Temperature regulation of seed dormancy and germination in *Arabidopsis thaliana*

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Abstract

The environmental regulation of seed dormancy and germination by temperature is an important process which allows the environmental conditions experienced by plants in the following generation to be controlled. Coping with temperature effects during seed maturation is essential for the consistent production of high quality seeds, but currently temperature signalling pathways in seeds are poorly understood. Previous work has shown that temperature during seed maturation regulates the levels of dormancy which are induced in the seed, although a mechanism for this pathway is currently unknown. Low temperature during imbibition promotes germination and although this is known to involve alterations to GA metabolism, again a mechanism is missing. Therefore, the aim of this study was to identify components of the mechanisms which regulate the temperature control of dormancy and germination in the model plant *Arabidopsis thaliana*.

Cool and warm seed maturation temperatures induce high and low levels of dormancy respectively in *Arabidopsis*. These changes to dormancy levels are coupled with altered ABA and GA levels and gene expression controlling hormone synthesis and breakdown. Changes in maturation temperature do not appear to be linked to altered seed coat morphology or embryo development.

During testing of cold-response mutants for dormancy phenotypes the expression of *CBFs*, a group of transcription factors which were characterised through the study of cold acclimation, was found to be necessary for dormancy. *CBF* RNAi and mutant seeds display reduced dormancy when matured at low temperature. However, the expression of *CBFs* is not promoted by exposure to low temperature in seeds,

suggesting that this is not an important mechanism for the temperature control of dormancy.

More strikingly, the E3 ligase HOS1 is absolutely required for high dormancy levels in response to low maturation temperature. *hos1* mutants show a complete loss of dormancy when matured at any temperature and this phenotype is maternally inherited. The germination of *hos1* seeds in the presence of PAC or ABA does not differ from wild-type, thus suggesting that sensitivity to GA and ABA is not altered in these seeds. However, levels of GA are increased in *hos1* mutant seeds, which could be important for the reduced dormancy phenotype. The expression of *TT* genes, which are responsible for the accumulation of anthocyanidins in the seed coat, is downregulated in *hos1* mutants and so could be involved in the regulation of dormancy by HOS1. Therefore; HOS1 defines a novel essential maternal pathway that regulates dormancy levels which involves the regulation of GA metabolism.

A forward genetic screen identified a number of *cold stratification insensitive* (*cosi*) mutants. A thorough characterisation of these mutants revealed interesting phenotypes, but phenotypic variation and a lack of robust segregation data meant that the *cosi* mutants were not mapped.

Table of contents

Table of contents

Table of contents

Table of contents

Table of contents

Table of contents

List of figures

List of figures

List of figures

List of figures

List of tables

Publications

Kendall, S., Hellwege, A., Marriot, P., Whalley, C., Graham, I. & Penfield, S. (2011). Induction of Dormancy in Arabidopsis Summer Annuals Requires Parallel Regulation of DOG1 and Hormone Metabolism by Low Temperature and CBF Transcription Factors. *The Plant Cell*, **23**:2568-2580.

The microarray data in this publication were provided by Steve Penfield and, therefore,

will be cited in this thesis as Kendall et al (2011) when referred to.

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Authors Declaration

I declare that I am the sole author of the work in this thesis and that it is original except where indicated by special reference in the text. No part of this degree has been submitted for any other degree to any other institution.

Seed fixing and sectioning outlined in section 2.8 was performed by Meg Stark.

The phytohormone analyses outlined in section 2.10 was performed by Anja Hellwege. The oxylipin analyses outlined in section 2.11 was performed by Anuja Dave. Fatty Acid Methyl Ester extractions and analysis outlined in section 2.12 was performed by Anuja Dave.

Running of RNA samples for RNA-Sequencing on the Bioanalyser (section 2.16.5) was performed by Celina Whalley. RNA-Sequencing and preliminary analyses (section 2.16.5) was performed by Konrad Paszkiewicz.

The *cosi* mutant screen and initial characterisation detailed in section 6.2.1 was performed by Steve Penfield.

Experiments in sections 6.2.5.2, 6.2.6.2 and 6.2.6.3 were performed by Natalie Palmer, an undergraduate project student under my supervision. I was responsible for the design of the experiments and the collection of data in her absence.

