.2 Mean number of leaves present in wild-type and mutant seedlings following treatment with 0, 100, 200, 300 mM NaCl. Results are shown for wild-type and mutant genotypes in (a) cv Himalaya, (b) cv H930-36, (c) cv Herta genotypes.

(a)

NaCl conc.	Genotype and mean leaf number					
	WT	*sln1c*	*sln1d*	*gse1a*	*gse1j*	*gse1n*
0	7	5	7	7	8	7
100	5	5	5	5	8	7
200	4	4	4	4	6	7
300	3	3	3	2	3	3

(b)

NaCl conc.	Genotype and mean leaf number	
	WT	*dwf2*
0	3	3
100	2	3
200	2	2
300	2	2

(c)

NaCl conc.	Genotype and mean leaf number	
	WT	*sln1-1*
0	8	5
100	5	4
200	4	3
300	3	3

Root number

As observed with mean leaf number, treatment at high salt (300 mM NaCl) caused a reduction in mean root number for both wild-type and the mutant genotypes that are predicted to have stabilised DELLA proteins (wild-type following salt stress, and *sln1d*, *gse1a*, *gse1j* and *gse1n*; Table 4.3). This reduction was seen in all cultivars tested. In contrast, seedlings containing the LoF alleles *sln1c* (Table 4.3a) and *sln1-1* (Table 4.3c) either showed little decrease or an increased root number, dependent on the cultivar. Root number was less affected at low salt with the root production being unaffected by treatment with 100 mM NaCl in the *gse1j* and *gse1n* lines, moreover, the mean number of roots remained constant in the *gse1n* mutant even at 200 mM NaCl. In contrast, root number was slightly reduced for all other cv Himalaya seedlings at 100 mM NaCl and increasingly affected at 200 mM NaCl (Figure 4.3a).

Table 4.3 Mean number of roots present following treatment of barley seedlings with 0 mM (control) or 100, 200, 300 mM NaCl. Results are shown for wild-type and mutant genotypes in (a) cv Himalaya, (b) cv H930-36, (c) cv Herta genotypes.

(a)

NaCl conc.	Genotype and mean root number					
	WT	*sln1c*	*sln1d*	*gse1a*	*gse1j*	*gse1n*
0	17	11	13	12	15	19
100	15	9	12	11	15	19
200	14	9	10	10	13	19
300	11	10	9	8	8	10

(b)

NaCl conc.	Genotype and mean root number	
	WT	*dwf2*
0	15	13
100	12	10
200	14	12
300	12	10

(c)

NaCl conc.	Genotype and mean root number	
	WT	*sln1-1*
0	14	9
100	11	10
200	12	11
300	10	12

Tiller number

Very few tillers were produced during the treatment period, such that significant differences between genotypes could not be discerned (data not shown).

Data obtained over three experiments for Himalaya wild-type and *sln1c* and *sln1d* mutants showed that few tillers were produced by *sln1c* LoF control (0 mM NaCl treatment) plants during the experimental period. The *sln1c* LoF mutant produced a tiller in only 4/15 plants tested whereas 13/15 and 10/15 of the wild-type and *sln1d* GoF plants, respectively, were able to do so. Notably, two tillers had emerged on one wild-type plant and two *sln1d* plants. At 100 mM NaCl only wild-type plants produced a tiller (4/10 plants). In the single experiment in which the *gse1* mutants *gse1a, gse1j, gse1n* were analysed, plants responded in a manner similar to the wild-type, with most producing a tiller at 0 mM NaCl (8/10, 7/8 and 8/8, respectively). The *gse1* mutants differed from the *Sln1* mutants in that all *gse1n* plants produced a tiller at 100 mM NaCl as did 3/4 of *gse1j* plants (with one plant producing 2 tillers), and 1/5 *gse1a* plants. Tiller production was completely inhibited for wild-type and all (*Sln1* and *gse1*) mutant plants at 250 mM NaCl.

4.4.6 Further observations of seedling phenotypes in response to salt

Root phenotypes

The root morphology of all lines was affected by salt treatment; limited visual assessment was carried out in two experiments and was based on 4 - 10 seedlings (cv Himalaya) per genotype per treatment. Root necrosis, scored based on root discoloration, was predominantly seen in seedlings grown under saline conditions (100 – 300 mM NaCl), however a low level of discolouration was observed in plants growing under control (0 mM NaCl) conditions; this was classified as minor root necrosis (Table 4.4). Root necrosis was seen at 100 mM in the wild-type, *sln1c*, and *sln1d* genotypes (Table 4.4), but was not observed in the other genotypes until salt treatment at 200 mM NaCl and even at this concentration the roots of the *gse1n* mutant remained healthy. The *gse1n* mutant only showed necrosis at the highest level of salinity (300 mM NaCl).

Table 4.4 Median root necrosis in cv Himalaya seedlings after 10 days growth in control (0 mM NaCl) and saline (100, 200, 300 mM NaCl) conditions. '-'represents minor root necrosis, '+' represents moderate root necrosis, '++' extensive root necrosis.

	NaCl concentration			
Genotype	**0 mM**	**100 mM**	**200 mM**	**300 mM**
WT	-	+	++	++
sln1c	-	+	+	++
sln1d	-	+	+	+
gse1a	-	-	+	+
gse1j	-	-	+	++
gse1n	-	-	-	++

During the first of these experiments it was noted that root hair formation was affected by salt and this was assessed visually in a single experiment consisting of 5 seedlings per genotype (cv Himalaya) per treatment. Although root hair formation was extensive in wild-type and *sln1c* (*Sln1* LoF) seedlings under control (0 mM NaCl) and low salinity (100 mM NaCl) conditions, fewer root hairs were seen in *sln1d* (*Sln1* GoF) mutant seedlings grown in the absence of salt and their appearance remained similar ("moderate root hair formation") following all salt treatments (100, 200 and 300 mM NaCl) (Table 4.5). In contrast, the wild-type and *sln1c* mutant seedlings were unable to produce extensive root hair formation at 300 mM NaCl, although the wild-type was less affected at 200 mM NaCl (Table 4.5).

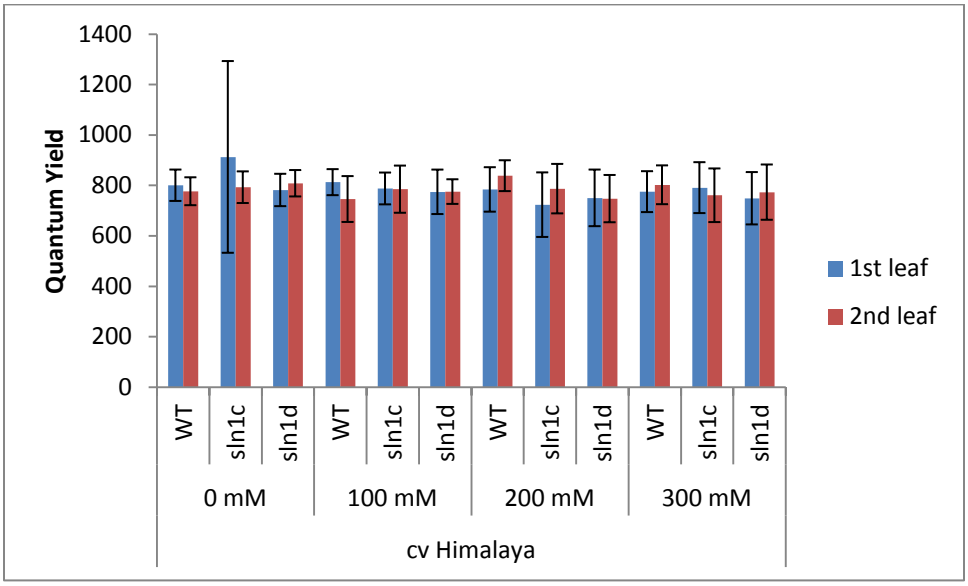
Table 4.5 Median root hair formation in cv Himalaya seedlings after 10 days growth in control (0 mM NaCl) and saline (100, 200, 300 NaCl) conditions. '+' represents moderate root hair formation, '++' extensive root hair formation.

	NaCl concentration			
Genotype	**0 mM**	**100 mM**	**200 mM**	**300 mM**
WT	++	++	++	+
sln1c	++	++	+	+
sln1d	+	+	+	+

Photosynthetic activity

It was noted that leaves of seedlings subjected to salt treatment became chlorotic, especially those subjected to 300 mM NaCl, but the dark green leaves typical of the dwarf and semi-dwarf mutant seedlings (cv Himalaya *sln1d*, *gse1a*, *gse1j*, *gse1n*) appeared less chlorotic than the wild-type leaves. Photosynthetic activity was similar between both the wild-type and mutant genotypes in the Himalaya background. Interestingly no notable differences in photosynthetic activity were observed between treatments (Figure 4.5a). A significant reduction in photosynthetic activity was observed in the second leaf of the cv Herta *sln1-1* mutant compared to the wild-type at 100 mM NaCl (Figure 4.5b).

(a)



(b)

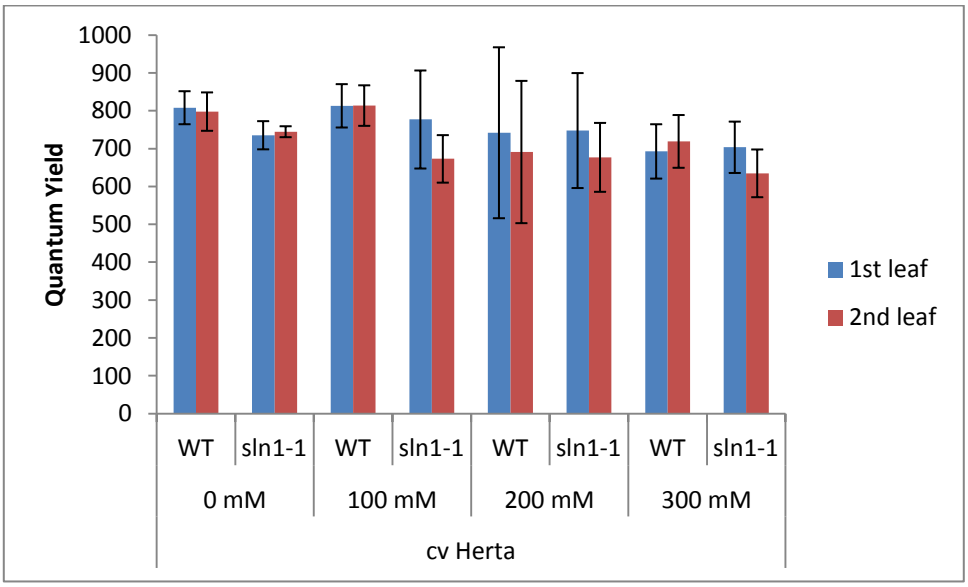


Figure 4.5 Photosynthesis yield analysis of first and second leaves of seedlings after 2 days treatment. Results are shown for (a) cv Himalaya and (b) cv Herta. Bars represent standard deviation.

4.4.7 The effect of DELLA on ion element accumulation under salt stress

A preliminary study was undertaken to determine whether DELLA is important in determining ion uptake through the roots in the presence, or absence, of salt. Ion element analysis was undertaken on cv Himalaya wild-type and the DELLA LoF and GoF mutants (*sln1c* and *sln1d*, respectively) at a single time point (two days) following incubation at 0 mM or 100 mM NaCl. Low salt concentration was used to minimise erroneous measurements arising from extensively damaged roots or shoot material. Although the data are limited to one tissue sample for each genotype per treatment, the analysis revealed differences between the genotypes in terms of sodium (Na^+), phosphorous (P^+), and calcium (Ca^{2+}) accumulation. Element accumulation is shown in ppm in Table 4.6a, and K^+:Na^+ ratios shown in Table 4.6b. The accumulation of Magnesium (Mg^{2+}) and Potassium (K^+) was similar between the genotypes in both root and shoots; for all genotypes levels of Mg^{2+} in the shoots were higher than in the roots in control and NaCl treated seedlings (Table 4.6a). In contrast, the K^+ accumulation in roots and shoots was similar within each genotype although there was an indication that K^+ accumulated to a greater extent in shoots of salt treated *sln1c* plants. In all cases, K^+ levels in roots and shoots were lowered by salt treatment. Na^+ levels in roots and shoots were, as expected, low in the control plants and no clear genotype-specific differences were observed. Following NaCl treatment the Na^+ values increased greatly in both roots and shoot material although both the roots and shoots of *sln1c* plants accumulated much less than those of the wild-type and *sln1d* plants (approx. 8,000 ppm Na^+ compared to more than 30,000 ppm) (Table 4.6a). Genotype-specific differences in the accumulation of P^+ were also identified; of particular note was the low level (approx. 200 ppm) in both roots and shoots of *sln1c* plants treated with 100 mM NaCl. Although this low level was also seen in shoots of *sln1c* control plants, a higher level of P^+ was seen in the roots of these plants (Table 4.6a). For all genotypes the P^+ accumulation was higher in roots than shoots in plants grown under control (0 mM) conditions. Again, the wild-type and *sln1d* plants showed similar patterns of accumulation irrespective of NaCl conditions. Accumulation of Ca^{2+} was higher in the shoots than the roots for the *sln1d* mutant in control (0 mM) and NaCl-treated plants. However, this was not the case in tissues of *sln1c* plants irrespective of the treatment; for this genotype Ca^{2+} levels were lower in the shoots than the roots. For all genotypes salt treatment resulted in lower Ca^{2+} in the shoots, but the difference was much less marked in the

roots of the *sln1c* mutant. Again, the *sln1c* mutant appeared to respond differently from the wild-type and *sln1d* mutant.

Table 4.6 Ion element accumulation in root and shoots in cv Himalaya. Seedlings were either grown in 0.5 x Hoagland's medium (control, 0 mM NaCl) or medium containing 100 mM NaCl. [1] Genotype; [2] No result. Results are presented as (a) parts per million and (b) K^+:Na^+ ratios.

(a)

		Root					Shoot				
Gen.[1]	**NaCl Conc.**	**Na^+**	**Mg^{2+}**	**P^+**	**K^+**	**Ca^{2+}**	**Na^+**	**Mg^{2+}**	**P^+**	**K+**	**Ca^{2+}**
WT	0 mM	156	1211	12013	64840	1468	13	1707	7956	63052	3743
	100 mM	31716	857	7956	63052	3743	33082	936	9241	33151	1458
sln1c	0 mM	469	914	8646	48769	2483	NR[2]	1676	270	49644	1115
	100 mM	7823	807	226	20975	2323	7884	1269	201	30470	956
sln1d	0 mM	506	1262	15208	82545	1281	287	2209	12368	74140	4352
	100 mM	42393	1117	15323	35942	766	31194	1257	12300	38350	1924

(b)

		Root	Shoot
Gen.[1]	**NaCl Conc.**	**K^+: Na^+ ratio**	**K^+: Na^+ ratio**
WT	0 mM	416 : 1	4850 : 1
	100 mM	2 : 1	1 : 1
sln1c	0 mM	104 : 1	NR[2]
	100 mM	3 : 1	4 : 1
sln1d	0 mM	163 : 1	258 : 1
	100 mM	1 : 1	1: 1

4.5 Discussion

The experiments described in this chapter were designed to determine whether the finding (Achard *et al*., 2006) that DELLA proteins were important for survival of salt by *Arabidopsis* was true also in a cereal species. Barley was chosen because it has a single DELLA and appropriate mutants were available. The aim was to replicate, as far as possible, the conditions used by Achard *et al.* (2006), but several modifications had to be made because of the longer life cycle and larger seedlings of barley compared with *Arabidopsis*. Notably, in the Achard study *Arabidopsis* plants were grown in culture plates on agar medium, and this was not feasible for the larger (barley) seedlings (Dr. M. Boulton, personal communication). Instead, hydroponic culture was used to allow investigation of both root and shoot responses. Hydroponic culture has been widely used to investigate salt tolerance and element uptake in cereals (Witzel *et al*., 2009). Space and equipment limitations, along with the longer life cycle and physical size of barley seedlings (which makes downstream processing of samples more difficult) meant that sample sizes were lower than those in the study of Achard *et al.* (2006). These limitations complicated the statistical analyses and advice was sought from Mr James Gallagher, Statistical Services Centre, University of Reading, UK). GA biosynthesis mutants were used in the *Arabidopsis* study, however these were unavailable in the barley backgrounds used in this study. To assess the effect of non-DELLA mutants on salt stress response, GSE1 mutants (equivalent to *Arabidopsis* GID1) were used in the study. This gene is also part of the DELLA signalling pathway, and although other characterised mutants of differing stature were sought, no taller plants were available and other dwarf mutants were either not characterised or were modified in pathways known to impinge on DELLA regulation.

Wild-type and mutant seedlings of all tested genotypes survived treatment at 0 mM NaCl, showing that the hydroponic treatment used in this study was suitable for barley cultivation. Despite a decrease in seedling survival for the *sln1c* genotype at 300 mM NaCl, no statistically significant differences in survival were observed between the wild-type, SLN1 LoF and putative SLN1 stabilising mutant genotypes (Figure 4.2a). This contrasts the results of the Achard study (Achard *et al.*, 2006), in which the *Arabidopsis* DELLA mutant lacking 4 of 5 DELLAs (GAI, RGA, RGL1 and RGL2) showed decreased survival under saline conditions compared to wild-type seedlings (5 and 36 % respectively). In the current study a survival differential was not observed

between wild-type and the putative LoF mutant *sln1-1* (see Section 4.4.2). The absence of a survival differential may be explained by differences in varietal background (cv Herta versus cv Himalaya) or by the different mutations resulting in a SLN1 protein with differing function or stability. Certainly the *sln1-1* plants are sturdier than the *sln1c* plants (see Chapter 3). Experimental conditions were designed based on preliminary experiments designed to optimise experimental (seedling survival) conditions in the Himalaya background, whereas cv Herta seedlings appear to be more salt tolerant. Differential salt tolerance of barley cultivars has been reported widely (Chen *et al*., 2007; Mahmood, 2011). Accordingly, further experiments, not possible within the time constraints of this project, using higher NaCl concentration are needed to determine whether the *sln1-1* mutant differs in salt tolerance from its wild-type parent. Furthermore, the *dwf2* mutant in the H930-36 background exhibited no significant difference in survival after salt treatment compared to the wild-type seedlings (Figure 4.2b). This data is again, inconsistent with the results in the Achard study (Achard *et al.*, 2006), in which the *Arabidopsis* DELLA GoF mutant (stabilised GAI) exhibited a greater level of survival under saline conditions compared to the wild-type (82 and 36% respectively). The survival data obtained in this study therefore suggest SLN1 has no significant effect on plant survival, supporting the null hypothesis (H_0), however the difficulty in obtaining reproducible data between every experiment and the limited sample numbers may account for the lack of statistical significance in the survival data.

Increases in salinity generally resulted in reduced root and shoot growth (both dry mass and length), for all genotypes, with only the root dry mass of *sln1c* and *gse1a* seedlings grown under 150 and 100 mM NaCl conditions respectively, defying this trend (Figure 4.3a, b). Salinity therefore has a detrimental effect on plant growth independent of SLN1 function, as has been widely reported (Munns *et al.*, 2006; Taghipour & Salehi, 2008). Significant differences were observed between the *dwf2* GoF mutant and the wild-type in the H930-36 background, with *dwf2* root and shoot mass significantly less inhibited than the wild-type under saline conditions (Figure 4.3c). Furthermore, shoot growth (length) for the putative SLN1 stabilising mutant *gse1n*, was significantly less inhibited, and root growth (length) for the *sln1c* LoF mutant, was significantly inhibited under saline conditions compared to the wild-type (Figure 4.4b). This provides strong evidence to support the alternative hypothesis (H_1).

The extent to which plant development is governed by DELLA function is likely determined by the interplay between two factors. DELLA inhibits growth under saline conditions, whilst simultaneously protecting plants from the harmful effects of salt, thereby promoting plant survival (and therefore, indirectly, growth). This interplay likely accounts for the difficulty in discerning DELLA-related trends for tissue mass and length. Salinity reduced root number for all tested cultivars (see Section 4.4.5). The *sln1c* (*Sln1* LoF) mutant showed reduced root number compared to wild-type and putative SLN1 stabilising mutants at 0 – 200 mM NaCl conditions (Table 4.3a). Fine root hair formation in both wild-type and mutant genotypes was reduced in line with increasing salinity. Interestingly, fine root hair formation was least prevalent in *sln1d* GoF mutant roots, which is consistent with the DELLA-mediated inhibition of growth observed in *Arabidopsis* (Achard *et al.*, 2006). The limited tiller production during the experimental period, and the low number of *gse1* mutant plants tested prevented statistical analysis to identify genotype-specific responses, but it is tempting to speculate that tiller production in *gse1j* and *gse1n* mutant plants was less affected than for the LoF *Sln1* mutants. A further difference between this study and that of the *Arabidopsis* study was the nature of the genotypes used. The LoF DELLA mutant in the *Arabidopsis* study lacked four of the five DELLAs, meaning that a single DELLA, RGL3, was functional in the mutant, whereas the LoF mutant in barley, *sln1c*, lacks the final 17 aa of a functional single DELLA protein (SLN1) and the protein has no growth repression activity. Although the *Arabidopsis* RGL3 has been implicated in stress tolerance (Achard *et al.*, 2008b), the *Arabidopsis* LoF plants were, like the *sln1c* plants, unable to survive high levels of NaCl.

Ion element accumulation analysis suggest Mg^{2+}, P^{+} and K^{+} uptake by roots is limited in roots under both control and salt stress conditions in the *sln1c* (LoF) mutant compared to the wild-type and *sln1d* (GoF) genotype, suggesting the uptake of essential plant nutrients is limited for the *sln1c* genotype. DELLA LoF mutants have been characterised as having elongated cells (Chandler *et al.*, 2002). If the number of ion channels remains unchanged compared to the wild-type, then the potential for ion uptake may be limited compared to uptake in wild-type root cells. This is largely consistent with observations of uptake by the *sln1d* (GoF) mutant, which has reduced cell size, and largely showed increased ion element accumulation compared to the wild-type under control conditions. Ion accumulation results were part of a preliminary

experiment, and data needs to be verified in additional experiments before definitive conclusions can be drawn.

Despite the increase in chlorosis observed in the SLN1 LoF mutants (*sln1c*, cv Himalaya; *sln1-1*, cv Herta) compared to the wild-type and SLN1 GoF mutants, there was largely no difference in photosynthetic activity between the genotypes. The similarity in photosynthetic activity suggests the decrease in the survival of *sln1c* seedlings is not due to loss of photosynthetic activity. Furthermore, given the low levels of Na^+ in shoot material in the wild-type and *sln1d* GoF mutant, it is similarly doubtful that sodium toxicity *per se* is the cause of decreased survival. The lowest levels of root necrosis were observed in the *sln1d* (SLN1 GoF) and GSE1 mutants, suggesting SLN1 stabilisation provides a high level of protection to seedling roots. It is therefore proposed that the primary cause of plant death is damage to roots.

Chapter 5: The Effect of Transient Extreme Heat Stress on *Sln1* Mutants

5.1 Aims

The aim of the work described in this chapter was to determine whether the salt stress tolerance conferred by stabilised DELLA observed by Achard *et al.* (2006) and in this study (Chapter 4), extended to other forms of abiotic stress. Transient "extreme" heat stress (heat shock, 50 °C, 1 - 4 h) was used to assess the immediate role of SLN1 in stress response, rather than its potential role in acquired tolerance. In addition to the use of the GA signal transduction mutants used in the salt stress experiments, additional height mutants were tested to establish whether plant stature determines survival to heat shock.

The following hypothesise were formulated. H_1: *Sln1* GoF mutants exhibit increased survival and are less susceptible to heat shock compared to the wild-type; Conversely, *Sln1* LoF mutants exhibit decreased survival and increased susceptibility to heat shock compared to the wild-type. H_0: SLN1 has no effect on plant survival or susceptibility to heat shock.

5.2 Introduction

Heat stress due to increased temperature is a growing agricultural problem worldwide, with an increase in the frequency, intensity and duration of seasonal heatwaves predicted as a result of global climate change (Wahid *et al.*, 2007; Ainsworth & Ort, 2010; Christensen *et al.*, 2007). Plants can be preconditioned to tolerate heat stress either through the application of osmoprotectants (e.g. glycinebetaine and proline), or the exposure of plants to environmental stress during the early stages of the plant life cycle (Wahid *et al.*, 2007). Tolerance of long term heat stress requires the integration of genetic heat tolerance traits into agricultural lines (Maestri *et al.*, 2002).

The role of temperature in plant development

Temperature plays an integral role in plant development, acting as an environmental cue for the transition between different developmental stages. Germination is highly sensitive to temperature in many species, with initiation triggered by the destruction of germination

inhibitors in response to either low or high temperature (Walbot, 2011). Transition from the vegetative to the reproductive phase is similarly affected, with temperature being the most important environmental cue for plant flowering (Huijser & Schmid, 2011). Increased temperature triggers the expression of flower inductive pathways (e.g. photoperiod and GA) that increase the expression of a small number of floral integrator genes such as *FT* (*Flowering locus T)*, *SOC1* (*Suppressor of constans1*) and *LFY* (*Leafy*) in *Arabidopsis*. When the expression of floral integrators exceeds the required threshold, plant flowering is initiated (Tooke *et al.*, 2005).

Effect of high temperature on plant development

In contrast to mammalian systems which maintain a constant temperature by homeostasis, plants must be able to function at a range of temperatures (Walbot, 2011). Both short and long term exposure of plants to high temperatures results in a range of morphological and biochemical changes affecting plant growth and development, with the impact of heat stress greatly dependent upon the stage of plant development at which the temperature stress occurs (Wahid *et al.*, 2007). Seedlings in the early vegetative phase (e.g. during early leaf emergence) are less tolerant to high temperatures than more well established plants, as the structures conferring heat tolerance in mature plants are undeveloped in young seedlings (Karim *et al.*, 1999; Walbot, 2011). Furthermore, the enzymes implicated in the breakdown of starch are inactivated under high temperature conditions, preventing the mobilisation of the metabolite reserves required for the seedling to develop (Essemine, 2010). Mature plants are most susceptible to heat damage at the point of transition between the vegetative and reproductive phases, with male pollen development being highly sensitive to even short-term extremes of temperature (Zinn *et al.*, 2010; Walbot, 2011). A moderate increase in temperature above optimal conditions was shown to greatly reduce the number of functional pollen grains in tomato (*Lycopersicon esculentum*), thereby reducing fertility (Sato *et al.*, 2006). In cereals, exposure to supraoptimal temperatures commonly results in a reduction in grain yield due to induced morphological changes, reduced photosynthesis, and early flowering coupled with pollen sterility (Wahid *et al.*, 2007; Essemine, 2010). High temperature reduced basal tillering, the numbers of grains per inflorescence, and single grain weight in pearl millet (*Pennisetum americanum*) (Fussel *et al.*, 1980). In wheat (*Triticum aestivum*), high temperature reduced photosynthetic activity and leaf area, and resulted in a decrease in shoot and grain mass, as well as the mass and starch content of the kernel (Shah & Paulsen, 2003).

Heat tolerance and phytohormones

High temperature disrupts cellular metabolic processes, resulting in the production and accumulation of toxic compounds including ROS, which interfere with the processes required for both photosynthesis and respiration (Essemine, 2010). Although the GA pathway is implicated in heat tolerance in plants, more investigation is required to understand the mechanisms involved (Vettakkorumakankav *et al.*, 1999; Qin *et al*., 2008), however, GA is thought to have a role in the antioxidant pathway that is induced during heat stress (Sarkar *et al*., 2004; Wigoda *et al.,* 2006) and there is evidence for heat-induced changes in expression of GA biosynthetic and signalling genes in both dicots and cereals. Indeed, Qin *et al*. (2008) reported the downregulation of expression of a "RGA homologue" in heat stressed wheat.

DELLAs are believed to act as integrators of heat stress signals, responding to ABA and ethylene signals. ABA is antagonistic to GA function (Weiss & Ori, 2007), inhibiting growth whilst GA promotes growth via DELLA degradation. Transient accumulation of ABA was observed after heat treatment in pea plants (*Pisum sativum*) (Liu *et al.*, 2006) whilst ABA has been shown to confer thermotolerance in maize (Gong *et al.*, 1998) and bromegrass (Robertson *et al.*, 1994). Furthermore the exogenous addition of ABA has been shown to induce heat tolerance in *Arabidopsis* and maize seedlings (Larkindale & Knight, 2002; Bonham-Smith *et al.*, 1988). The importance of ABA function in mediating response to heat stress is illustrated by the response of the *abi-1* mutant of *Arabidopsis*, which lacks a protein phosphatase required for sensing ABA, rendering the mutant ABA-insensitive. When subjected to heat stress, the *abi-1* mutant exhibited decreased tolerance to heat stress compared to the wild-type plants (Larkindale & Knight, 2002). These findings are in agreement with the report (Qin *et al.*, 2008) that the wheat homologues of the *Arabidopsis Arac7* and *Arac10* genes (the negative regulators of ABA-mediated signalling) were downregulated in heat treated wheat. A link between ethylene and heat susceptibility in wheat was reported by Hays *et al*. (2007*)*, with a heat susceptible cultivar showing increased ethylene levels in kernels, embryos and flag leaves whereas a heat tolerant variety showed no change in ethylene production. Other reports have shown ethylene levels to increase in wheat (Balota, 2004) and creeping bentgrass (Larkindale & Huang, 2005) in response to heat stress, with levels rising during the recovery phase post heat treatment. Furthermore, the ethylene-insensitive *Arabidopsis* mutant *etr-1* showed increased susceptibility to heat

damage (Larkindale & Knight, 2002). Achard *et al.* (2003) showed that ethylene has a stabilising effect on DELLA in root cells of *Arabidopsis*, even in the presence of bioactive GA, leading the authors to hypothesise that ethylene confers heat stress tolerance via a DELLA-mediated response. Further investigations have highlighted the importance of DELLAs as integrators of multiple plant growth regulatory inputs converging ethylene, ABA and auxin signals (reviewed by Van Der Straeten *et al.*, 2007). Yet the author accepted that the mechanism was still largely unknown. Considerable research is ongoing which emphasises the cross-regulatory mechanisms in hormonal signalling which in many cases take place at the level of transcriptional regulation (Kuppusamy *et al*., 2008*)*. The integral importance of the (transcription factor) function of the DELLA proteins in these pathways was recently shown by transcriptomic analysis of DELLA responsive genes in *Arabidopsis* seedlings which revealed that the GA pathway directly influenced both the ethylene and auxin pathways and, through additional effects on gene expression, other transcriptional networks (Gallego-Bartoleme *et al*., 2011).

5.3 Materials and methods

5.3.1 Plant material

In addition to barley lines used in salt stress experiments (Chapter 4; cv Himalaya: *sln1c*, *sln1d*, *gse1a*, *gse1j*, *gse1n*; cv H930-36: *dwf2*; cv Herta: *sln1-1*), cv Bowman mutants were used to determine whether seedling height determines survival of heat stress. Four cv Bowman mutants were used in this study, two uncharacterised mutants exhibiting a tall phenotype (*M380*, *M382*; Dr. A. Druka, SCRI, Dundee, personal communication), and two semi-dwarf mutants, a GA-20 oxidase mutant (*M827*; Dr. L. Ramsay, The James Hutton Institute (JHI), Dundee, personal communication) and a brassinosteroid receptor mutant (*M855*; Chono *et al.*, 2003).

5.3.2 Plant growth and heat shock

Growth conditions

Seeds for the mutant barley genotypes listed in Section 5.3.1, and their corresponding wild-types were stratified as described in Section 2.1.3.1, and planted and grown in barley mix soil under CER or growth cabinet conditions (Section 2.1.2).

Heat shock

The duration of the heat shock treatment used in each experiment, ranging from 1 - 4 h, was dependent upon the barley line tested and based on preliminary data collected by Dr. Andrey Korolev (JIC). Seedlings were not watered before heat shock treatment, although soil moisture was checked by touch to ensure soil was not dry. Seedlings were subjected to heat shock when at the 2-3 leaf stage, with one or no tillers emerging (Zadoks stage 12-13/20-21; Zadoks, 1974). Heat shock conditions (50 °C, Humidity 60%, irradiance ~150 µmol m^{-2} s^{-1}) were generated in growth cabinets (MLR Plant Growth Chamber, Sanyo). Cabinet temperature was monitored by observing the cabinet's external electronic temperature display, and by periodic observation of an alcohol thermometer (Russel Scientific, Dereham, UK) within the cabinet. Heat shock was conducted at 6 h into the light cycle. Seedlings from each line tested were placed (positions were randomised) on trays, and during heat shock treatment trays were repositioned on the growth cabinet shelves on an hourly basis to overcome potential positional bias. Seedlings were returned to the CER after heat shock, and were watered at least 1 h after heat shock, to allow time for the soil to cool. Seedlings were grown for a further 3 wks post heat shock under CER conditions (Figure 5.1) and inspected visually during this time.

Figure 5.1 A representative tray of cv Himalaya seedlings (wild-type, *sln1c*, *sln1d*, *gse1a*, *gse1j*, *gse1n*) showing plant development 3 wks post heat shock treatment. Seedlings were randomised on the tray before heat shock and during subsequent growth to negate positional effects.

Sample size

Survival, final plant height, and shoot dry mass were measured three weeks after heat shock using 8 - 48 seedlings of each genotype per treatment. Roots could not be sampled because plants were grown in compost.

5.3.3 Assessment of plant growth

Survival was assessed visually 3 wks after heat shock treatment, with plants defined as dead if leaf and stem necrosis was extensive or total. Prediction values were generated from survival data using general linear models. 3 wks after heat shock treatment, shoot lengths were measured before being separately sampled and frozen in liquid nitrogen and freeze dried (Edwards Modulyo Freeze-Dryer, Edwards Lab, Sandusky, OH, USA) for five days. Samples were weighed to four decimal places and dry mass recorded. Shoot dry mass and length data was collected from individual seedlings, with dead seedlings excluded from analysis. Mean values

were then calculated for each genotype under each treatment, and normalised against the mean value of the control group for each genotype, by expressing growth as a percentage of growth under control conditions (control group growth being equal to 100%). Individual values were used to obtain standard deviation values for shoot dry mass and length in the H930-36 and Herta backgrounds.

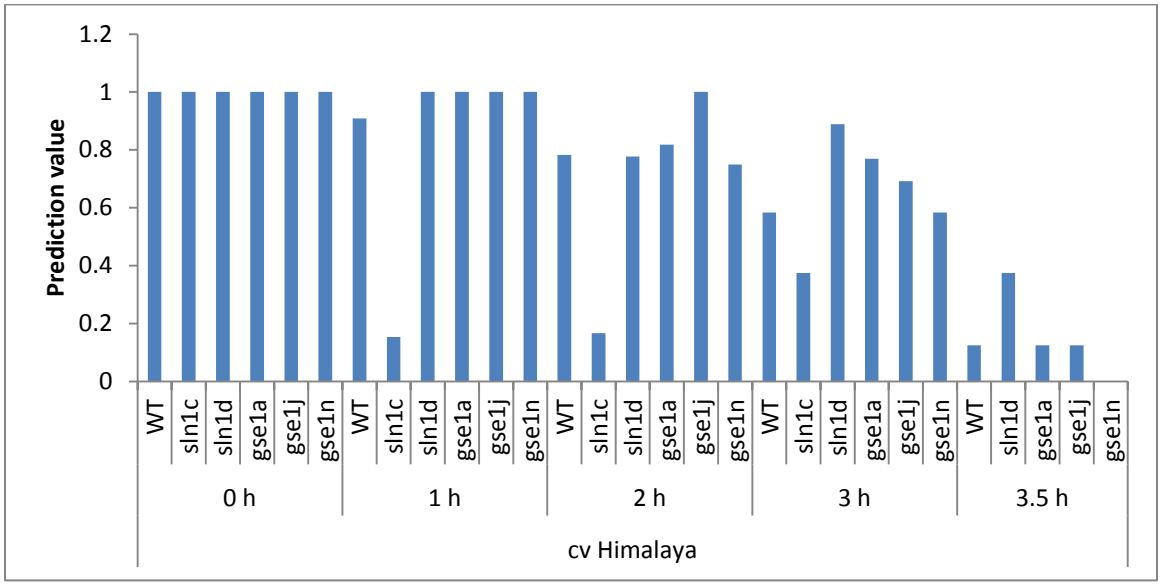
5.4 Results

5.4.1 Seedling survival

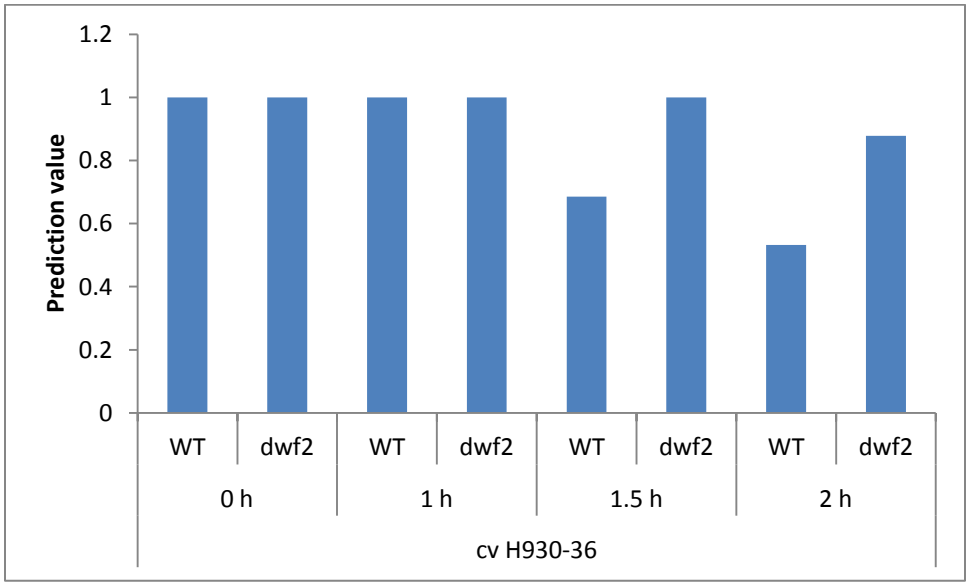
Preliminary data generated by Dr. Andrey Korolev was used to determine the experimental conditions required to generate approximately 50% survival in the wild-type lines of cv Himalaya. Plant survival was measured in further experiments using cv Himalaya (WT, *sln1c*, *sln1d*, *gse1a*, *gse1j*, *gse1n*), cv H930-36 (WT, *dwf2*), cv Herta (WT, *sln1-1*), and cv Bowman (WT, M380, M382, M820, M827). Survival was assessed using general linear models and Mann-Whitney U testing as described for salt treatment (Section 4.4.2). All seedlings, independent of background and genotype, survived control conditions (0 h heat shock). Increasing the duration of heat shock resulted in a general decrease in survival for all genotypes. Survival results for cv Himalaya (Figure 5.2a) were obtained using 8-29 seedlings per genotype per treatment, and heat shock treatments of 1, 2, 3 and 3.5 h. The LoF mutant *sln1c* showed significantly lower survival compared to the wild-type after 1 h heat shock (*P*: 0.014), however differences after 2 and 3 h treatments were not significant (*P*: 0.071 and 0.5 respectively). The *sln1c* mutants were not subjected to 3.5 h heat shock, as seedling numbers were limited. Of the putative SLN1 stabilising mutants, only the *sln1d* mutant at 1 h exhibited significantly increased survival compared to the wild-type (*P*: 0.014). Survival results for genotypes of the H930-36 background (Figure 5.2b) were obtained using 35-47 seedlings per genotype per treatment, and heat shock treatments of 1, 1.5 and 2 h. All of the wild-type and *dwf2* (GoF) mutant seedlings survived 1 h heat shock, therefore no survival differential was observed. Furthermore, no significant differences in survival were observed between the wild-type and *dwf2* GoF mutant genotypes after 1.5 and 2 h heat shocks (*P*: 0.5 and 0.129 respectively). Survival results for cv Herta (Figure 5.2c) were obtained using 6-58 seedlings per genotype per treatment, and heat shock treatments of 1, 1.5 and 2 h. The *sln1-1* LoF mutant showed significantly lower levels of survival

after 1 h heat shock compared to the wild-type (*P*: 0.008), however an equivalently significant survival differential was not observed between the *sln1-1* and wild-type genotypes at 1.5 h (*P*: 0.15). No seedlings in the Herta background survived 2 h heat shock. Survival results for cv Bowman (Figure 5.2d) were obtained using 8-18 seedlings per genotype per treatment. No significant differences in survival were observed between the Bowman genotypes after 3 and 4 h heat shock (*P*: 0.3 – 0.5).