Chapter 1 Introduction

1.1 Temperature

Temperature acts as an important cue to allow plants to sense and respond to changes in environmental conditions, enabling them to co-ordinate their life cycle with the changing of the seasons. Temperature does not only change according to the season, but also on a diurnal basis and it is important that plants are able to anticipate these changes. The ability of plants to perceive both high and low temperatures is key to their survival, but is also used to regulate a number of developmental processes (Penfield, 2008).

As plants are unable to move when conditions become unfavourable they have developed the ability to readily adapt their growth and developmental processes to changing conditions, i.e. developmental plasticity. Increases in mean global temperature and extremes of temperature are predicted for the future and these predictions are significantly larger than those that have occurred so far, suggesting that there will be large disruptions to the behaviour of wild plants and crops (Kumar and Wigge, 2010). It has been predicted that temperature alone could contribute to the extinction of up to one third of all European plant species (Thuiller et al., 2005). The importance of plant responses to temperature in terms of surviving climate change is highlighted by Willis et al.,(2008). Evidence is presented to suggest that species which are able to shift flowering time in response to temperature change are less prone to extinction than those species which cannot.

1.1.1 Molecular mechanisms of temperature perception

To date, the majority of research investigating the molecular mechanisms that underlie temperature perception has focused on the responses of plants to extreme temperatures (i.e. low and high temperatures), such as the cold acclimation and vernalisation pathways. However, more recently the mechanisms that regulate the responses of plants to ambient temperatures are starting to be uncovered (Koini et al., 2009, Saidi et al., 2009 Kumar and Wigge, 2010, Sidaway-Lee et al., 2010, Finka et al., 2012, Gao et al., 2012, Kumar et al., 2012).

Relatively small changes to temperature in the ambient range have been shown to have significant effects on plant development. For example, hypocotyl elongation is much more prominent at 28°C in comparison to 22°C and this is mediated by PHYTOCHROME INTERACTION FACTOR 4 (PIF4) and involves the hormone auxin (Franklin, 2009, Koini et al., 2009, Franklin et al., 2011). A role for PIF4 has also been identified in the pathway that is responsible for the promotion of flowering under short day conditions by increasing temperature (Kumar et al., 2012). The promotion of flowering by increasing temperature from 22°C to 27°C in wild-type plants was abolished in *pif4-101* plants and this was due to a decrease in *FLOWERING LOCUS T* (*FT*) expression. PIF4 binds to the *FT* promoter to activate expression in a temperature dependent manner and this binding is thought to be regulated by H2A.Z nucleosome dynamics (Kumar et al., 2012).

Ambient temperature sensing has been shown to involve changes in the H2A.Z occupancy of nucleosomes (Kumar and Wigge, 2010). Increases in temperature lead to a decrease in H2A.Z occupancy, allowing DNA to become less tightly wound and more

accessible to RNA polymerase II. The transcriptome of the *actin related protein* (*arp6*) mutant is constitutively 'warm' and this is due to a decrease in H2A.Z occupancy in *arp6* mutants in comparison to wild-type at 17°C.

Calcium signalling has been associated with temperature signalling in plants for a number of years. Cold shock of *Arabidopsis* seedlings leads to an increase in cytosolic Ca^{2+} levels (Knight et al., 1996). Tobacco plants which overexpress *Arabidopsis CATION EXCHANGER1* (*CAX1*) are Ca^{2+} deficient and show increased sensitivity to cold shock (Hirschi, 1999). Building evidence suggests that Ca^{2+} permeable channels could act as a temperature sensor (Monroy and Dhindsa, 1995, Plieth et al., 1999, Saidi et al., 2009, Finka et al., 2012, Gao et al., 2012). Disruption to the *Arabidopsis CYCLIC NUCLEOTIDE-GATED ION CHANNEL2* (*CNGC2*) gene, which encodes a component of a nucleotide gated Ca^{2+} channel, leads to the heat shock response at milder temperatures (Finka et al., 2012). In another study, *Arabidopsis* CNGC6 was shown to mediate heat-induced Ca^{2+} influx and regulates the expression of *HEAT SHOCK PROTEIN* genes, which in turn leads to thermotolerance (Gao et al., 2012).