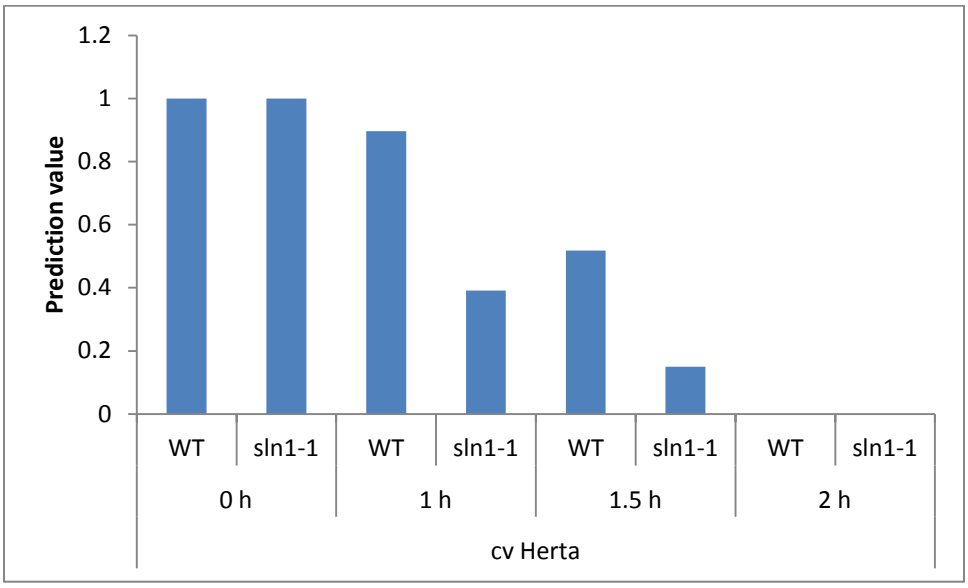
(a)


Prediction value
1.2
1
0.8
0.6
0.4
0.2
0
WT
sln1c
sln1d
gse1a
gse1j
gse1n
0 h
1 h
2 h
3 h
3.5 h
cv Himalaya

(b)


Prediction value
1.2
1
0.8
0.6
0.4
0.2
0
WT
dwf2
0 h
1 h
1.5 h
2 h
cv H930-36

(c)



(d)

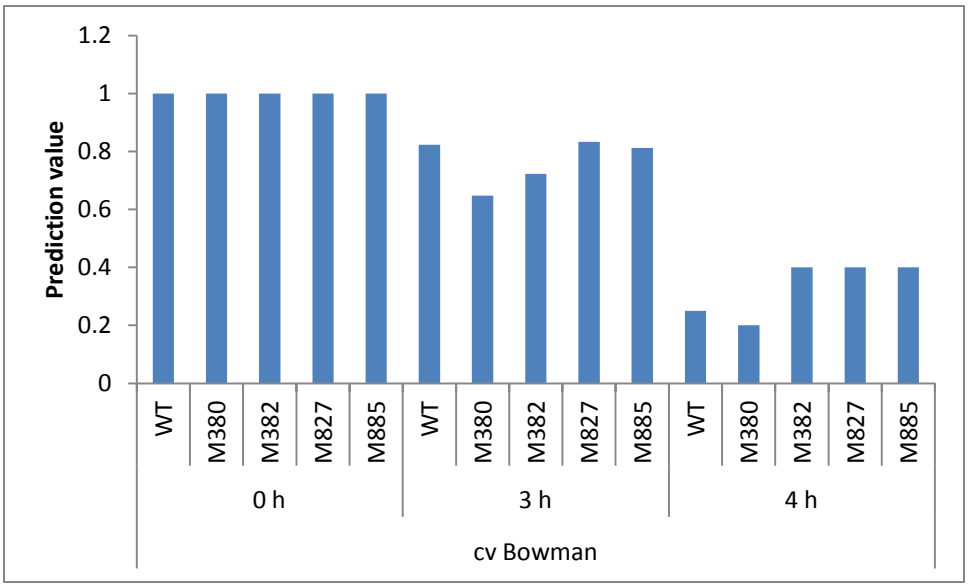
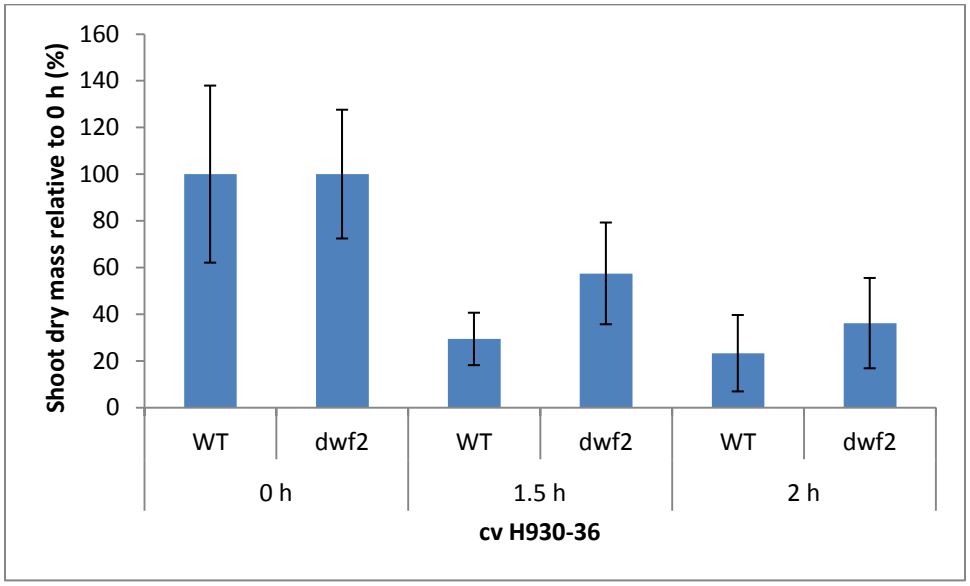


Figure 5.2 Survival values for plants subjected to heat shock or control (0 h heat shock) conditions. Prediction values were generated from survival data using general linear models. Prediction values are shown on the y-axes. Values of 1 represent the prediction of total survival, and 0 the death of all samples of the genotype under the stated treatment condition. Genotype and heat shock duration are on the x-axes. Results are presented for (a) cv Himalaya, (b) cv H930-36, (c) cv Herta, (d) cv Bowman.

5.4.2 Shoot mass

Shoot mass was measured in the H930-36 and Herta backgrounds. Data were obtained for cv H930-36 using 20-38 samples per genotype per treatment and 0, 1.5 and 2 h heat shock treatments (Figure 5.3a). Heat shock led to a decrease in dry mass for both the wild-type and *dwf2* plants. The results suggest that the *dwf2* (GoF) mutant has significantly greater shoot dry mass than the wild-type after 1.5 h heat shock (*P*: <0.001), however there was no significant difference at 2 h. Data were not collected after 1 h treatment. Data were obtained for the Herta background using 17-44 samples per genotype per treatment and 0 and 1 h heat shock treatments (Figure 5.3b). The number of surviving *sln1-1* mutant seedlings was too low after 1.5 and 2 h heat shock treatments to make meaningful comparisons between the mutant and wild-type genotypes. The *sln1-1* mutant showed significantly lower dry mass compared the wild-type after 1 h treatment (*P*: <0.001). At this time point the wild-type showed a relatively low decrease in dry mass after heat shock treatment.

(a)



(b)

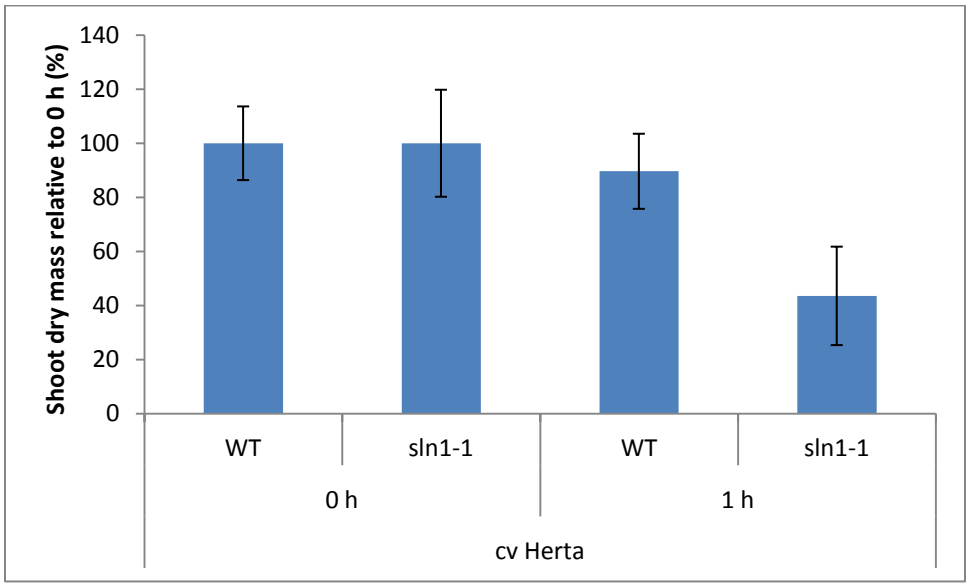
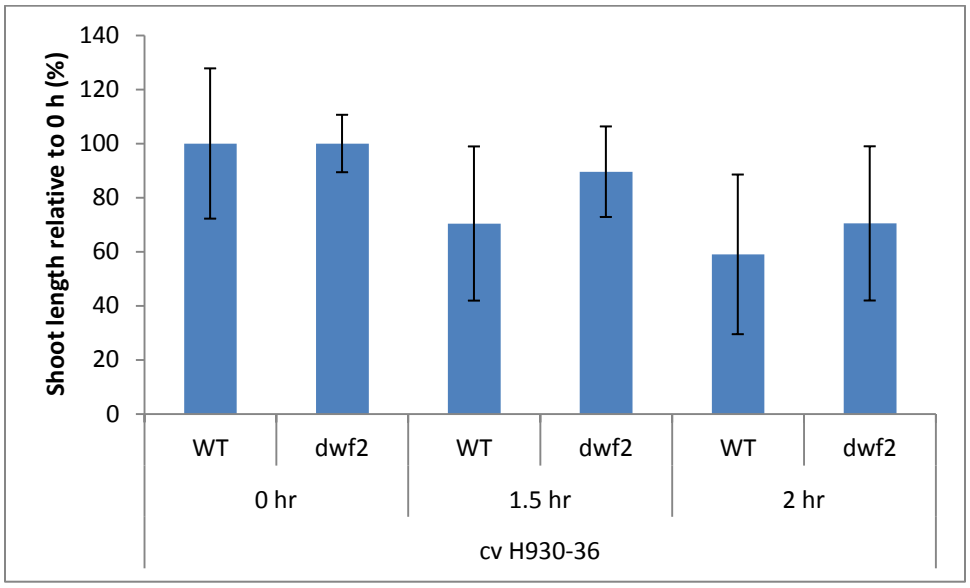


Figure 5.3 Relative shoot dry mass of wild-type and mutant seedlings treated with 0, 1, 1.5 or 2 h heat shock (50 °C). Growth (as represented by biomass) is shown as a percentage of the biomass under control conditions (0 h) for each genotype with values on the y-axes. The duration of heat shock and the seedling genotype are shown on the x-axes. (a) data from cv H930-36 experiment, (b) cv Herta. Bars represent standard deviation.

5.4.3 Shoot lengths

Shoot length was measured in experiments using seedlings of the H930-36 and Herta backgrounds. Data were obtained for the H930-36 background using 24-45 samples per genotype per treatment and 0, 1.5 and 2 h heat shock treatments (Figure 5.4a). Heat shock produced a decrease in mean height for both the GoF *dwf2* and wild-type genotypes. There was no significant difference in shoot length between the *dwf2* and wild-type genotypes after 1.5 and 2 h heat shock (*P*: 0.431 and 0.886 respectively). Data were not collected after 1 h treatment. Data were obtained for the Herta background using 18-52 samples per genotype per treatment and 0 and 1 h heat shock treatments (Figure 5.4b). The surviving LoF *sln1-1* mutant seedlings showed significantly reduced shoot length compared to the wild-type after the 1 h heat shock treatment (*P*: <0.001).

(a)



(b)

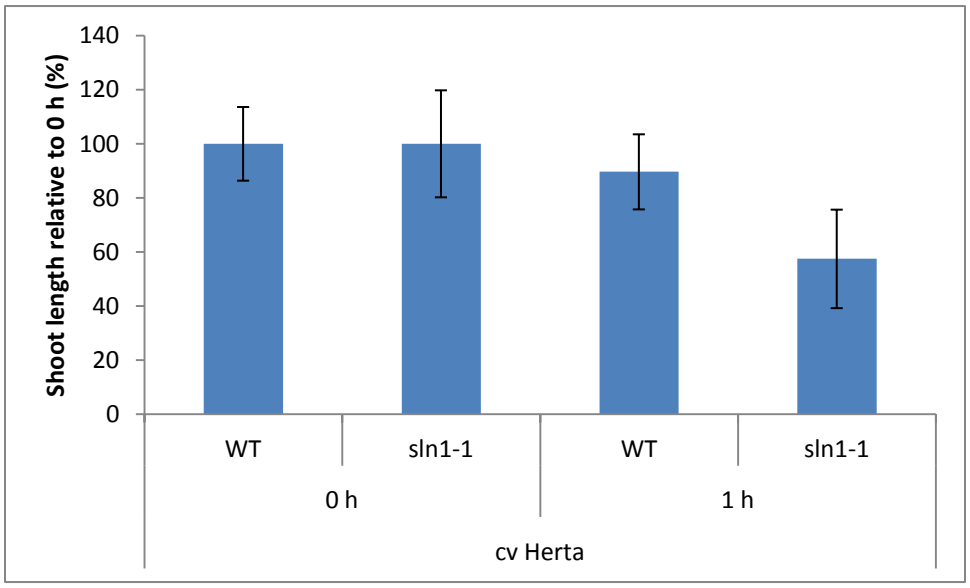


Figure 5.4 Relative shoot length of wild-type and mutant seedlings treated at 0, 1, 1.5 or 2 h heat shock (50 °C). Growth (as represented by length) is shown as a percentage of height under control conditions (0 h) for each genotype. The duration of heat shock is shown on the x-axes, along with the genotype. (a) data from cv H930-36 experiment, (b) cv Herta. Bars represent standard deviation.

5.5 Discussion

In order to extend the investigation of the role of DELLA proteins in abiotic stress tolerance in cereals, heat shock treatment was selected in order to produce high levels of ROS within plant cells (Mittler, 2002) and with the aim of identifying differential survival between genotypes. High levels of ROS were required as DELLAs are believed to confer abiotic stress tolerance by reducing ROS via upregulation of ROS detoxifying enzyme expression (Achard *et al.*, 2006). The high-temperature (50 °C), transient heat stress described in this study was favoured over a prolonged exposure to elevated temperature as long-term exposure to lower temperature could have allowed plants to adapt to the abiotic stress conditions and was unlikely to produce 50% death of wild-type plants (Dr. A. Korolev, personal communication). Furthermore longer term heat stress was likely to result in increased stabilisation of DELLA in wild-type plants, perhaps thereby resulting in levels close to those in the DELLA GoF mutants. The conditions also parallel those used by Sarkar *et al.* (2004), and allowed comparison of their results (where LoF mutants were not used) with the results obtained in this study. The use of heat shock had the advantage of ease of application and for a relatively high number of samples to be tested simultaneously, providing a strong basis for statistical analysis of the results. Other forms of induced oxidative stress were considered, but were rejected based on practicality (e.g. cold stress) or concerns regarding the instigation of non-ROS related DELLA interactions (e.g. UV light treatment and DELLA-PIF interaction).

Heat shock was conducted on seedlings, as young plants were reported to be more prone to heat stress than older, more established plants (Karim *et al.*, 1999; Walbot, 2011). However, a small scale experiment conducted on mature plants (Zadoks stage: 55+; Zadoks, 1974), suggested the mature plants responded to heat stress in a similar way as the young seedlings of the same genotype (cv Himalaya: wild-type, *sln1c*, *sln1d*), (data not shown). The use of seedlings was also desirable due to practical constraints imposed by the size of the heat shock cabinets, and the availability of growth space. Constant environment space restrictions also meant that the number of SLN1 LoF plants (cv Himalaya: *sln1c*; cv Herta: *sln1-1*) was always limited due to the recessive nature of the mutants (see Chapter 3), and the low germination of the mutant seedlings, which required large numbers of seed from heterozygous plants to be germinated.

The focus of the work in this chapter was to establish whether SLN1 function confers heat shock tolerance at the cellular level, therefore efforts were made to limit the impact of plant stature and phenotype when assessing plant survival. As SLN1 function is intrinsically linked to plant phenotype, this was difficult to achieve. Furthermore there is limited availability of height mutants with lesions not implicated in the GA pathway. Plant stature affects tolerance to heat and abiotic stress (Patel & Franklin, 2009; Sarkar *et al*., 2004), as does leaf morphology (Chaves *et al.*, 2003), and the use of seedlings went some way to negating the effect of plant stature. The cv Bowman mutants of differing stature showed no significant differences in survival as a result of heat shock (Figure 5.2d), suggesting plant stature *per se* does not determine survival under the conditions used in this study. Caution must however be applied when comparing the results of the Bowman background with results of stature mutant survival in the Himalaya, H930-36 and Herta backgrounds. None of the cv Bowman mutants were severely dwarfed (M827 and M885 were semi-dwarfs), nor did the "tall" exhibit a true slender phenotype (M380 and M382 were tall rather than slender). The genetic basis for the M827 and M885 mutants are known (GA-20 oxidase and brassinosteroid receptor mutants respectively), with both likely to result in stabilised SLN1 through mediation of GA levels (Dr. L. Ramsey, JHI, personal communication; Chono *et al.*, 2003), however, the data do not suggest the mutations confer a statistically increased tolerance to heat shock under the conditions used (Figure 5.2d). Although these findings tend to support the conclusion of Sarkar *et al.* (2004) that short stature in barley resulting from reduced GA levels (or from reduced sensitivity to GA) leads to abiotic stress tolerance, the link of the M827 and M885 mutants to the GA – DELLA pathway and the lack of characterisation of the lesions for the tall phenotypes exhibited by M380 and M382 mean that the importance of stature versus DELLA function in heat stress tolerance cannot be clearly dissected.

Based on the survival under salt stress of DELLA GoF and LoF mutants of *Arabidopsis*, it was hypothesised that SLN1 stability and function would confer increased survival to heat stress. The results of the heat shock experiments support this, with the *sln1d* GoF mutant exhibiting increased survival, and the *sln1c* LoF mutant exhibiting significantly decreased survival (*P*: 0.014) after 1 h heat shock compared to the wild-type (Figure 5.2a). Furthermore, the *sln1-1* LoF mutant showed significantly decreased survival after 1 h heat shock compared to the wild-type (Figure 5.2c; *P*: 0.008). A greater survival differential was observed in heat shock experiments

compared to the salt stress experiments, perhaps because of the larger number of plants tested, or because the smaller experiments resulted in fewer variables such as position effects in the cabinet. The survival data supports the alternative hypothesis (H_1). The need to process large numbers of samples meant that shoot length and dry mass analysis had to be limited and was focused on the H930-36 and Herta backgrounds, for which there is a single GoF and LoF *sln1* mutant genotype, respectively, in each background. Furthermore, the *sln1-1* phenotype was less severe than that of the s*ln1c* plants (plants grew slightly more strongly and appeared to have slightly thicker leaves, Chapter 3), which would go some way to diminishing the effect of plant morphology on the data. Growth data (dry mass and length) was collected only from surviving samples in order to investigate how SLN1 affects plant recovery and growth following transient heat stress. The *dwf2* GoF mutant showed significantly increased shoot biomass accumulation at 1.5 h compared to the wild-type (*P*: <0.001; Figure 5.3a), suggesting the stabilised DELLA in these plants protected them from heat shock and allowed them to better maintain their growth characteristics. Conversely, the *sln1-1* LoF mutant showed significantly reduced biomass accumulation and shoot length at 1 h compared to the wild-type (*P*: <0.001; Figures 5.3b, 5.4b), suggesting that even when these plants survived heat stress, they showed limited recovery and were unable to "benefit" from the lack of DELLA to recommence growth. These findings support the alternative hypothesis (H_1).

It is possible that DELLA confers stress tolerance in DELLA stabilising mutants such as *dwf2*, by reducing the effects of oxidative damage, whereas potential stabilisation of DELLA in wild-type plants is relatively delayed and unable to provide rapid protection. However, it is likely that the potential stabilisation was then also a cause of the reduced growth in these plants compared to non-treated controls. It is unfortunate that anti-SLN1 antiserum was no longer available (and attempts by collaborators to produce more failed) since direct measurement of the protein levels would have been informative. It is likely that the removal of dead samples from the analyses reduced the significance of genotype differences even for the *dwf2* (cv H930-36) GoF mutant. For example, removal of dead samples from cv H930-36 1.5 and 2 h treatment groups reduced the total sample size for analysis from 35 to 24 seedlings, and 47 to 25 seedlings respectively, with most dead plants being from the wild-type group. It was interesting to note that highly damaged seedlings of the wild-type and GoF (but not LoF) mutants produced new shoots from the meristem or from emerging tillers. Since SLN1 is localised in growing tissue

(Chandler *et al.*, 2002), the presence of high levels of DELLA, even in wild-type plants, may protect meristem tissue from heat shock damage, allowing some seedlings to survive despite widespread death to older leaf and stem tissue.

Chapter 6: Silencing *Sln1* Expression

6.1 Aims

The aim of the work described in this chapter was to produce transgenic lines with downregulated expression of *Sln1* in order to assess the effect of reduced SLN1 on plant growth and development. This work was undertaken as there were no barley plants available which had either a complete knockout of the wild-type gene or known reduced expression. This chapter describes the construction and use of RNAi (hairpin construct) designed to give a range of silencing levels for the *Sln1* gene in barley.

The following hypothesise were formulated. H_1: significant downregulation or the complete knockout of *Sln1* expression results in altered plant development; H_0: significant downregulation or complete knockout of *Sln1* expression has no effect on plant development.

6.2 Introduction

6.2.1 Applications of transgenic technology

Plant transformation has allowed both greater insight into the fundamental mechanisms of plant function and the direct improvement of commercial crop species (Bartlett *et al.*, 2008). Economic and agriculturally beneficial traits including reduced stature, increased yield, altered plant and seed composition, and biotic and abiotic stress tolerance have been introduced to crop lines (Dayan *et al.*, 2010; Dunwell, 2000). Modification of flowering time has allowed growth patterns to be exploited to increase seasonal yield, change growing season to secure better market opportunities, or to extend the plant vegetative stage if the desired product is foliage rather than grain or fruit (Richards, 2000; Salehi *et al.*, 2005). Silencing of GA signalling pathway genes has been used in monocots, and extensively in dicots, to determine how the loss of pathway components affects plant growth and development. Barley is an ideal system for translating transgenic research from dicots (*Arabidopsis* and tobacco), to cereals, as barley is amenable to transformation, and barley DELLA (SLN1) is encoded by a single gene (*Sln1*).

6.2.2 Transgenic adaptation of the GA-DELLA signal transduction pathway

Studies using transgenic plants have provided a greater insight into DELLA function and structure. Overexpression of DELLA in wild-type and mutant lines allows the affect of altered DELLA levels on plant development to be observed, whilst the expression of mutant DELLA genes allows the identification of key protein functional regions and motifs. The tagging of DELLA proteins using fluorescent proteins (GFP) has allowed the sites of intracellular DELLA accumulation and degradation to be visualised.

Transgenic regulation of GA-DELLA signal transduction pathway components

The effect of DELLA on plant growth has been investigated through the expression of the *Arabidopsis* DELLA gene (*RGA*) tagged with Green Fluorescent Protein (GFP) expressed in wild-type, and the DELLA *rga* LoF mutant. Constitutive expression of the GFP-RGA fusion protein under the 35S promoter in wild-type plants repressed GA signalling more efficiently than expression under the native promoter (Silverstone *et al.*, 2001), whilst expression in the mutant lines (lacking functional RGA) resulted in a reversion from the mutant to the wild-type phenotype, thereby confirming RGA as a GA-mediated inhibitor of growth. An analogous study was conducted in rice by Itoh *et al.*, 2002. Expression of SLR1-GFP in transgenic rice resulted in the development of mild dwarf plants (60 – 80% of the height of wild-type plants), which were responsive only to the addition of GA_3 at high concentration (100 µM). SLR1-GFP expression in the *slr1-1* slender mutant rescued the mutant phenotype to that of the wild-type (Itoh *et al.*, 2002). Expression of *GAI*, the *Arabidopsis* DELLA orthologous to *SLR1*, also resulted in a dwarf phenotype when expressed in wild-type rice (Fu *et al.*, 2001). Furthermore *GAI* expression was linked to the extent of the dwarfism, with higher expression (conferred by the ubiquitin promoter as opposed to the 35S promoter) resulting in a more extreme phenotype, supporting the relief of restraint model proposed by Harberd *et al.*, 2003 (Figure 1.2).

In addition to overexpression of DELLA, the effect of increased GID1 expression has been explored in rice. Overexpression of GID1 which mediates DELLA degradation in the presence of GA (see Section 1.4.2) produces a GA hypersensitive phenotype. The GA hypersensitive response was identified through the growth of second leaf material, which was highly responsive to the addition of exogenous GA compared to control plants. The slender-like

phenotype characterised by the development of long, light green leaves, poor fertility, and decreased tiller formation, likely results from increased degradation of DELLA, and the subsequent loss of growth inhibition (Ueguchi-Tanaka *et al.*, 2005).

Components of the GA-DELLA signal transduction pathway can be silenced using transgenic methods. Antisense silencing of the single endogenous DELLA in tomato, *SIDELLA*, produced plants with a slender-like phenotype, elongated flower trusses and parthenocarpic small fruit (Martí *et al.*, 2007).

Expression of DELLA mutant genes

The importance of conserved motifs in GA recognition in cereals was identified in rice (*Oryza sativa*) by Itoh *et al.*, 2005. The expression of transgenes with altered DELLA and TVHYNP motifs, and alteration to the space between these motifs, resulted in a GA insensitive severe dwarf phenotype. Transgenic expression of mutant GA-insensitive DELLA GoF mutant proteins in transgenic plants results in growth inhibition. Expression of the *rgl1* and *Atgai*^{del}, in *Arabidopsis* and tomato (*Solanum Lycopersicum* L.) respectively, produced severe GA-insensitive dwarf phenotypes, with the growth restraint in tomato equivalent to that of GA-deficient mutants (Marti *et al.*, 2007). Transgenic *Arabidopsis* exhibited delayed bolting and under-developed trichomes and flowers compared to control lines (Wen & Chang, 2002), whilst tomato lines were partially sterile with compacted inflorescences. Expression of the GA-insensitive GoF mutant *Arabidopsis* DELLA allele *gai* in transgenic rice resulted in a GA insensitive dwarf phenotype. As with *GAI* expression, phenotype was linked to the strength of expression, with *gai* driven by the ubiquitin promoter resulting in a more severe dwarf phenotype than expression under the 35S promoter (Fu *et al.*, 2001).

6.2.3 Post-transcriptional gene silencing

Mechanism of RNA interference (RNAi) based silencing

Post-transcriptional gene silencing (PTGS) is ubiquitous to eukaryotic organisms, providing roles in defence against viruses, condensation of chromatin into heterochromatin and regulation of

gene expression (Qi & Hannon, 2005; Sharp, 2001; Baulcombe, 2004). PTGS allows the translation of one or more genes to be downregulated or suppressed entirely via the targeted action of sequence specific antisense RNA. A common mechanism of PTGS is RNA interference (RNAi), which is initiated when sense and antisense strands form a double helix of long double-stranded RNA (dsRNA), and interact with RNA Dicer, (a ribonuclease (RNase) III enzyme). The dicer cuts dsRNA (typically >200 nt) into small interfering RNAs (siRNAs, typically 20-25 nt) and micro RNA (miRNA) that target homologous mRNAs for destruction via an RNA-induced silencing complex (RISC).

Transgenic application of gene silencing

Elucidation of the RNA interference (RNAi) silencing pathway has allowed specific target genes to be downregulated or suppressed entirely through the expression of silencing constructs. Dicer activity is dependent on a stem loop structure in the secondary structure of RNA, formed by the expression of inverted repeat sequences of the gene separated by an intron. These hairpin forming structures are naturally encoded in genomic DNA, however artificial structures can be expressed transgenically in order to silence a gene of interest.

Transgenic induction of the RNAi silencing mechanism has been shown to work in both dicots and cereals, including hexaploid wheat and barley, and has been used extensively as a tool for functional genomics, allowing the linkage of gene and protein function to plant phenotype (McGinnis *et al.*, 2005). RNAi has advantages over GoF and LoF approaches in the study of gene function, as it allows silencing of multigene families, and in polyploids such as hexaploid wheat it allows homeologous genes to be silenced (Travella *et al.*, 2006).

Transgenic utilisation of the RNAi silencing pathway has provided agricultural and commercial benefits. One practical application for the RNAi silencing pathway has been in conferring resistance to disease, as shown by the immunity conferred to barley yellow dwarf virus through the expression of the virus derived transgene in a silencing hairpin structure (Wang *et al.*, 2000). Further examples of RNAi induced resistance to disease are seen in papaya with resistance to Papaya Ringspot Virus (PRSV), and transgenic potatoes with resistance to Potato Leafroll Virus and Potato Virus Y (PVY), (Fuchs & Gonsalves, 2007; Lawson *et al.*, 1990; Eamens *et al.*, 2008). In

addition to virus resistance, RNAi has been used to enhance crop qualities, highlighting the commercial applications of the RNAi silencing mechanism.

6.3 Materials and Methods

6.3.1 Production and cloning of the *Sln1* fragment

PCR amplification of the *Sln1* fragment

The hairpin construct designed to downregulate endogenous *Sln1* gene expression required an insert of 150-500 bp, consisting of 5' untranslated region (UTR) and 5' ORF sequence. 5' sequence was required to ensure SLN1 specificity (i.e. to prevent silencing of orthologous, non-target GRAS proteins). Additionally, 5' prime sequence has been shown to produce a lower efficiency of silencing compared to 3' sequence (Helliwell & Waterhouse, 2005), which was most appropriate, as total silencing was not desired due to concerns that full *Sln1* silencing would be lethal to seedling development. To produce an appropriate *Sln1* product, PCR amplification and a *Sln1* specific primer pair (Table 6.1) were used. Barley (cv Himalaya) genomic DNA, isolated using DNeasy Plant Minikit (Qiagen), was used as template DNA. PCR was conducted using GoTaq® DNA Polymerase (Promega) with the following cycling conditions: 94 °C for 3 min; then 10 cycles of 94 °C for 15 s, 59 °C for 15 s (decreasing by 0.5 °C per cycle) and 72 °C for 30 s. This was followed by 25 cycles of 94 °C for 15 s, 54 °C for 15 s, 72 °C for 30 s, followed by a final extension of 72 °C for 10 min. The expected product should be 468 bp, consisting of 274 bp of 5' UTR and 194 bp of 5' ORF sequence. The success of the amplification and the size of the *Sln1* product were assessed by electrophoresis and comparison against Quick-Load® 100 bp DNA ladder (New England BioLabs).

Table 6.1 Primers used to produce the *Sln1* insert. [(a)]'F' denotes forward, and 'R' reverse primer orientation. [(b)]Primer binding site coordinates are presented relative to the first nucleotide of the *Sln1* initiation codon ('A' = co-ordinate 1), '-' refers to the number of bases upstream of this point. The reference sequence used was *Sln1* of Himalaya (see Appendix 2).

Orientation [(a)]	Primer binding site [(b)]	Sequence (5'-3')
F	-274 to -255	CACACCACTATGCCCAGATG
R	175 to 194	TCGAGCTGCTCCAGCTTCTG

The *Sln1* silencing insert was cloned into pCR®8/GW/TOPO® TA using the pCR®8/GW/TOPO Cloning Kit (Invitrogen) and Library Efficiency® DH5α™ cells (Invitrogen). Cloning was conducted according to the manufacturer's instructions, with the following modifications: the ligation reaction was incubated at rt for 30 min instead of 5 min, and cells were heat-shocked for 35 s instead of 45 s. Transformed cells were propagated on spectinomycin (50 mM) selective LB-G plates (see Appendix 1.4) at 37 °C overnight.

The presence and orientation of the *Sln1* insert was confirmed by colony PCR (see Section 2.2.5) using the vector specific forward primer M13F-20 (GTAAAACGACGGCCAGT), and the reverse primer used to produce the silencing insert (Table 6.1). The following cycling conditions were used: 95 °C for 5 min; then 35 cycles of 95 °C for 30 s, 47 °C for 30 s, 72 °C for 30 s, followed by a final extension of 72 °C for 10 min. The size of the product was assessed against Quick-Load® 100 bp DNA ladder (New England BioLabs), with a product of 612 bp indicative of successful cloning of an insert in the appropriate orientation. It was estimated that 50% of the colonies would contain the insert in the desired orientation.

Selected colonies were grown with shaking in overnight culture (LB-G, spectinomycin 50 mM) at 37 °C, before plasmids were isolated (see Section 2.2.6), and the integrity of the insert confirmed by sequencing using vector specific primers M13F-20 and M13R (CAGGAAACAGCTATGACC) and the primers used to produce the *Sln1* insert (Table 6.1). Sequencing was carried out by the The Genome Analysis Centre (TGAC). The selected clone was designated pENTRYSln1.

6.3.2 *Sln1* plant gene silencing vector construction

The *Sln1* silencing vector for plant transformation was created using the Gateway® system (Invitrogen). The LR reaction in which the entry vector was recombined in a site-specific reaction (based on attL recombination sites) with the destination vector pBract207 was performed according to manufacturer's instructions, with the exception that the LR reaction was incubated for a minimum of 2 h at rt instead of 1 h. pENTRYSln1 DNA was combined with the destination

plasmid (http://www.bract.org) designed to provide a hairpin-based silencing vector in the LR clonase mix, with the resulting construct being designated pBract207Sln1-SC1 (Figure 6.1)

E. coli (DH5α) was transformed with the mix and recombinants selected by plating on LB-G containing 50 µg/mL kanamycin. Insertion of two copies in the destination vector in opposite orientations results in the deletion of both copies of the *ccdB* gene which prevents growth in *E. coli*, allowing selection of the desired clone. Selected colonies were grown with shaking in overnight culture (LB-G, spectinomycin 50 mM) at 37 °C, before plasmids were isolated (see Section 2.2.6).

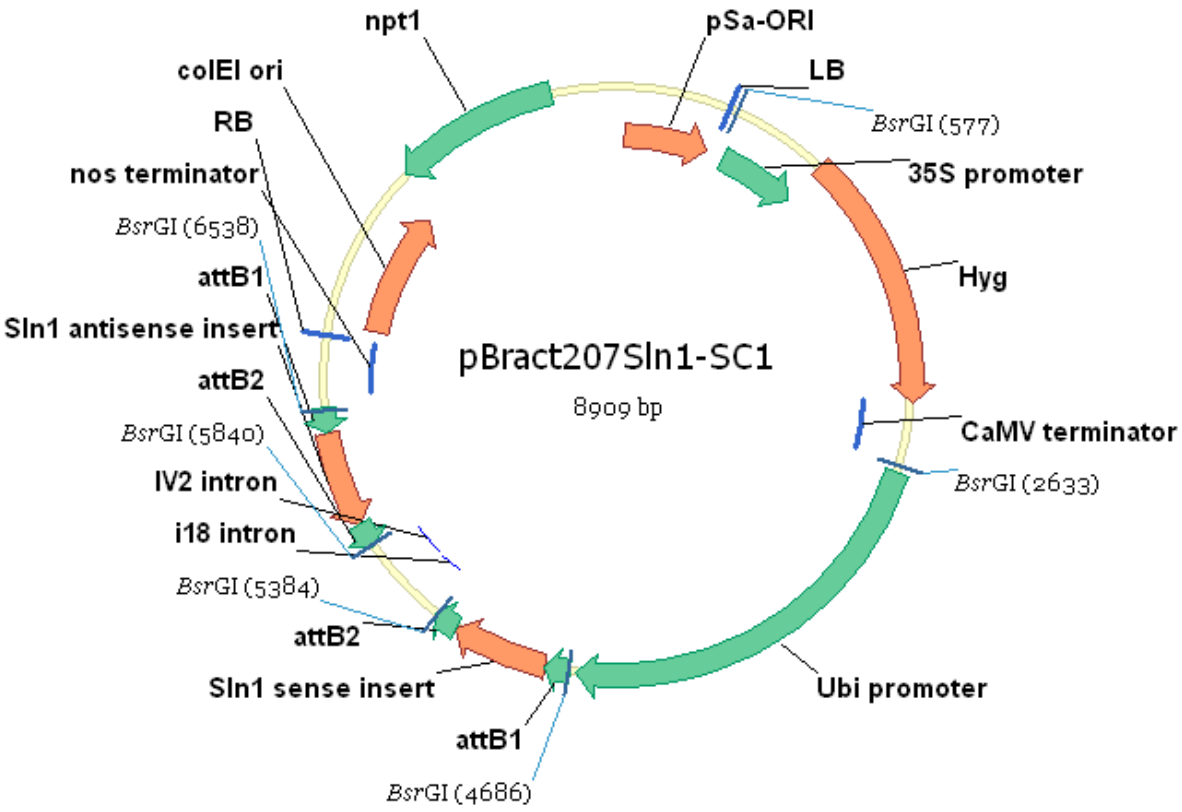


Figure 6.1 RNAi silencing vector pBract207Sln1-SC1. RNAi cassette expression is driven by the ubiquitin promoter, providing strong, constitutive expression. The *Hyg* resistance gene is under constitutive expression by the 35S promoter. Arrows denote gene promoter orientation.

6.3.3 Transformation of *Agrobacterium tumefaciens*

The pBract vectors are based on pGreen and were therefore co-transformed into *Agrobacterium* with helper plasmid pSoup (Hellens *et al.*, 2000). The pBract202 (containing the hygromycin resistance gene, *Hyg*) was used as a transformation control (Figure 6.2).

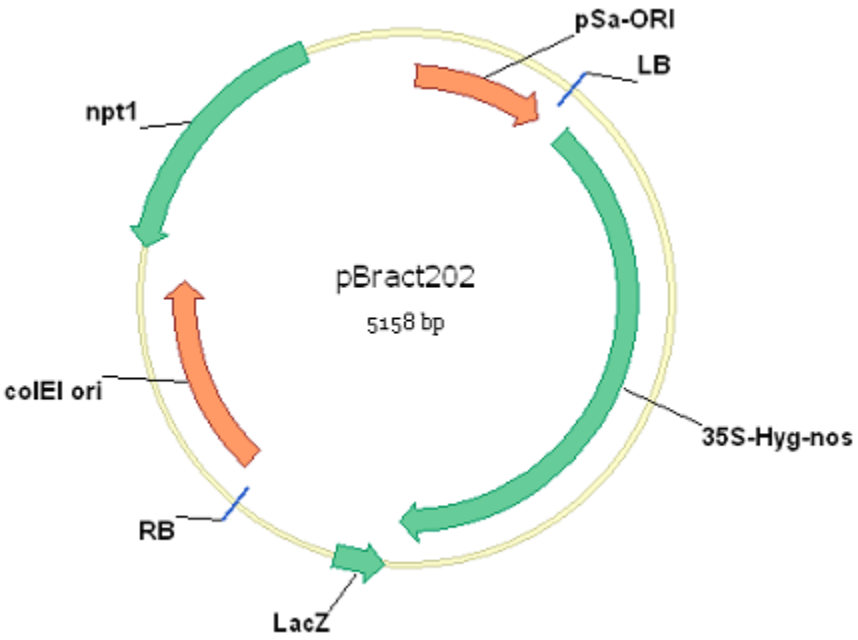


Figure 6.2 The pBract202 construct used in the production of control lines. pBract202 contains the *Hyg* resistance gene under constitutive expression by the 35S promoter. Arrows denote gene orientation.