1.1.2 Cold Acclimation

The process of cold acclimation, which is used by plants to survive adverse conditions, involves plants experiencing a period of exposure to low temperature that allows them to survive subsequent exposures to freezing temperatures (Guy, 1990). Central players in this pathway are a small group of APETALA2 (AP2) domain transcription factors known as C-REPEAT BINDING FACTORS (CBFs) (Stockinger et al., 1997, Gilmour et al., 1998) (Figure 1.1). This group comprises three genes; *CBF1, CBF2* and *CBF3* which have a large amount of homology (Medina et al., 1999). *CBF* expression increases transiently in response to exposure to low temperature with peaks in

expression occurring approximately one hour after transfer to 2.5°C and expression diminishes after twenty four hours (Gilmour et al., 1998). The overexpression of *CBFs* confers freezing tolerance in the absence of cold acclimation due to the increase in expression of a suite of genes that are involved in metabolic and physiological changes that aid resistance to freezing temperatures (Jaglo-Ottosen et al., 1998, Gilmour et al., 2000, Vogel et al., 2005).

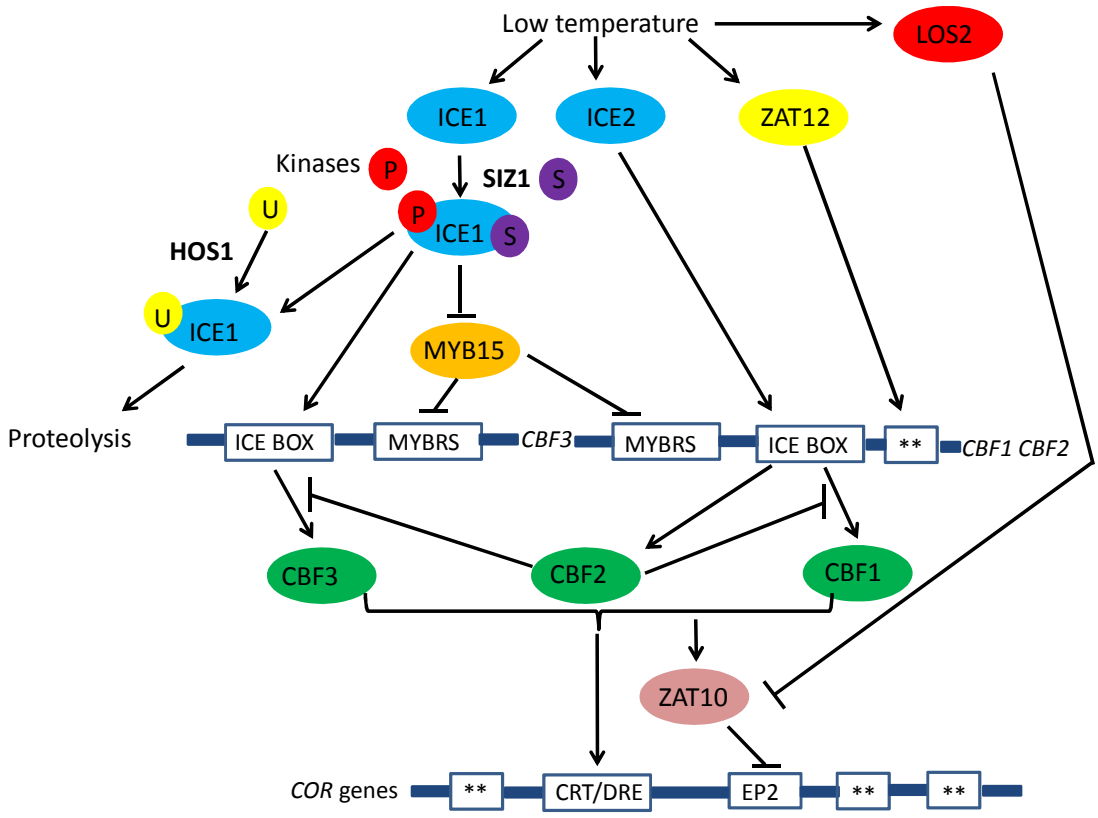

Figure 1.1: Schematic of cold acclimation pathway.
Constitutively expressed ICE1 is activated by low temperature through sumoylation and phosphorylation, which is able to promote *CBF3* expression and repress *MYB15* expression. The ubiquitination of ICE1 by HOS1 targets it for proteolysis. *CBF3* expression is negatively regulated by ZAT12 and MYB15. *CBF1* expression is promoted by ICE2. CBF2 negatively regulates the expression of *CBF1* and *CBF3*. The CBFs promote expression of the *COR* genes. *ZAT10* is induced by the CBFs, which may negatively regulate *COR* gene expression. Cold upregulation of LOS2 negatively regulates *ZAT10* expression. P, phosphorylation; U, ubiquitination; S, sumoylation. Adapted from Chinnusamy et al., (2007).