***Agrobacterium* transformation**

Electroporation competent *A. tumefaciens* (strain AGL1) cells, prepared as described by Harwood *et al.*, 2008 were provided by Mr. M. Smedley, JIC. Transformation of *Agrobacterium* was performed using electroporation. 100 ng of pBract207Sln1-SC1 and 1 µL pSoup (50 ng) was gently mixed with 45 µL competent cells in a pre-chilled electroporation cuvette (2mm gapped, AnaSpec, Fremont, CA, USA), and electroporated at 2.5 V. The electroporation mix was immediately added to 250 µL LB (Invitrogen), at rt (and gently shaken at rt for 4 h. An aliquot (100 µL) of the suspension was spread on LB-G plates (see Appendix 1.4) with rifampicin and

kanamycin selection (both at 50 µg mL^{-1}). Plates were incubated at 28 °C for two days to allow recombinant *Agrobacterium* clones to grow.

Confirmation of pBract207Sln1-SC1 status in *Agrobacterium*

In order to confirm the presence and orientation of the inserts, and the entirety of the pBract207Sln1-SC1 vector in *Agrobacterium*, extracted plasmid was back-transformed into *E. coli*. Plasmids were isolated from *Agrobacterium*, and transformed into DH5α™ (see Section 2.2.3), and propagated on spectinomycin (50 mM) selective LB-G plates (see Appendix 1.4) at 37 °C overnight. Selected colonies were grown with shaking in overnight culture (LB-G, spectinomycin 50 mM) at 37 °C, before plasmids were isolated (see Section 2.2.6). To confirm that all parts of the pBract207Sln1-SC1 construct were present, a restriction digest was run using the restriction enzyme *Bsr*GI. Confirmation of insert orientation was provided by colony PCR using sense (UbiProF1, i18intronR1) and antisense (IV2intronF1, NostermR1) primer sets (Table 6.2). The following cycling conditions were used: 95 °C for 5 min; then 38 cycles of 95 °C for 30 s, 59 °C for 40 s, 72 °C for 45 s, followed by a final extension of 72 °C for 10 min. Product size was assessed by agarose gel electrophoresis and comparison with HyperLadder™ I (Bioline), with a band of 903 bp produced by sense primers and a band of 820 bp produced by antisense primers indicative of a successful LR reaction and cloning.

Table 6.2 Primers used to confirm the presence and orientation of the *Sln1* insert. [(a)]Orientation of the *Sln1* insert in the pBract207Sln1-SC1 vector. [(b)]'F' denotes forward, and 'R' reverse primer orientation.

Primer	Insert orientation [(a)]	Primer orientation [(b)]	Sequence (5'-3')
UbiProF1	Sense	F	ATGCTCACCCTGTTGTTTGG
i18intronR1	Sense	R	CATCGTTGTATGCCACTGGA
IV2intronF1	Antisense	F	CCAAAATTTGTTGATGTGCAG
NostermR1	Antisense	R	TGTTTGAACGATCCTGCTTG

Preparation of standard *Agrobacterium* inoculums

The method of Tingay *et al.* (1997) was used to prepare a standard inoculum for transformation. Standard *Agrobacterium* inoculum was produced from single colonies in modified MG/L medium (with rifampicin and kanamycin selection, Appendix 1.4; Garfinkel & Nester, 1980) incubated for 40 h at 27 °C. A standard inoculum was prepared by adding 200 µL of culture to 200 µL of 15% aqueous glycerol in a microcentrifuge tube, and kept at rt for 6 h before being transferred to -80 °C. A 400 µL aliquot of standard inoculum was removed from -80 °C storage, added to 10 mL of MG/L medium without antibiotics, and incubated overnight at 28 °C with shaking at 200 rpm to produce *Agrobacterium* suspension.

6.3.4 Production of embryos for transformation

Immature seed sterilisation

Immature barley seed was collected from donor plants grown as described in Section 2.1.2. Seed sterilisation was performed as described by Harwood *et al.* (2008). Briefly, barley spikes were collected when the immature embryos were approximately 1.5 – 2 mm in diameter. All subsequent steps were performed in a laminar flow hood. Immature seeds were removed from the spike and sterilised by washing in 70% ethanol for 30 s followed by three washes in sterile distilled water. A 50% (v/v) solution of sodium hypochlorite (sodium hypochlorite solution, Sigma Aldrich) was then added and seeds incubated for 4 min. Finally, seeds were washed four times in sterile distilled water, and left wet in a sterile screw top jar ready for the isolation of embryos.

Isolation of immature embryos and induction of callus

Under sterile conditions, sterilised immature seeds were opened with a pair of fine forceps. The embryonic axis was removed from the immature embryo, before the immature embryo was plated scutellum side up on BCI medium (Appendix 1.3). Up to 25 embryos were placed on each 9 cm Petri plate. The immature embryos were stored overnight in an incubator (24 °C), before transformation with *Agrobacterium*.

6.3.5 Barley transformation

Transformation of embryos

Barley transformation was performed as described by Bartlett *et al.* (2008). A small drop of *Agrobacterium* suspension was added to each of the immature embryos on a plate. The plate was then tilted to allow any excess *Agrobacterium* suspension to run off. Immature embryos were then gently drawn across the surface of the medium (to remove excess *Agrobacterium*), before transfer to a fresh BCI medium plate, scutellum side down. Embryos and *Agrobacterium* were co-cultivated in the dark for 3 days at 23 – 24 °C.

Selection of Transformed Material

Selection of transformed material was performed as described by Harwood *et al.* (2008). Hygromycin-resistant transformants were selected by transferring embryos to selective BCI medium plates containing 50 mg L^{-1} hygromycin and 160 mg L^{-1} Timentin (Duchefa). Inoculated embryos were cultured for 2 wks at 23 – 24 °C in the dark (selection 1), then transferred to fresh selective BCI medium plates on a 2 week basis (selection 2 and 3) and cultured under the same conditions as selection 1. During this 6 wk period, callus showing no development or severe *Agrobacterium* contamination, were discarded. After 6 wks selection on BCI medium, developing callus was transferred to selective BT medium (50 mg L^{-1} hygromycin and 160 mg L^{-1} Timentin), (Appendix 1.3), and developed for 2 wks under low light conditions, achieved by covering plates with a thin sheet of paper in the tissue-culture room (conditions described in Section 2.1.2).

Regeneration of transgenic plants

Regeneration of transgenic plants was performed as described by Harwood *et al.* (2008). After 2 wks on BTM, embryo-derived material was transferred to selective BR medium (50 mg L^{-1} hygromycin and 160 mg L^{-1} Timentin) in deep Petri dishes (Appendix 1.3). Callus was observed on a bi-weekly basis; with callus development efficiency calculated as the number of calli exhibiting shoot initiation during development on BR medium, as a proportion of the original number of inoculated embryos. Plantlets were grown in tissue culture rooms, under normal light conditions, as described in Section 2.1.2. Once shoots were 2-3 cm in length and roots had developed, the small plantlets were transferred to glass culture tubes (Sigma Aldrich), containing approximately 12 mL of selective BCI medium (with hygromycin and Timentin

selection as above but without dicamba or growth regulators). Transformation efficiency was determined as the number of independently transformed lines (which successfully produced adult plants), as a proportion of the original number of inoculated embryos. Once a root system had developed, roots were gently washed in water, and seedlings transplanted into Barley Mix soil (see Appendix 1.1), and grown under the same conditions as donor plants under CER conditions as described in Section 2.1.2. Plant phenotype was observed on a bi-weekly basis. T_1 seed was harvested from mature T_0 plants, with the germination rate calculated as the number of germinating seeds as a proportion of the total seed harvested from unbagged T_0 heads.

Transgenic nomenclature

For each experiment the inoculations were given a laboratory experiment number, such that in the first experiment embryos inoculated with pBract207Sln1-SC1 were designated 271 and those inoculate with pBract202 were designated 272. Regenerants generated from callus from original embryos were given the suffix -01, -02. Individual T_0 plantlets developing from these separate transformation events were assigned a further suffix e.g. -01, -02. Plants in the T_1 and T_2 generation were assigned further suffixes -01, -02 etc. to denote separate individual plants developing from the previous generation.

Screening of transgenic plants

T_0, T_1, and T_2 lines of plants transformed with pBract207Sln1-SC1 and pBract202 were screened for the presence of the hygromycin resistance gene (*Hyg*) and both sense and antisense components of SC1. Genomic DNA was extracted from leaves of seedlings of each of the putative transgenic lines using a modification (see Section 2.1.4.1) of the protocol described by Edwards *et al*. (1991).

Screening for *Hyg* was conducted using *Hyg* specific primers: HygF (ACTCACCGCGACGTCTGTCG) and HygR (GCGCGTCTGCTGCTCCATA) provided by Dr. Wendy Harwood. Cycling conditions were: 95 °C for 5 min; then 35 cycles of 95 °C for 30 s, 60 °C for 40 s, 72 °C for 1 min, followed by a final extension of 72 °C for 10 min. The product was assessed by agarose gel electrophoresis and by comparison with a DNA marker. A product of 917 bp indicated the presence of *Hyg*.

The sense and antisense specific primer sets used to determine the presence of the silencing insert in transformed *E. coli* (Section 6.3.1), were used in a PCR based screen for the presence of SC1 in transgenic lines. Cycling conditions were: 95 °C for 5 min; then 38 cycles of 95 °C for 30 s, 60 °C for 40 s, 72 °C for 45 s, followed by a final extension of 72 °C for 10 min. Products were assessed following electrophoresis and comparison with a DNA marker. Products of 903 bp (using sense primers) or 820 bp (using antisense primers) indicated the presence of the *Sln1* sequences.

6.3.6 Indentifying homozygotes in T_1 lines

Identification of homozygote lines was conducted so that fair comparisons could be made between putatively silenced lines, in addition to establishing whether Sln1-SC1 copy number impacted on the degree of silencing for each line. Estimation of copy number in the T_1 seedlings was performed by iDNA Genetics (Norwich, UK, http://www.idnagenetics.com), by quantitative PCR (qPCR) of the *Hyg* gene, using *Hyg* specific TaqMan™ primers and a TaqMan™ probe designed by Dr. Peter Isaac (iDNA Genetics, Norwich). Between 6 - 11 *Hyg* positive T_1 samples per line were submitted for copy number analysis. With the exception of a single null control, no other null samples were submitted for analysis. Consequently, a segregation ratio of 2:1 hemizygous to homozygous for *Hyg* was expected, with plants with the highest *Hyg* copy number per line identified as likely homozygotes.

6.3.7 Assessing silencing levels in barley lines transformed with pBract207Sln1-SC1

Silencing levels were assessed using the procedure described in Section 2.4. RNA was extracted from young developing tiller leaves of T_0 and T_1 plants. Care was taken to ensure that leaves of a similar developmental stage were selected. Due to time constraints, and a desire to sample plants at contemporaneous stages of development, T_2 samples were generated from seed by embryo rescue. First leaf material was sampled and combined from 2-7 rescued T_2 embryos for each selected T_1 plant after approximately one week of development. The embryo rescue procedure was similar to that of the isolation of immature embryos described in Section 6.3.4,

however embryonic axes were not removed and immature embryos were plated on 0.8% MS medium (Murashige & Skoog, 1962) instead of BCI medium.

Sln1 transcript levels were assessed by qRT-PCR in T_0, T_1, T_2 transgenic lines, with Golden Promise WT and pBract202 lines used as controls. Low *Sln1* transcript levels (compared to control lines) were indicative of *Sln1* silencing.

6.4 Results

6.4.1 Production of the silencing construct

6.4.1.1 Production of the *Sln1* insert

The *Sln1* insert (comprised of *Sln1* ORF and upstream sequence), was successfully amplified using the methods described in Section 6.3.1. Assessment of PCR product against a ladder marker suggested a product size of 468 bp was produced; the correct size for the desired insert (Figure 6.3).

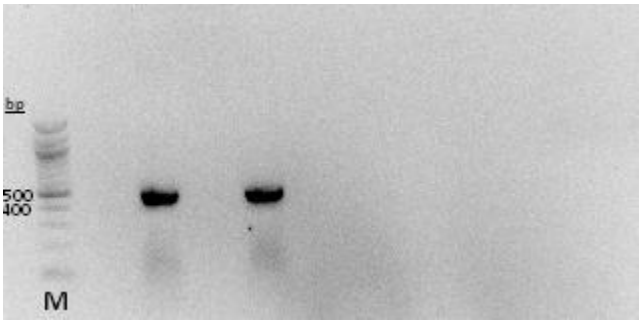


Figure 6.3 PCR amplification of the *Sln1* insert used in the production of the silencing construct. Both lanes were loaded with 2 µL of PCR product, with 1 µL x5 loading buffer and 2 µL SDW. Samples were run on a 0.8% agarose gel (TBE buffer) at 120 V. (M) 2 µL Quick-Load® 100 bp DNA ladder (NEB) was used as a ladder marker.

6.4.1.2 Assessment of the pCR®8 vector

PCR product of 612 nt was used to indicate that the *Sln1* insert was present in the correct orientation in the pCR®8 vector (Figure 6.4). Of the 10 colonies analysed, 6 (60 %; lanes 1, 2, 4, 6, 8, 10, Figure 6.4) contained the insert in the correct orientation, which correlated with the pre-screen estimate of 50% (see Section 6.3.1).

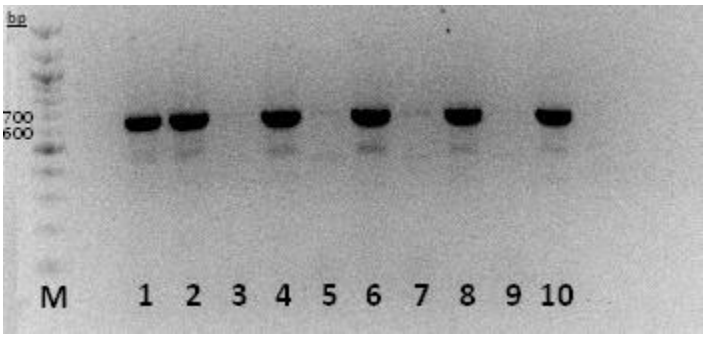


Figure 6.4 Colony PCR to determine the presence and orientation of the *Sln1* insert in the Gateway vector. Sample lanes were loaded with 5 µL of PCR product. Samples were run on a 0.8% agarose gel (TBE buffer) at 100 V. (M) 2 µL Quick-Load® 100 bp DNA ladder (NEB) was used as a ladder marker.

Sequencing of 4 of the 6 colonies confirmed that the insert was in the correct orientation, and was completely homologous to the Himalaya wild-type upstream and ORF sequence (Figure 6.5).

$^{-274}$CACACCACTATGCCCAGATGCCTTCCCCTCCCATCACCCGATGCCGTCTCGCAATCTCCTCC
CTCCCCCCCTCCCCTACAACTACTCCCAGTTGCTCCCGCTGCCGCTCGCTCGCTGCTTTGCCAGT
TTGCCCGCTCGCTCCCCTCCTCCTCCCCCCTTTCCCAACCCTGGATCCAAATCCCGACCCTCCCC
GCACCCGAAACCGAGGCAAGCAAAAGCTTCCCGCGATTATTGGCTAGGTAGAGAGCGAGGTAGCT
CGCTCGCGGCGAGGATCATGAAGCGCGAGTACCAGGACGGCGGCGGGAGCGGCGGTGGGGGTGAT
GAGATGGGGTCGTCGAGGGACAAGATGATGGTGTCGTCGTCGGAGGCGGGGGAGGGGGAGGAGGT
G**GACGAGCTGCTGGCG**GCGCTCGGGTACAAGGTGCGGGCGTCCGACATGGCGGACGTGGCGCAGA
AGCTGGAGCAGCTCGA^{194}

Figure 6.5 Sequence of the *Sln1* silencing insert sequenced from the pCR®8 vector. The methionine encoding 'ATG' denoting the start of the *Sln1* ORF is underlined, and the 'GACGAGCTGCTGGCG' region encoding the 'DELLA' motif in underlined in bold. Coordinates denote the position of the sequence relative to the start codon (ATG) of the Himalaya ORF, which is 0.

6.4.1.3 Assessment of the pBract207Sln1-SC1 construct

Restriction digest

Restriction digests were conducted on two *E. coli* colonies transformed with the pBract207Sln1-SC1 plasmid derived from *Agrobacterium* as described in Section 6.3.3, in order to confirm that no key regions had been lost from the construct (see Figure 6.1). The presence of bands of 2948 bp, 2056 bp, 2053 bp, 698 bp, 456 bp in size indicated that the construct was in its whole form (Figure 6.6).

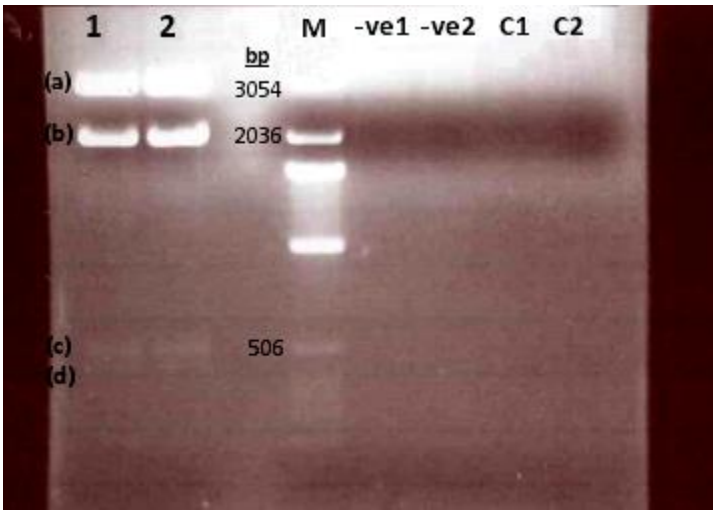


Figure 6.6 Restriction digest to confirm the presence of the *Sln1* insert in the pBract207Sln1-SC1 construct. The two colonies produced identical digest patterns. [(a)]Vector backbone fragments are visible at 2948 bp. [(b)]35S-*Hyg* CaMV terminator fragments are visible at 2056 bp, and the *Ubi* promoter at 2053 bp. [(c)]*Sln1* insert fragments (including part of the recombination site) are visible at 698 bp (one for sense and one for antisense). [(d)]Intron fragments are visible at 456 bp. Sample lanes were loaded with 3 µL of digest product, with 3 µL x6 loading buffer and 9 µL SDW. Samples were run on a 1% agarose gel (TBE buffer) at 100 V. (M) 1 µL 1kb DNA ladder (Invitrogen) was used as a ladder marker.

PCR

The two samples used in the restriction digest were also used in a PCR to confirm the presence of the sense and antisense components of the silencing cassette. PCR amplification produced products of 903 bp and 820 bp, confirming the presences and correct orientation of the silencing insert (Figure 6.7).

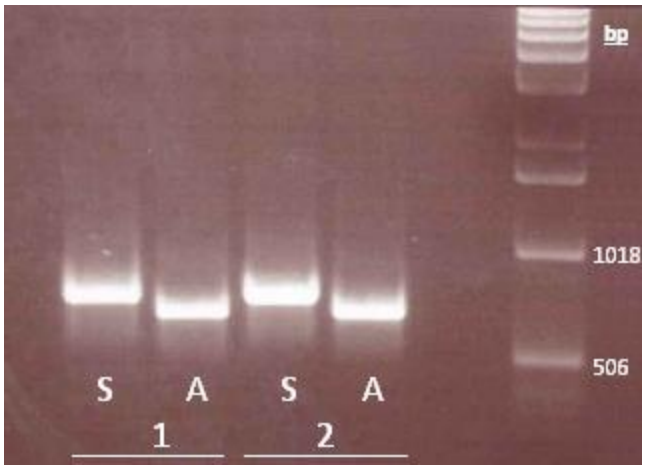


Figure 6.7 Colony PCR to determine the presence and orientation of the *Sln1* insert in the pBract207Sln1-SC1 construct. Two samples were used in PCR (1, 2). Amplification of the sense (S), and antisense (A), components of the silencing construct produced PCR products 903 and 820 bp in length respectively. Sample lanes were loaded with 15 µL of PCR product, and run on a 1% agarose gel (1x TBE buffer) at 100 V. (M) 1 µL 1 Kb DNA Ladder (Invitrogen), was used as a ladder marker.

6.4.2 Characterisation of T_0 transformants

Embryo transformation

Embryo-derived callus putatively transformed with silencing construct (pBract207Sln1-SC1) inoculum developed into plantlets, as did callus transformed with pBract202 control vector inoculum (Table 6.3, Figure 6.8). Callus development efficiency of pBract207Sln1-SC1 inoculated lines was almost equal to those inoculated with pBract202 (8% and 10.2% respectively). Callus inoculated with pBract207Sln1-SC1 exhibited difficulties in regeneration compared to pBract202 inoculated callus, characterised by increased levels of chlorosis, and reduced vegetative growth (Figure 6.9).

Transformation efficiency of T_0 lines

A total of 27 transgenic plants from 16 independent lines were generated (9 putative silencing plants from 5 independent lines, 18 control plants from 11 independently transformed lines). Of these 27 plants, 16 were transferred to soil. The transformation efficiency of silencing construct lines (1.3%) was less than half that of pBract202 control lines (4.9%). Both callus development efficiency and transformation efficiency varied greatly between experiments, irrespective of the inoculum used (Table 6.3).

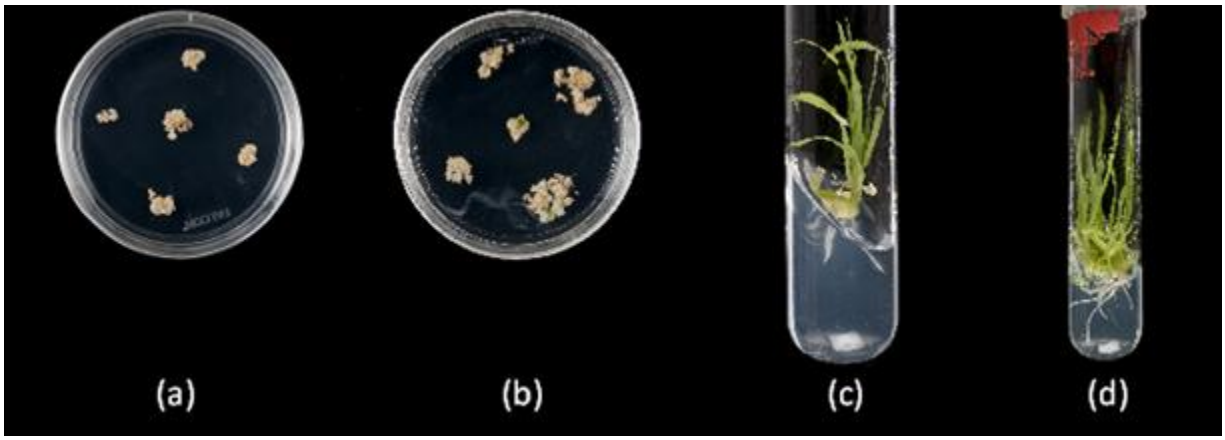


Figure 6.8 *Agrobacterium*-mediated transformation of Golden Promise. The stages of transformant development from embryo-derived callus to transgenic plantlet (a) callus development on BCI medium (selection 1) 2 wks post inoculation; (b) regeneration of callus on BR medium 8 wks post inoculation; (c) plantlet development on BCI medium 12 wks post inoculation; (d) plantlet development on BCI medium 16 wks post inoculation.

Table 6.3 Efficiency of *Agrobacterium*-mediated transformation of Golden Promise embryos with the silencing construct pBract207Sln1-SC1 and pBract202 control vector. [(a)]Individual embryo isolation event. [(b)]Construct used for transformation. [(c)]Callus development efficiency was calculated as the number of calli exhibiting shoot initiation during development on BR medium, as a proportion of the original number of inoculated embryos. [(d)]Transformation efficiency was determined as the number of independently transformed lines that successfully produced adult plants, as a proportion of the original number of inoculated embryos.

Iso. [(a)]	Exp.	Construct [(b)]	No. Embryos	Independent *Hyg* resistant callus	Callus dev. efficiency (%) [(c)]	No. of independent lines	Trans. plants produced	Trans. efficiency (%) [(d)]
1	271	pBract207Sln1-SC1	75	4	5.3	1	3	1.3
	272	pBract202	75	6	8	3	10	4
2	273	pBract207Sln1-SC1	76	6	7.9	1	1	1.3
	274	pBract202	75	7	9.3	6	6	8
	275	pBract207Sln1-SC1	75	0	0	0	0	0
	276	pBract202	75	2	2.7	2	2	2.7
3	296	pBract207Sln1-SC1	75	12	16	3	5	4
	299	pBract207Sln1-SC1	75	8	10.7	0	0	0
	Totals	pBract207Sln1-SC1	376	30	8	5	9	1.3
		pBract202	225	23	10.2	11	18	4.9

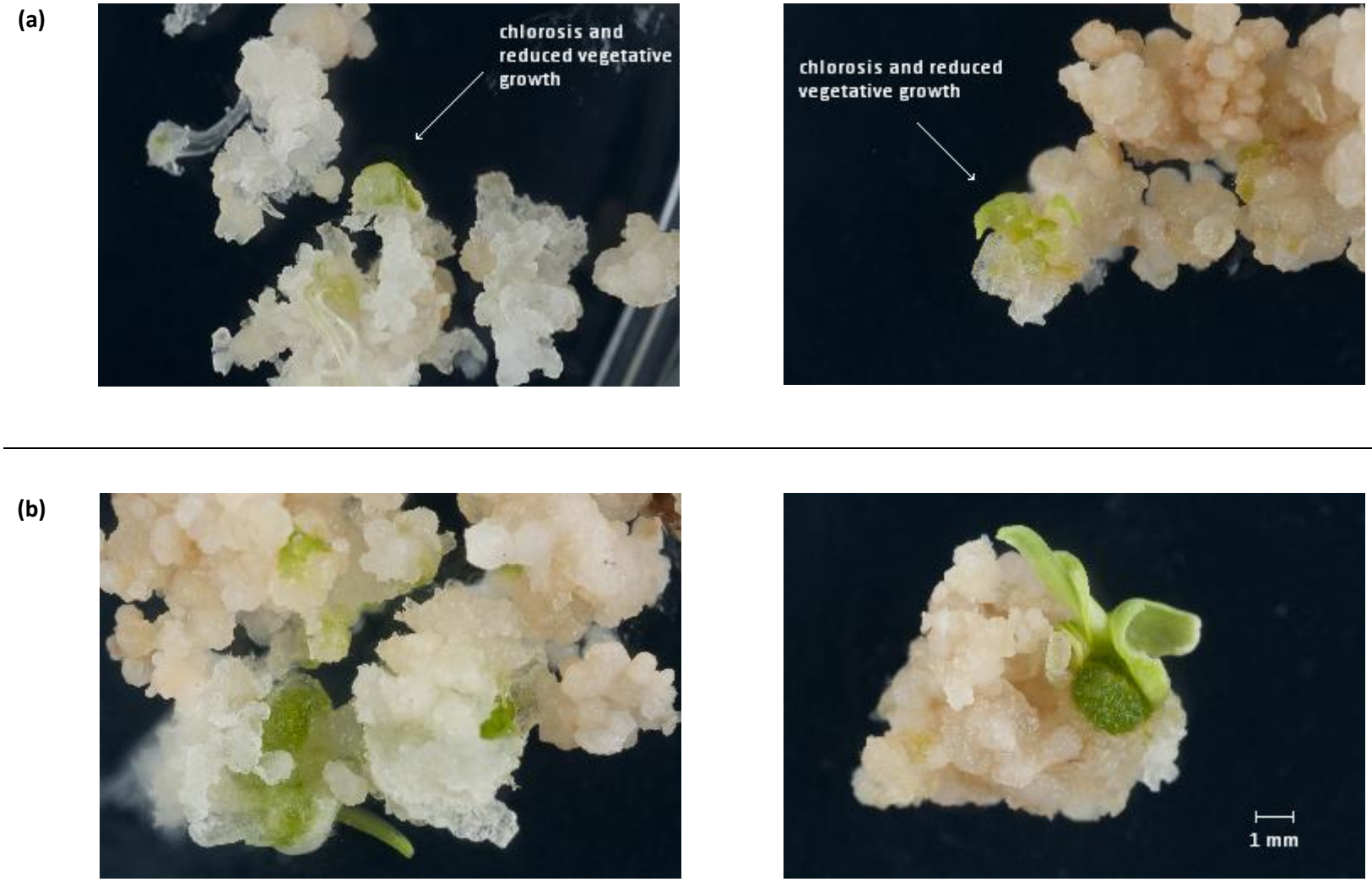


Figure 6.9 Typical development of transformed callus (8 wks post inoculation) on BR medium (a) callus transformed with silencing construct (pBract207Sln1-SC1) and (b) control construct (pBract202). pBract207Sln1-SC1 inoculated lines exhibit difficulties in regeneration characterised by increased levels of chlorosis and reduced vegetative growth. All pictures are taken at the same magnification.

PCR screening of T_0 lines

T_0 plantlets were screened for the presence of *Hyg* and, when appropriate, both sense and antisense components of the pBract207Sln1-SC1 as described in Section 6.3.5. All 16 transgenic lines (both silencing construct and control) were positive for the *Hyg* gene (Figure 6.10 (a)). Of the five putative silenced lines, three contained both sense and anti-sense components of the silencing cassette (Figure 6.10 (b), 273-01, 296-01, 296-02, lanes 4 –7), one contained the sense component alone (Figure 6.10 (b), 271-01, lanes 1 –3), and one contained neither sense nor anti-sense components of the silencing cassette (Figure 6.10 (b), 296-03, lanes 8 - 9), (Table 6.4). The later line was termed '*Hyg*-only', due to the presence of *Hyg*, but absence of the silencing cassette. Identical results were obtained for all plantlets within each line. A secondary screen using a new genomic DNA preparation confirmed these results.

Table 6.4 Screening of putative pBract207Sln1-SC1 T_0 plants. The results of PCR screens for the presence of *Hyg*, and both sense and antisense components of the silencing cassette. [(a)]Lane reference for agarose electrophoresis gel results shown in Figure 6.10.

Line	Transformation Event	Plants	*Hyg*	SC1 sense	SC1 antisense	Gel reference [(a)]
271	01	01	+	+	-	1
		02	+	+	-	2
		03	+	+	-	3
273	01	01	+	+	+	4
296	01	01	+	+	+	5
		02	+	+	+	6
	02	01	+	+	+	7
	03	01	+	-	-	8
		02	+	-	-	9

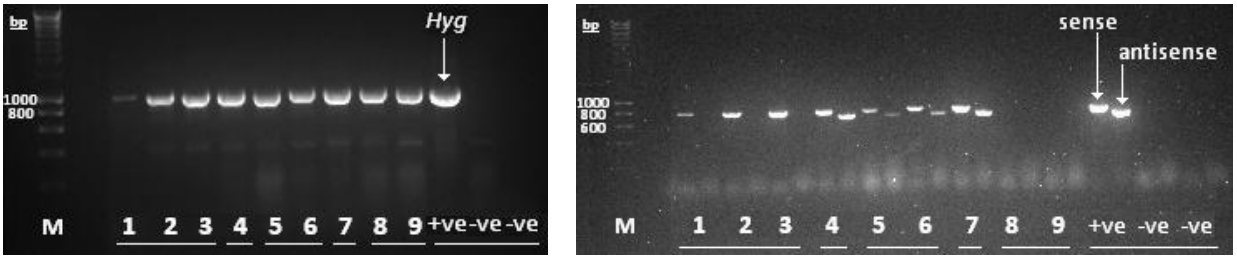


Figure 6.10 Screening of putative pBract207Sln1-SC1 T_0 plantlets for (a) *Hyg*, and (b) sense and antisense components of the silencing cassette. Lanes 1-9 contain products obtained using DNA extracted from plantlets developing from embryo inoculated with the pBract207Sln1-SC1 construct. Diluted (1:10) minipreps of pBract207Sln1-SC1 cloned cells were used for the (+ve) control. DNA extract from a non-transgenic donor plant (left), and SDW (right) were used for the negative controls (-ve). (M) 2 µL HyperLadder™ I (Bioline). *Hyg* specific primers were used to generate the product shown in (a), these were (HygF and HygR, Section 6.3.5), which produce a product of 917 bp. Each sample was separately amplified with a sense-specific primer set (UbiProF1 and i18intronR1, Section 6.3.3), which produces a product of 903 bp, and an antisense specific primer set (IV2intronF1 and NosTermR1, Section 6.3.3), which produces a product of 820 bp.

T_0 plant development

The development of T_0 lines in soil was observed until plants reached maturity. Transgenic plants expressing the partial construct appeared shorter at the time of flowering than full construct or *Hyg*-only lines (Table 6.5). No differences were observed in anthocyanin accumulation or stem thickness in any of the transgenic lines. All pBract207Sln1-SC1 containing plants produced seed, and no consistent difference in germination of T_1 seed from unbagged T_0 heads was seen between partial and full Sln1-SC1 construct containing and control transgenic lines (varying between 38 and 63%), however the *Hyg*-only line showed a higher germination rate (94%) than the other transgenic lines.

Table 6.5 Germination and final plant height of T_0 plants at maturity. [(a)]Each line combines individual plant data. [(b)]Height was measured from the stem base to the top of the grain. [(c)]Seed germination rate was calculated from 32-48 seeds. [(d)]This line produced no seed. NR denotes data was not recorded, NA indicates data was not analysed.

Line [(a)]	SC1 status	Height (cm) [(b)]	Mean	Germination (T_1 seed (%))[(c)]
273-01	Full	72.7	NA	38
296-01	Full	69.5; 72.4	71.0	44
296-02	Full	70.3	NA	75
271-01	Partial	66.9; 67.2; 67.5	67.2	38
296-03	*Hyg*-only	70.0; 71.2	70.6	94
272-01	Control	NR	NA	63
272-02[(d)]	Control	55.0	NA	0
272-03	Control	72.8; 74.4; 75.6	74.3	52

Assessment of silencing in T_0 lines

Sln1 transcript levels in young, single leaf material was measured using qRT-PCR as described in Section 6.3.7. Lines containing the full SC1 construct (273-01, 296-01 and 296-02), a *Hyg*-only line (296-03) and the transgenic control line (272-01) had *Sln1* transcript levels between 69 and 145% of those seen in the non-transformed Golden Promise plant (taken as 100%; Figure 6.11). *Sln1* transcript levels were highest (288%) compared to the untransformed plant(s) in the 271-01 transformant, which contained only part of the SC1 construct.

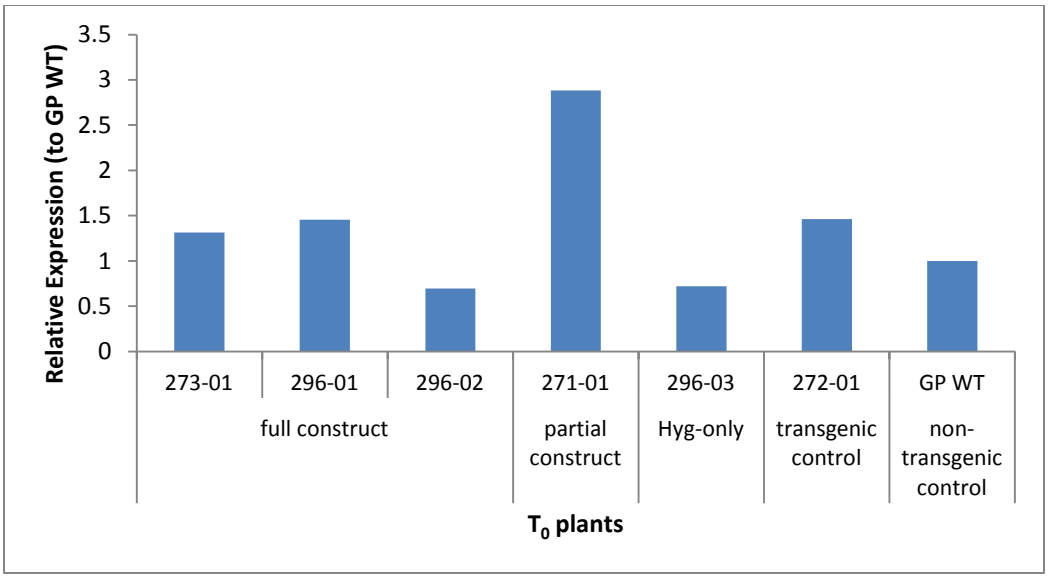


Figure 6.11 *Sln1* expression analysis in T_0 lines. *Sln1* transcript levels for transgenic plantlets containing the full (273-01, 296-01, 296-02) and partial form (271-01) of the Sln1-SC1 construct are shown beside transgenic control (272-01; *Hyg* positive without Sln1-SC1 construct), a *Hyg*-only plant (296-03; *Hyg* positive and Sln1-SC1 negative), and non-transgenic control (Golden Promise wild-type) lines. Expression is presented relative to the Golden Promise wild-type (GP WT) control that was set to 1.

6.4.3 Characterisation of T_1 transformants

Identification of homozygotes in T_1 lines

PCR screening of genomic DNA with primers specific for *Hyg* and the Sln1-SC1 was conducted as described in Section 6.3.5 to establish the transgenic status of the T_1 generation, and the presence of Sln1-SC1 in the T_1 lines. 61 *Hyg* positive plant samples were submitted for *Hyg* copy number analysis, along with one control plant sample, as described in Section 6.3.6. Of the 62 *Hyg* positive plant samples, two lines (totalling 14 plants) contained the full Sln1-SC1 cassette, one line (9 plants) contained the partial Sln1-SC1 cassette, three transgenic control lines (totalling 29 plants), and one *Hyg*-only line (10 plants) were submitted (Table 6.6).

Table 6.6 Results of *Hyg* copy number analysis on the T_1 generation. T_1 plants listed were retained for T_2 expression analysis. Homozygous lines were identified based on *Hyg* copy number. [(a)]Denotes the T_0 plant the T_1 plant was derived from, line information is derived by removing the final two letter suffix from the T_0 plant number. [(b)]Suffix given to denote individual T_1 plants e.g. 273-01-01-xx, where xx denotes the suffix listed in this column.

Line (T_0 plant) [(a)]	SC1 status	*Hyg* copy no.	No. of plants with copy no.	T_1 plants with copy no. [(b)]
273-01-01	full construct	5	1	-01
		4	5	-02 to -06
		3	1	-07
	null control	0	1	-08
296-02-01	full construct	10	1	-01
		8	2	-02, -03
		7	1	-04
		6	1	-05
		3	1	-06
271-01-03	partial construct	4	1	-01
		2	3	-02 to -04
		1	5	-05
296-03-02	*Hyg*-only	8	1	-01
		4	2	-02, -03
		2	7	-04, -10
272-01-01	transgenic control	16	4	-01 to -04
		8	6	-05 to -10
272-03-05	transgenic control	12	2	-01, -02
		8	4	-03 to -06
		6	2	-07, -08
		4	1	-09
		2	1	-10
272-03-08	transgenic control	3	2	-01, -02
		2	2	-03, -04
		1	5	-05 to -09

Assessment of silencing in T_1 lines

Sln1 transcript levels in young, single leaf material was measured using qRT-PCR as described in Section 6.3.7. The T_1 plants analysed are shown in Table 6.7. Golden Promise leaf material at an equivalent stage of development was not available; therefore comparisons were made with the null control plant, 272-03-07-01.

Table 6.7 Plants selected for T_1 expression analysis. Zygosity was determined from the *Hyg* copy number ratios for each line, as described in Section 6.3.6.