The CBFs bind to a low temperature responsive DNA regulatory element called C-repeat (CRT)/ dehydration response element (DRE) in the promoters of *COLD RESPONSIVE* (*COR*) genes (Yamaguchi-Shinozaki and Shinozaki, 1994, Stockinger et al., 1997). These motifs contain the conserved CCGAC core sequence. *CBF3* expression is promoted by the MYC-type basic helix–loop–helix (bHLH) transcription factor INDUCER OF CBF EXPRESSION1 (ICE1) (Gilmour et al., 1998, Chinnusamy et al., 2003). An ICE1 homologue, ICE2, is involved in regulating expression of *CBF1* (Fursova et al., 2009). The *ice1-2* mutant is incapable of inducing expression of *CBF3* and plants are freezing sensitive as the cold acclimation pathway cannot be employed (Chinnusamy et al., 2003).

The regulation of an optimal cold-induced transcriptome relies on feedback repression of transcription factors that regulate cold-responsive gene expression (Chinnusamy et al., 2007). Analysis of the *cbf2* mutant showed that in the absence of *CBF2* expression, levels of *CBF1* and *CBF3* expression was elevated, thus suggesting that CBF2 acts as a negative regulator of *CBF1* and *CBF3* expression (Novillo et al., 2004). CBF1 and CBF3 have been shown to act additively in cold acclimation (Novillo et al., 2007).

An important negative regulator of cold induced gene expression is the transcription factor MYB15, which represses *CBF3* expression by binding to the MYB recognition sequence in the promoter of *CBF3* (Agarwal et al., 2006). *myb15* mutant plants display increased freezing tolerance, whilst the overexpression of *MYB15* leads to increased freezing sensitivity (Agarwal et al., 2006). Interestingly, ICE1 is able to negatively regulate MYB15 and this is indicated by elevated *MYB15* expression levels in the *ice1-2* mutant (Chinnusamy et al., 2003, Agarwal et al., 2006).

A number of additional negative regulators of the cold acclimation pathway has been identified. The cold induced C2H2 zinc finger protein ZAT12 has also been demonstrated as a negative regulator of *CBF* expression (Vogel et al., 2005). Studies using the *los2* mutant identified another C2H2 zinc finger protein, ZAT10 as being a negative regulator of CBF target gene expression (Lee et al., 2002a). LOS2 is a bifunctional enolase which binds to the MYC recognition elements in the ZAT10 promoter *in vitro* (Lee et al., 2002a). LOS2 is a negative regulator of ZAT10, since the *los2* mutant displays increased levels of expression of *ZAT10* (Lee et al., 2002a).

Post-translational regulation of cold acclimation is also important. In response to low temperature, ICE1 is sumoylated by the SUMO E3 ligase SIZ1 (Miura et al., 2005) which leads to the activation or stabilisation of the ICE1 protein (Miura et al., 2007). Sumoylation is a post-translational protein modification whereby SUMO (a small ubiquitin-related modifier) proteins are conjugated to protein substrates and this relies on SUMO E3 ligases. The sumoylation of ICE1 allows it to bind to the MYC recognition elements in the promoter of *CBF3* to promote expression (Chinnusamy et al., 2003).

Another component of the cold acclimation pathway which is involved in post-translational regulation is HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1 (HOS1). *HOS1* encodes a Ring E3 ligase that is involved in the ubiquitination of ICE1 (Lee et al., 2001, Dong et al., 2006a). The *hos1-1* mutant allele was isolated from a screen using *Arabidopsis* plants that expressed the Luciferase reporter gene, which was driven by the *RD29A* promoter (Ishitani et al., 1998). The promoter of *RD29* contains the CRT/DRE element and its expression is induced by cold, Abscisic Acid (ABA), drought and salt (Gilmour and Thomashow, 1991). Luciferase luminescence was greatly increased in *hos1-1* plants in response to cold in comparison to wild-type,

whereas there was only a small increase in luminescence in response to ABA (Ishitani et al., 1998). Expression of a number of cold regulated genes, such as *COR15A* and *COR47* was increased in *hos1-1* mutants and often the peak in expression of these genes was earlier than in wild-type (Ishitani et al., 1998). The increase in expression of cold-responsive genes is a consequence of elevated *CBF* expression in *hos1-1* (Lee et al., 2001). The *hos1-1* mutant displayed increased electrolyte leakage in non-acclimated plants, whereas freezing tolerance was unaltered in acclimated plants (Ishitani et al., 1998). This result is surprising given the increase in expression of the *CBFs* and downstream target genes and that overexpression of the *CBFs* leads to an increase in freezing tolerance. In contrast, Dong et al., (2006a) found that plants overexpressing *HOS1* had decreased freezing tolerance, which was presumably due to the decrease in expression of the low temperature regulon.

Expression of *HOS1* was found to decrease quickly and transiently in response to low temperature (Lee et al., 2001). HOS1 has been shown to physically interact with ICE1 and mediates the ubiquitination both *in vitro* and *in vivo* (Dong et al., 2006a). Plants overexpressing *HOS1* display increased levels of GREEN FLUORESCENT PROTEIN (GFP)-ICE1 levels and a corresponding increase in *CBF* expression. Using HOS1-GFP, HOS1 was found to be exclusively localised to the nucleus following cold treatment, whereas under control conditions there was only very faint fluorescence that was localised to the cytoplasm (Lee et al., 2001).

The overexpression of *CBF3* leads to a dwarfed plant phenotype and stunted growth in *Arabidopsis* (Liu et al., 1998, Gilmour et al., 2000). Achard et al., (2008) link the dwarfed phenotype of 35S::*CBF1 Arabidopsis* plants to the phenotype of Gibberellin (GA) deficient mutants described by Richards et al., (2001). Achard et al., (2008) show

that the exogenous application of GA to 35S::*CBF1* plants rescued the stunted growth phenotype. Additionally, when *GA INSENSITIVE* (*GAI*) and *REPRESSOR OF ga1-3* (*RGA*), genes encoding two members of the five DELLA proteins in *Arabidopsis* are knocked down alongside overexpression of *CBF1*, growth is also restored to wild-type. Therefore, *CBFs* restrain growth in a DELLA dependent manner via the effect on GA metabolism (Achard et al., 2008). Additionally, expression of two genes involved in GA catabolism, *GA 2-oxidase3* (*GA2ox3)* and to a lesser extent *GA2ox6* are upregulated in *CBF1*-overexpressing plants and lead to a decrease in bioactive GA levels.

A recent microarray study revealed that *CBF2*-overexpressing plants showed an increase in expression of genes associated with ABA biosynthesis and response genes (Sharabi-Schwager et al., 2010). Increases in both *CBF* transcript and protein levels have been observed in response to ABA treatment (Knight et al., 2004). Therefore, there is evidence to suggest that *CBFs* have a role in the regulation of both ABA and GA levels.

1.1.3 Vernalisation

The process of vernalisation involves the promotion of flowering in winter annuals in response to prolonged cold, ensuring that flowering occurs in the spring. The MADS box transcription factor FLOWERING LOCUS C (FLC) plays a central role in the pathway and is responsible for the repression of the expression of the floral promoters *FT*, *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*) and *LEAFY* (*LFY*). FT is a floral promoter which integrates both photoperiod and temperature signals. FT is produced in the leaves and moves through the vascular tissues to the shoot apex where it induces flowering (Jaeger and Wigge, 2007).