Line	SC1 status	T1 plant	*Hyg* copy number	Zygosity
273-01	full construct	273-01-01-02	4	
296-02	full construct	296-02-01-01	10	Homozygous
271-01	partial construct	271-01-03-01	4	Homozygous
		271-01-03-02	2	Hemizygous
296-03	*Hyg*-only	296-03-02-02	4	
272-01	transgenic control	272-01-01-02	16	Homozygous
272-03	transgenic control	272-03-05-01	12	Homozygous
272-03	transgenic control	272-03-08-02	3	
272-03	null control	272-03-07-01	0	
296-03	null control	296-03-01-01	0	

Sln1 transcript levels in plants containing the full or partial Sln1-SC1 construct were similar (37 – 55%, and 52 – 55% respectively), compared to that of the null control plant 272-03-07-01 (taken as 100%). *Sln1* transcript levels in the full and partial Sln1-SC1 construct plants, appeared generally lower than the transgenic control (21 – 79%), approximately half that of null control plants (100 – 300%), but higher than the transcript level in the *Hyg*-only plant, 296-03-02-02 (39%). The similarity in *Sln1* transcript level between 271-01-03-01 (homozygous; 55%), and 271-01-03-02 (hemizygous; 52%), produced from the same T1 parent, suggests consistency in *Sln1* transcript levels between directly related plants, regardless of zygosity (Figure 6.12).

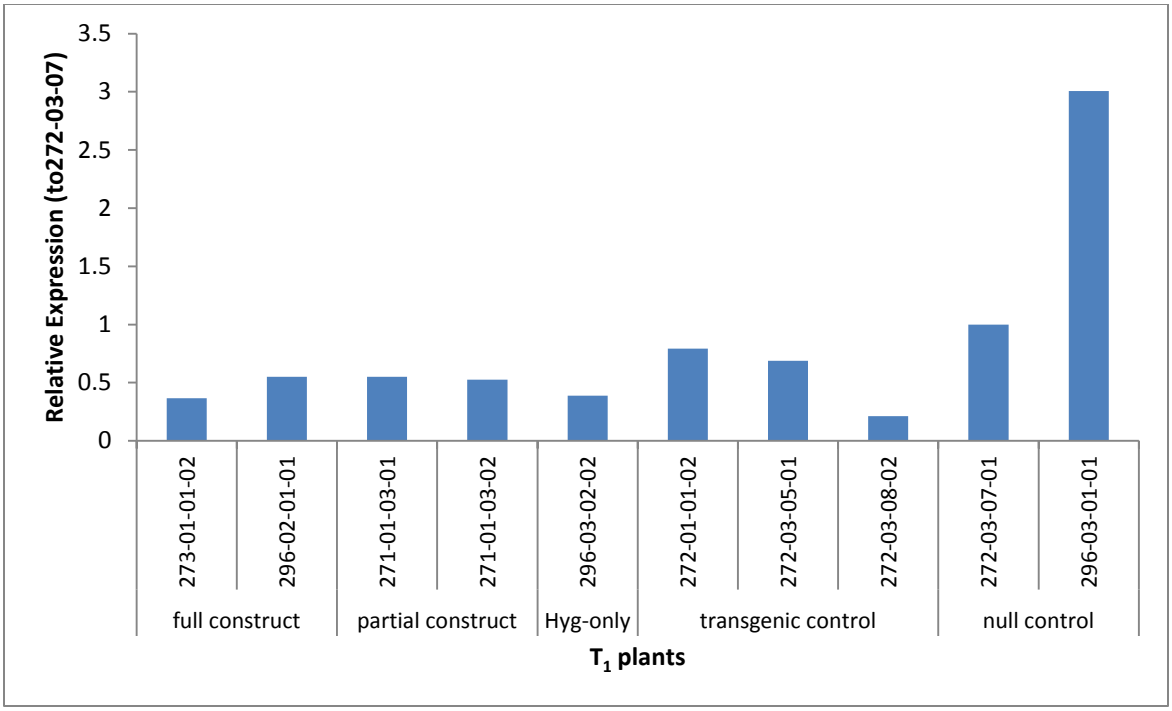


Figure 6.12 *Sln1* expression analysis in T_1 lines. *Sln1* transcript levels for transgenic plants containing *Hyg* and the full (273-01-01-02, 296-02-01-01) or partial form (271-01-03-01, 271-01-03-02) of the Sln1-SC1 construct are shown beside transgenic control (272-01-01-02, 272-03-05-01, 272-03-08-02), *Hyg*-only (296-03-02-02), and null control (273-03-07-01, 296-03-01-01) plants. Expression is presented relative to the 272-03-07-01 null control that was set to 1.

T1 plant development

22 plants from the seven independent transformation lines were grown to maturity. These 22 plants comprised of two likely homozygotes and one likely hemizygote from each line, with a further single null control from the 273-01-01 line. No clear phenotypic differences were noted between plants in this generation. Immature T_2 seed was collected for embryo rescue to speed up obtaining the T_2 generation.

6.4.4 Characterisation of T_2 transformants

Analysis of *Sln1* transcript levels was conducted in pBract207Sln1-SC1 T_2 lines containing the full silencing construct. Although not ideal due to the effects of tissue culture, time constraints meant that embryo rescue was used to generate the T_2 plants used in *Sln1* expression analysis. T_2 seed was at an early stage of development, which may account for the resulting low number

of plantlets that were successfully regenerated. Lines of interest, including those containing the partial Sln1-SC1 construct were consequently unavailable for T_2 expression analysis. PCR screening of genomic DNA with primers specific for *Hyg* and Sln1-SC1 was conducted as described in Section 6.3.5 to establish the transgenic status of the T_2 generation, and the presence of Sln1-SC1 in the T_2 lines. Expression analysis was conducted as described in Section 2.2.4. Expression values for Golden Promise wild-type plants were disregarded as the geNorm software identified them as being unsuitable for further analysis. Comparisons were therefore made with the transgenic control plants derived from 272-01-01-04 (pBract202), and a single null control plant (Table 6.8).

Table 6.8 Plants selected for T_2 expression analysis. Zygosity was determined from the *Hyg* copy number ratios for each line, as described in Section 6.3.6.

Line	SC1 status	T1 plant	*Hyg* copy no.	Zygosity	T_2 plant analysed
273-01	full construct	273-01-01-01	5	Homozygous	273-01-01-01-01
273-01	full construct	273-01-01-05	4	Homozygous	273-01-01-05-01
273-01	full construct	273-01-01-07	3	Hemizygous	273-01-01-07-01
296-02	full construct	296-02-01-01	10	Homozygous	296-02-01-01-01
296-02	full construct	296-02-01-02	8	Homozygous	296-02-01-02-01
296-02	full construct	296-02-01-06	3	Hemizygous	296-02-01-06-01
272-01	transgenic control	272-01-01-04	16	Homozygous	272-01-01-04-01
272-03	transgenic control	272-03-05-01	12	Homozygous	272-03-05-01-01
272-03	transgenic control	272-03-05-08	6	Hemizygous	272-03-05-08-01
273-01	null control	273-01-01-08	0	NA	273-01-01-08-01

Sln1 transcript levels were similar in plants containing the full Sln1-SC1 construct (22 – 66%) compared to the transgenic control plants (16 - 50%), and the null control (28%), (Figure 6.13).

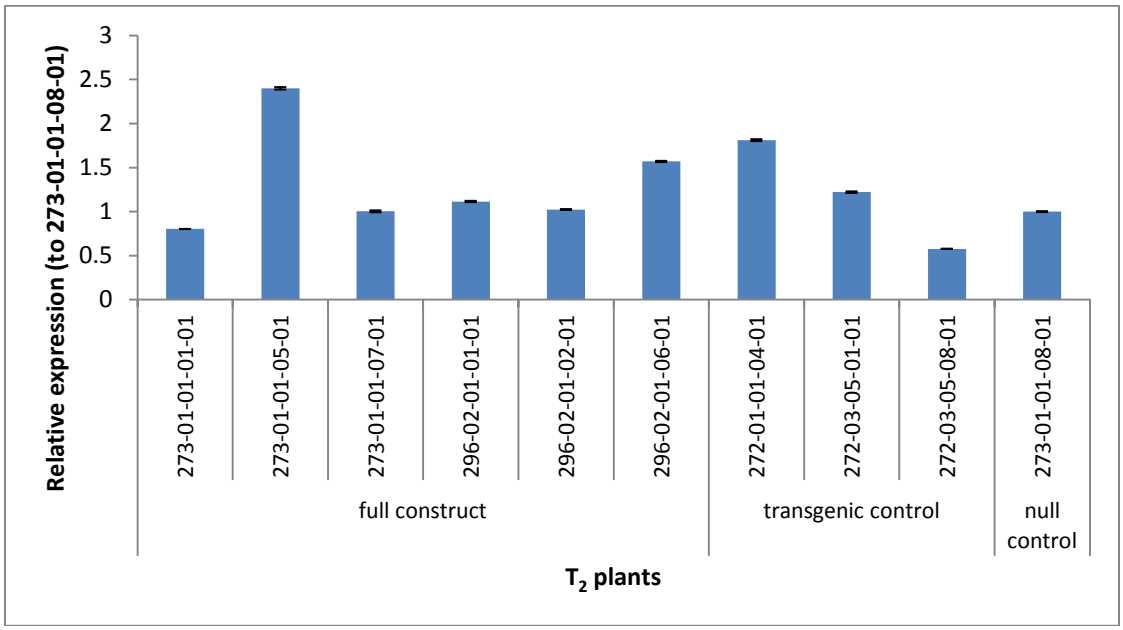


Figure 6.13 *Sln1* expression analysis in whole plant T_2 lines. Error bars denote standard deviation between technical repeats. Expression is presented relative to the 273-01-01-08-01 null control (*Hyg* and Sln1-SC1 negative) that was set to 1.

6.5 Discussion

Sln1 regulates growth and development at all stages of the barley life cycle (Achard *et al.*, 2009). Due to the potentially lethal effect on plant develop that complete *Sln1* silencing could cause, barley was transformed using the pBract207Sln1-SC1 construct, which previous experimental evidence suggested was likely to provide a range of silencing levels. It is likely that lines with high levels of silencing were non-recoverable. 5' sequence was selected to ensure that RNAi silencing was DELLA specific, as the 3' region is conserved amongst members of the GRAS family (Pysh *et al.*, 1999). Expression of 3' *Sln1* sequence in an RNAi construct could conceivably result in the silencing of non-target members of the GRAS family of regulatory proteins, affecting non-DELLA-mediated plant growth and development (Engstrom, 2011).

Development of Sln1-SC1 lines during the early stages of callus regeneration and plantlet development was closely monitored in case expression of the RNAi construct proved lethal to embryo development. Data showed that although callus development efficiency was similar between Sln1-SC1 lines and control lines, the transformation efficiency of Sln1-SC1 lines was

much lower. The difference between callus development efficiency and transformation efficiency suggests Sln1-SC1 expression did not affect callus initiation from immature embryos, nor the very early stages of embryo development, but resulted in plant death at the early stages of plantlet development. Phenotype observations during the regeneration stage support this view, with Sln1-SC1 lines exhibiting early signs of plantlet death. Commonly in Sln1-SC1 lines, green areas were observed on callus, but green shoots failed to develop. Differences in development and transformation efficiency may result from the response of Sln1-SC1 lines to tissue culture induced abiotic stress. As DELLAs are implicated in conferring resistance to abiotic stress (Achard *et al.*, 2008c), silencing of DELLA expression would likely result in increased susceptibility to the stress induced by tissue culture. Sln1-SC1 lines exhibited stress phenotypes at the regeneration stage, characterised by high levels of chlorosis and reduced vegetative growth. It is likely that the most highly stress susceptible Sln1-SC1 lines fail to develop beyond the regeneration stage, accounting for the difference in transformation efficiency compared to control lines.

A slender phenotype similar to that of the *sln1c* (cv Himalaya) or *sln1-1* (cv Herta) LoF mutants (Chandler *et al.*, 2002) was expected in lines expressing the Sln1-SC1 construct. Characteristics including anthocyanin accumulation and slender phenotype, in addition to sterility were not seen in the Sln1-SC1 lines. No clear differences in phenotype were apparent between the Sln1-SC1 and control lines from the callus regeneration stage onwards. Furthermore, all Sln1-SC1 lines produced viable seed, with a germination rate of T_1 seed similar to that of the control lines. Fundamental difference between silencing lines and LoF lines could account the lack of a slender-like phenotype. As the silencing construct lines were knockdown rather than knockout lines, functional SLN1 would still be produced. This functional SLN1 would inhibit growth, preventing the extreme slender phenotypes seen in *Sln1* LoF mutants. Furthermore, functional SLN1 could interact with other signalling pathways (see Section 1.4.3), promoting other developmental and growth inhibitory mechanisms.

Hyg copy number analysis identified potentially complex transgene insertion patterns and an unusually high copy number in the majority of transgenic lines generated in this study. A study of the efficacy of the *Agrobacterium* transformation method (Bartlett *et al.*, 2008), suggests that 45% of transgenic plants should have a *Hyg* copy number of one, and that high copy numbers

are rare, with only 3% of transformants containing a transgene copy number of over seven. *Hyg* copy numbers appear high in both Sln1-SC1 and control lines, suggesting high *Hyg* copy number is not linked to construct type. The concentration of genomic DNA submitted for *Hyg* count analysis was lower than that usually submitted, which may have reduced the usual precision of the method, producing more variable and higher copy number reads.

Based on previous experimental evidence, it was expected that half the developing Sln1-SC1 lines would contain the full Sln1-SC1 cassette, with the remainder containing only part of the cassette, and a small proportion containing no part of the cassette at all (Wendy Harwood, JIC, personal communication). Accordingly, one line (296-03) appeared to contain only the *Hyg* component, whilst another (271-01) appeared to contain only the *Hyg* and the sense component of the cassette. PCR is unable to elucidate the size of the missing sequence, raising the possibility that regions as large as the Sln1-SC1 cassette, or as small as the sequence necessary for primer binding, may have been recombined out. If the latter is true, the RNAi construct may be fully functional despite the negative results suggested by PCR. This may be the case in the 296-03 (*Hyg*-only) line, which showed reduced *Sln1* transcript levels in both the T_0 and T_1 generations compared to the respective control lines, suggesting *Sln1* is silenced in this line. Similarly, *Sln1* transcript levels varied greatly between the T_0 and T_1 generations in the 271-01 (partial construct) line compared to the respective control lines. In the T_1 generation (in which two plants generated from seed were analysed for *Sln1* silencing), both 271-01 plants showed decreased *Sln1* transcript levels compared to the control lines, suggesting *Sln1* silencing is functional in this line, and that antisense primer biding sites are likely to have been lost. Whilst expression of the antisense component may result in silencing through complementary binding to *Sln1* mRNA, expression of the sense component alone would likely have no silencing effect. Furthermore, expression of the sense component alone is unlikely to produce a protein with DELLA function, as only a small component of the ORF would be expressed, and such a protein would lack the GRAS domain required for SLN1 function, and the TVHYNP motif required for GID1-GA recognition (Itoh *et al.*, 2005).

Investigation of the full impact of *Sln1* silencing in barley was limited by the low numbers of silencing line transformants, despite the high number of inoculations conducted in this study. Silencing levels were assessed in T_0 lines to determine if the Sln1-SC1 construct conferred

functional *Sln1* silencing, and whether silencing was readily apparent. Such analysis would normally be conducted post T_0 generations; however, concerns that *Sln1* silenced lines would be sterile due to loss of developmental function made it important to assess possible silencing at the earliest feasible stage. A clear pattern of silencing was not observed in the T_0 generation, likely due to the effects of tissue culture, which produces variability between regenerating tissues, and also imposes abiotic stress likely to directly affect *Sln1* transcript levels. Project time constraints meant that analysis of the T_2 generation had to be conducted on material generated from embryo rescue rather than from germinated seed, which would have been preferable. As with the T_0 analysis, although there was some indication of silencing in the Sln1-SC1 lines, no definitive pattern of silencing was observed in this generation. Analysis of *Sln1* silencing in the T_1 generation provided a more stable basis for analysis compared to the T_0 and T_2 generations, as samples are generated from seed rather than tissue culture. Analysis of the T_1 generation indicated that Sln1-SC1 lines contained reduced *Sln1* transcript levels compared to null control lines, although similar results were observed in the transgenic control lines. Further analysis will be required to confirm the degree of silencing in the Sln1-SC1 lines, however the data presented suggests expression of the RNAi construct confers some silencing in transgenic barley. Further confirmatory work is required on the T_2 or subsequent generations to determine whether the null hypothesis (H_0) is to be rejected or accepted.

This study is the first reported example of DELLA silencing in cereal species, with only one preceding example reported in tomato (*Solanum lycopersicum* L.), (Martí *et al.*, 2007). The slender-like elongated phenotype and parthenocarpy reported in DELLA gene homologue (*SlDELLA*) antisense silenced lines was not apparent in the Sln1-SC1 lines observed in this study. The ability of *SlDELLA* lines to develop to maturity may be due to functional redundancy caused by the function of orthologous DELLA genes present in tomato, but absent in barley.

Chapter 7: General Discussion

The primary aim of the work described in this study is summarised by the following hypothesise. H_1: DELLA function is equivalent between barley and *Arabidopsis*; H_0: DELLA function is not equivalent between barley and *Arabidopsis*. Barley was selected for this translational work, as DELLA is expressed in barley by a single gene, unlike *Arabidopsis* DELLAs, which are expressed by five genes with overlapping function. Barley also represents a simpler system for the study of a temperate cereal than wheat which is less genetically tractable because DELLA is expressed by three homeologous genes. Several characterised *DELLA* and *GID1* mutants were available and additional putative mutants were stored in the laboratory or JIC genetic resources unit although these stocks were poorly labelled and required characterisation. An additional benefit of using barley was the availability of established platforms for barley transformation and regeneration at the John Innes Centre (JIC), thereby facilitating the transgenic study of *Sln1* expression and protein function. Although rice also possesses only one fully functional *DELLA* (*SLR1*), and has the advantage of a sequenced genome, it has proven difficult to grow at the JIC (Dr. P. Vain, JIC, personal communication). Barley is also an important crop to UK agriculture, and the fourth most abundant cereal crop in the world (Bartlett *et al*., 2008), therefore a greater understanding of DELLA function has the potential to be advantageous for world agriculture, and to form the basis for translation to wheat, the UK's major cereal crop.

The current work commenced some time after the cessation of studies of DELLA function in barley (carried out by Fu *et al.,* 2002) with limited stocks and poorly labelled packets of some seed being available. Thus, it was necessary to characterise at the molecular level, the *DELLA* sequence present in stocks labelled *sln1* and *dwf2*. This proved unexpectedly challenging with both sequencing and PCR being relatively inefficient because of the high GC content of *DELLA* and the resulting complex secondary structure (Chapter 3). Other groups have reported similar difficulties with analysis of the wheat and barley DELLAs (Pearce *et al*., 2011; Saville, 2011; Dr. P. Chandler, personal communication). However, optimisation of the protocols enabled these analyses and identified the lesions in the mutant stocks. Although the lesion in the DELLA domain of *dwf2* was similar to that in *sln1d* (Chandler *et al.,* 2002), the premature stop codon in *sln1-1* produced a protein truncated upstream of the lesion in *sln1c*, and a slightly less severe phenotype which was useful for stress tolerance studies. The similarity in mutant phenotype

and the location of the lesions within the DELLA genes is indicative of the high degree of DELLA homology, both genetic and functional, between barley and *Arabidopsis*; further supporting the alternative hypothesis (H_1) of the shared role of DELLA. Sequencing of the wild-type parental cultivars (cv Himalaya, cv H930-36, Herta and Triumph, all of which are modern breeding lines) revealed a lack of diversity in the gene with very few SNPs present (Chapter 3). While this could reflect the need for sequence conservation of a gene integral to plant growth and development, the finding is in agreement with Comadran *et al*. (2011) who found limited diversity in conserved genes of modern varieties.

The findings of Achard *et al.* (2006) formed the basis for the translational studies of abiotic stress tolerance described in this study. Initial assessment of the importance of SLN1 in tolerance to abiotic stress was conducted using salinity, with the protocol following, as far as possible, that described in the Achard study. The advantage of using *Arabidopsis* as a model plant was apparent when comparing the sample throughput of *Arabidopsis* and barley. The space requirements and the manipulations required for setting up, and then analysing the data from, the hydroponics study limited seedling numbers to 28 per treatment per experiment, compared to the Achard study which used small seedlings and agar culture meaning that there was no major limit to the number of samples that could be treated simultaneously. Low sample number can give undue prominence to outliers, whilst the sample numbers were insufficient in the hydroponic experiments for statistically meaningful conclusions to be made for survival results, statistically meaningful differences were identified for root and shoot growth, supporting the alternative hypothesis (H_1). The use of heat shock as a source of abiotic stress allowed sample numbers to be increased, with the result that clearer conclusions could be drawn. The abiotic stress tests used in this study provided results consistent with those obtained for *Arabidopsis*, (supporting the alternative hypothesis, H_1), with SLN1 GoF mutants conferring increased survival to abiotic stress, and LoF mutants showing decreased survival to abiotic stress compared to the wild-type genotype. Achard *et al.* (2006) reported that DELLA-mediated growth inhibition was lacking in the DELLA LoF mutant (quadruple-DELLA) grown under salt conditions. This trend was not observed in this study, with the growth of the barley SLN1 LoF mutants (*sln1c*, *sln1-1*) generally showing reduced growth in response to salt and heat stress. The reduced growth in the barley SLN1 LoF mutants is likely due to reduced growth resulting from damage, perhaps as a consequence of the production of ROS, as suggested for *Arabidopsis* by

Achard *et al.* (2008). The effect of abiotic stress on the *Arabidopsis* quadruple-DELLA mutant may be diminished by the remaining single functional DELLA (RGL3) since RGL3 has been implicated in stress response (Alvey & Boulton, 20008). It would be interesting to determine whether the 'global' DELLA mutant that lacks all five DELLAs responds differently from the quadruple-DELLA mutant. However, overall the data presented in the current translational study suggest that a common DELLA-mediated response to abiotic stress is conserved in dicots and monocots.

Although the effect of SLN1 on plant phenotype, and in this study, on abiotic stress tolerance, has been observed at the whole-plant level, the elucidation of SLN1 localisation and accumulation remains an avenue of further investigation, which despite being an early aim of this study, was not achieved due to the recalcitrance of the *Sln1* gene to molecular techniques (Chapter 3). Chandler *et al*. (2002) used immunoblotting to show that SLN1 accumulated in the elongation zone of young 2nd leaves of barley, but no evidence for potential stabilisation of protein or altered *Sln1* expression during abiotic stress has been presented. To address this I aimed to produce reporter constructs for SLN1 protein accumulation and for *Sln1* gene expression. However this was not achieved; the envisaged SLN1:GFP fusion protein construct containing the *Sln1* promoter and the *nos* terminator could not be completed. Despite successful cloning of the *Sln1* promoter, it was not possible to clone the SLN1 ORF downstream of it (data not presented). Similarly, the *Sln1* promoter::insert could not be cloned into the GUS plant gene reporter vector (data not presented). Similar difficulties were experienced by Dr. Nadia Al-Kaff (JIC, personal communication) during the production of an *Rht:GFP* construct for the transformation of wheat, which ultimately had to be synthesized commercially and did not produce plants expressing the fusion protein, and resulted in many lines showing recombination of the transgene (personal communications, Dr. H. Jones, Rothamsted Research, Dr. A. Korolev, JIC). Cereal DELLA GUS reporter constructs for gene expression studies can however be made as Pearce *et al*. (2011) analysed *Rht* expression in transgenic wheat. The SLN1 antibody used by Chandler *et al*., (2002) was no longer available (F. Gubler, personal communication) and antibodies that would recognise the RHT or SLN1 proteins in plants could not be generated during the lifetime of this project, despite the assistance of two collaborating groups (personal communications, Dr. M. Boulton, JIC, S. Thomas, Rothamsted Research). Assessment of *Sln1*

transcript levels using qRT-PCR as part of the Triumph mutant characterisation during the current study found that DELLA transcript levels were highest in growing leaf material (Section 3.4.3), which is consistent with the findings of Chandler *et al*. (2002) who found *Sln1* mRNA to be preferentially expressed in elongating regions of the leaf.

There were no mutant lines available in which the SLN1 protein was absent or expressed at low levels. Given that the LoF mutants were capable of producing a truncated protein (and this protein, in the case of the *sln1c* mutant, was extremely stable *in planta*) I decided to attempt to produce transgenic barley lines in which *Sln1* was silenced, or partially silenced. This was to give me the opportunity to correlate the level of functional SLN1 protein with abiotic stress tolerance and plant phenotype. Although transgenic barley lines containing the *Sln1* RNAi construct were obtained, none of them showed clearly stable silencing of *Sln1* (Chapter 6). The low number of transformants containing an intact construct suggests that silencing of the gene may be detrimental to regeneration. I had envisaged this might be the case, and had designed a construct that was intended to give "inefficient" silencing. It is commonly found that it is not possible to obtain transgenic plants in which essential genes are silenced. For example, Liu & Makaroff (2006) were unable to obtain *Arabidopsis* plants transformed with a CaMV 35S promoter:*AESP* construct (AESP is necessary for embryo development), but were able to obtain them if the construct was expressed from a meiosis-specific promoter. Further attempts to decrease *Sln1* expression should therefore be based on weaker or inducible promoters.

The integration of DELLA mutant alleles into agricultural populations fuelled the Green Revolution, and as understanding of DELLA function increases, the potential benefits of further integration remain high. Anti-lodging characteristics and stress tolerance are clearly beneficial to agriculture, especially with the potential for climate change to increase the incidence of lodging. Further consideration needs to be given to negative linkages that may exist between DELLA genes and alleles encoding negative agricultural traits (e.g. reduced yield, increased disease susceptibility). Further elucidation of the barley genome should facilitate rapid and thorough haplotype analysis, whilst crop breeding programs seek to eliminate any possible negative linkages associated with DELLA genes. There remains an important trade-off between abiotic stress tolerance and reduced growth that must be considered when using DELLA mutants in an agricultural context. Thousand grain weight (TGW) appeared reduced in the DELLA stabilising

mutants (cv Himalaya: *sln1d*, *gse1a*) compared to the wild-type, suggesting DELLA stability results in diminished yield in barley. Whereas the integration of the *Rht* dwarfing genes (*Rht-B1b* and *Rht-D1b*) have been reported to increase grain yield at the cost of stem biomass production, *Rht* is present on each of the three homeologous pairs of chromosomes in wheat, so the wild-type allele is still functional in dwarf and semi-dwarf mutant lines. This is not the case for barley, where DELLA is expressed by *Sln1* alone, with the introduction of the known dwarfing alleles resulting in a severe dwarf phenotype, meaning the range of heights achievable using *Rht* mutants of hexaploid wheat is not currently possible for barley. However, useful mutants may be obtained by TILLING (Colbert *et al.*, 2001) of the DELLA gene, or of genes in the GA biosynthetic or degradation pathway, or potentially using transgenic approaches although this may currently not be acceptable to consumers. Certainly my data suggest that modification of the GA-DELLA pathway could be a feasible approach to increasing crop resistance to abiotic stress provided mutants can be identified that do not have the negative association of extreme reduction in plant height. An additional benefit of DELLA has recently been identified in biotic stress tolerance, where barley and wheat GoF DELLA mutants generally conferred increased resistance to necrotrophs and increased susceptibility to biotrophs compared to wild-type plants (Saville, 2011). Many studies aimed at increasing plant stress tolerance (e.g. through the overexpression of antioxidant components) can be detrimental to plant development and crop yield (see Section 1.5.6.1) and it is clear that further work, employing molecular manipulation and the identification of stress tolerant varieties is still required.

Nevertheless the data reported here, and the methodology established, provide a basis, and the tools, for further work towards the production of cereals with abiotic stress tolerance as well as providing additional characterised mutants to allow studies into the mechanism of that tolerance. They will also allow investigation of the interconnectivity between the signalling response pathways of which DELLA is an integrator via response to changes in GA levels.

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Appendix 1: Growth Media

1.1 Soil growth media

Barley Mix compost

375 L Levington M3 compost (Scotts professional)

100 L Perlite

200 L 4 mm grit

1.6 kg Osmocote Plus™

1.2 Hydroponic solution

Hoagland's solution (Hoagland & Arnon, 1950)

Formula for 1 litre of (x 0.5) modified Hoagland's medium

3 ml of 1 M potassium nitrate	(KNO_3)
2 ml of 1 M calcium nitrate	($Ca(NO_3)_2$)
0.5 ml of 1 M monoammonium phosphate	($NH_4H_2PO_4$)
1 ml of 1 M magnesium sulphate	($MgSO_4$)
0.5 ml of micronutrient solution	(see below)
0.125 ml of iron chelate stock solution	(see below)

sodium chloride added as required from a 5 M stock (JIC media supply)

Adjust to 1 litre using de-ionised water

Micronutrient stock solution

Formula for 1 litre

2.86 g boric acid	(H_3BO_3)
1.81 g manganese chloride 4-hydrate	($MnCl_2.4H_20$)
0.22 g zinc sulphate 7-hydrate	($ZnSO_4.7H_2O$)
0.08 g copper sulphate 5-hydrate	($ZnSO_4.5H_2O$)
0.02 g molybdic acid (assaying 85% MoO_3)	($H_2MoO_4.H_2O$)

Adjust to 1 litre using de-ionised water

Iron chelate stock solution

Formula for 1 litre

In 286 ml de-ionised water:

26.1 g EDTA

19 g potassium hydroxide (KOH)

In 500 ml de-ionised water

24.9 g iron sulphate 7-hydrate ($FeSO_4.7H_2O$)

Slowly add iron sulphate solution to potassium EDTA solution, aerate overnight with stirring. Adjusted to 1 litre using de-ionised water, and stored at 4 °C

1.3 Transgenics media

Barley callus induction media (Bartlett *et al.*, 2008)

Formula for 1 litre of medium

4.3 g Murashige & Skoog plant salt base (M0221, Melford Laboratories, UK)

30 g maltose

1.0 g casein hydrolysate

350 mg myo-inositol

690 mg proline

1.0 mg thiamine HCl

2.5 mg dicamba

1.25 mg copper sulphate 5-hydrate ($CuSO_4.5H_2O$)

pH 5.8

3.5 g Phytagel™

Barley transition media (Bartlett *et al*., 2008)

Formula for 1 litre of media

2.7 g Murashige & Skoog modified plant salt base (without NH_4NO_3)

20 g maltose

165 mg ammonium nitrate (NH_4NO_3)

1.25 mg copper sulphate 5-hydrate ($CuSO_4.5H_2O$)

750 mg glutamine

100 mg myo-inositol

0.4 mg thiamine HCl

2.5 mg 2,4D (2,4-Dichlorophenoxyacetic acid)

0.1 mg BAP

pH 5.8

3.5 g Phytagel™

Barley regeneration media (Bartlett *et al*., 2008)

Formula for 1 litre of media

2.7 g Murashige & Skoog modified plant salt base (without NH_4NO_3)

20 g maltose

165 mg ammonium nitrate (NH_4NO_3)

750 mg glutamine

100 mg myo-inositol

0.4 mg thiamine HCl

pH 5.8

3.5 g Phytagel™

1.4 Bacterial growth media

LB-G broth and agar

Formula for 1 litre of media

10 g tryptone

5 g yeast extract

10 g NaCl

pH 7.0

For solid medium (LB–G agar), 10 g Lam M No. 1 agar was added

MG/L medium (modified from Garfinkel & Nester, 1980)

5.0 g / L tryptone

5.0 g / L mannitol

2.5 g / L yeast extract

1.0 g / L glutamic acid

250 mg / L potassium phosphate monobasic (KH_2PO_4)

100 mg / L NaCl

100 mg / L magnesium sulphate 7-hydrate ($MgSO_4.7H_2O$)

10 µL biotin (0.1 mg / mL stock)

pH 7.0

SOC media

20 g Tryptone

5 g yeast extract

2 ml of 5M NaCl

2.5 ml of 1 M KCl

10 ml of 1 M $MgCl_2$

10 ml o 1 M $MgSO_4$

20 ml of 1 M glucose

Adjust to 1 litre using de-ionised water, sterilise by autoclaving

Appendix 2: *Sln1* ORF Sequences

WT (cv Himalaya, cv Herta, cv Triumph), *y-1* (cv Triumph)

Nucleotide sequence

atgaagcgcgagtaccaggacggcggcgggagcggcggtgggggtgatgagatggggtcgtcgag
ggacaagatgatggtgtcgtcgtcggaggcgggggaggggggaggaggtggacgagctgctggcgg
cgctcgggtacaaggtgcgggcgtccgacatggcggacgtggcgcagaagctggagcagctcgag
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ggagcgcacagagcggcacgagacactggggcagtggcggaaccggctgggcaacgccgggttcg
agaccgtgcacctgggctccaatgcctacaagcaggcgagcacgctgctggccctcttcgccggc
ggcgacgggtacaaggtggaagagaaggaagggtgcctgactctcgggtggcacacgcgcccgct
gatcgccacttccgcatggcgcctcgccgcgccgtga

Amino acid sequence

MKREYQDGGGSGGGGDEMGSSRDKMMVSSSEAGEGEEVDELLAALGYKVRASDMADVAQKLEQLE
MAMGMGGPAPDDGFATHLATDTVHYNPTDLSSWVESMLSELNAPPPPLPPAPPQLNASTSSTVTG
GGGYFDLPPSVDSSSSTYALRPIISPPVAPADLSADSVRDPKRMRTGGSSTSSSSSSSSSLGGGA
ARSSVVEAAPPVAAAAAAPALPVVVVDTQEAGIRLVHALLACAEAVQQENLSAAEALVKQIPLLA
ASQGGAMRKVAAYFGEALARRVFRFRPQPDSSLLDAAFADLLHAHFYESCPYLKFAHFTANQAIL
EAFAGCRRVHVVDFGIKQGMQWPALLQALALRPGGPPSFRLTGVGPPQPDETDALQQVGWKLAQF
AHTIRVDFQYRGLVAATLADLEPFMLQPEGEEDPNEEPEVIAVNSVFEMHRLLAQPGALEKVLGT
VRAVRPRIVTVVEQEANHNSGSFLDRFTESLHYYSTMFDSLEGGSSGGPSEVSSGGAAPAAAAGT
DQVMSEVYLGRQICNVVACEGTERTERHETLGQWRNRLGNAGFETVHLGSNAYKQASTLLALFAG
GDGYKVEEKEGCLTLGWHTRPLIATSAWRLAAP

WT (cv H930-36)

Nucleotide sequence

atgaagcgcgagtaccaggacggcggcgggagcggcggtgggggtgatgagatggggtcgtcgag
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ggcgacgggtacaaggtggaggagaaggaagggtgcctgactctcgggtggcacacgcgcccgct
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Amino acid sequence

MKREYQDGGGSGGGGDEMGSSRDKMMVSSSEAGEGEEVDELLAALGYKVRASDMADVAQKLEQLE
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GGGYFDLPPSVDSSSSTYALRPIISPPVAPADLSADSVRDPKRMRTGGSSTSSSSSSSSLGGGA
ARSSVVEAAPPVAAAAAAPALPVVVVDTQEAGIRLVHALLACAEAVQQENLSAAEALVKQIPLLA
ASQGGAMRKVAAYFGEALARRVFRFRPQPDSSLLDAAFADLLHAHFYESCPYLKFAHFTANQAIL
EAFAGCRRVHVVDFGIKQGMQWPALLQALALRPGGPPSFRLTGVGPPQPDETDALQQVGWKLAQF
AHTIRVDFQYRGLVAATLADLEPFMLQPEGEEDPNEEPEVIAVNSVFEMHRLLAQPGALEKVLGT
VRAVRPRIVTVVEQEANHNSGSFLDRFTESLHYYSTMFDSLEGGSSGGPSEVSSGGAAPAAAAGT
DQVMSEVYLGRQICNVVACEGTERTERHETLGQWRNRLGNAGFETVHLGSNAYKQASTLLALFAG
GDGYKVEEKEGCLTLGWHTRPLIATSAWRLAAP

***dwf2* (cv H930-36)**

Nucleotide sequence

atgaagcgcgagtaccaggacggcggcgggagcggcggtgggggtgatgagatggggtcgtcgag
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Amino acid sequence

MKREYQDGGGSGGGGDEMGSSRDKMMVSSSEAGEGEEVDELGYKVRASDMADVAQKLEQLEMAMG
MGGPAPDDGFATHLATDTVHYNPTDLSSWVESMLSELNAPPPPLPPAPPQLNASTSSTVTGGGGY
FDLPPSVDSSSSTYALRPIISPPVAPADLSADSVRDPKRMRTGGSSTSSSSSSSSLGGGAARSS
VVEAAPPVAAAAAAPALPVVVVDTQEAGIRLVHALLACAEAVQQENLSAAEALVKQIPLLAASQG
GAMRKVAAYFGEALARRVFRFRPQPDSSLLDAAFADLLHAHFYESCPYLKFAHFTANQAILEAFA
GCRRVHVVDFGIKQGMQWPALLQALALRPGGPPSFRLTGVGPPQPDETDALQQVGWKLAQFAHTI
RVDFQYRGLVAATLADLEPFMLQPEGEEDPNEEPEVIAVNSVFEMHRLLAQPGALEKVLGTVRAV
RPRIVTVVEQEANHNSGSFLDRFTESLHYYSTMFDSLEGGSSGGPSEVSSGGAAPAAAAGTDQVM
SEVYLGRQICNVVACEGTERTERHETLGQWRNRLGNAGFETVHLGSNAYKQASTLLALFAGGDGY
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***dwf2-1* (cv H930-36)**

Nucleotide sequence

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ggacaagatgatggtgtcgtcgtcggaggcgggggaggggggaggaggtggacgagctgctggcgg
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Amino acid sequence

MKREYQDGGGSGGGGDEMGSSRDKMMVSSSEAGEGEEVDELLAALGYKVRASDMADVAQKLEQLE
MAMGMGGPAPDDGFATHLATDTVHYNPTDLSSWVESMLSELNAPPPPLPPAPPQLNASTSSTVTG
GGGYFDLPPSVDSSSSTYALRPIISPPVAPADLSADSVRDPKRMRTGGSSTSSSSSSSSLGGGA
ARSSVVEAAPPVAAAAAAPALPVVVVDTQEAGIRLVHALLACAEAVQQENLSAAEALVKQIPLLA
ASQGGAMRKVAAYFGEALARRVFRFRPQPDSSLLDAAFADLLHAHFYESCPYLKFAHFTANQAIL
EAFAGCRRVHVVDFGIKQGMQWPALLQALALRPGGPPSFRLTGVGPPQPDETDALQQVG(W/**sto
p**)KLAQFAHTIRVDFQYRGLVAATLADLEPFMLQPEGEEDPNEEPEVIAVNSVFEMHRLLAQPGA
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PAAAAGTDQVMSEVYLGRQICNVVACEGTERTERHETLGQWRNRLGNAGFETVHLGSNAYKQAST
LLALFAGGDGYKVEEKEGCLTLGWHTRPLIATSAWRLAAP

***sln1-1* (cv Herta)**

Nucleotide sequence

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cgctcgggtacaaggtgcgggcgtccgacatggcggacgtggcgcagaagctggagcagctcgag
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ggcgacgggtacaaggtggaagagaaggaagggtgcctgactctcgggtggcacacgcgcccgct
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Amino acid sequence

MKREYQDGGGSGGGGDEMGSSRDKMMVSSSEAGEGEEVDELLAALGYKVRASDMADVAQKLEQLE
MAMGMGGPAPDDGFATHLATDTVHYNPTDLSSWVESMLSELNAPPPPLPPAPPQLNASTSSTVTG
GGGYFDLPPSVDSSSSTYALRPIISPPVAPADLSADSVRDPKRMRTGGSSTSSSSSSSSLGGGA
ARSSVVEAAPPVAAAAAAPALPVVVVDTQEAGIRLVHALLACAEAVQQENLSAA**stop**