The requirement for the repression of flowering by FLC in winter annuals is conferred by the presence of a dominant allele of *FRIGIDA* (*FRI*) (Michaels and Amasino, 1999, Sheldon et al., 1999, Johanson et al., 2000). In contrast, summer annuals flower readily without a requirement for vernalisation. This is due to the absence of an active *FRI* allele or the presence of a weak *FLC* allele (Johanson et al., 2000, Gazzani et al., 2003, Michaels et al., 2003). The flowering of winter annuals is promoted by the epigenetic silencing of *FLC*, which is regulated by the transcriptional activation of *VERNALISATION INSENSITIVE3* (*VIN3*) in response to long periods of cold (Sung and Amasino, 2004). VIN3 is able to distinguish the length of exposure to cold, since *VIN3* is only expressed after a duration of cold that is effective for vernalisation (Sung and Amasino, 2004). The repression of *FLC* remains stable when plants are transferred to warm conditions (Sung et al., 2006).

1.1.4 Temperature regulation of growth

Temperature has been identified as a regulator of growth for a number of years (Briggs et al., 1920, Grime and Hunt, 1975), although the genetic mechanism for this regulation has only started to be identified recently (Sidaway-Lee et al., 2010). The bHLH transcription factor SPATULA (SPT) has been shown to be important for reducing growth rate in response to low ambient temperatures, since *spt* mutants show increased growth at 15°C in comparison to wild-type (Sidaway-Lee et al., 2010). This regulation of growth rate in response to temperature by SPT is thought to be independent of GA signalling or Phytochrome B (PHYB). This study showed that only the morning temperature contributes to the regulation of growth rate and the regulation of growth rate by SPT is limited to the integration of morning temperature.

1.2 Seed dormancy and germination

The *Arabidopsis* mature dry seed consists of an embryo which is surrounded by a single layer of endosperm cells. A seed coat, which consists of five layers, surrounds the endosperm cells and this structure is derived from two ovular integuments and is therefore, maternal in origin (Beeckman et al., 2000). Cells of the seed coat in the dry mature *Arabidopsis* seed are not living, since these cells die during the late maturation stage following significant developmental changes. However, in other species, seed coat cells remaining alive at maturity. In contrast, the endosperm cells are physiologically active.

Freshly harvested mature *Arabidopsis* seeds can display primary dormancy. Primary dormancy will be referred to as dormancy throughout this thesis. Although a number of definitions for dormancy has been proposed, here it will be described simply as the inability of a viable seed to germinate when conditions are favourable (Bewley, 1997). Seed dormancy is an important adaptive trait that is used by plants to survive natural catastrophes, avoid competition between individuals of the same species or prevent germination out of season (Finkelstein et al., 2008). Not only does dormancy ensure that seeds do not germinate when conditions are unfavourable, but it also enables seeds be stored in the soil as a seed bank. The presence of seed banks in the soil allows germination to occur over several seasons, thus maximising the chances of long-term survival (Gubler et al., 2005).

Two forms of dormancy have been recognised. Firstly, dormancy in which the seed coat or other surrounding structure prevents the germination of the embryo, although the embryo is not dormant itself, is known as coat enhanced dormancy. Secondly, embryos themselves can be dormant and this is known as embryo dormancy (Bewley,

1997). Primary dormant seeds are able to enter into a second stage of dormancy during imbibition if conditions for germination are unfavourable and this is known as secondary dormancy (Bewley and Black, 1994). As an example, secondary dormancy can be induced by prolonged exposure to low temperature during imbibition in a number of accessions (Finch-Savage et al., 2007, Penfield and Springthorpe, 2012). Secondary dormancy can also be induced if germination is prevented by darkness during imbibition. Seasonal dormancy cycling occurs in seeds where dormancy is continually gained and lost until seeds germinate or die.

Defining dormancy is a difficult task since dormancy levels can only be quantified by analysing germination. The germination of a seed can be described as an all-or-nothing event whereas the dormancy of a seed can be anywhere between all (maximum dormancy) and nothing (non-dormancy) (Finch-Savage and Leubner-Metzger, 2006). Therefore, the levels of dormancy of a population of seeds can only be interpreted by measuring the germination frequency.

The germination of a seed begins with the uptake of water by imbibition of the dry seed, which is followed by embryo expansion. This is followed by rupture of the endosperm and seed coat and the emergence of the radical. The completion of germination is characterised by the protrusion of the radical through the seed coat.

1.2.1 Economical and Agronomical importance of dormancy and germination

Although low dormancy levels have been selected for in many domesticated plants to ensure fast and uniform germination and seedling establishment to lead to good crop yields, premature loss of dormancy can be problematic. Pre-harvest sprouting is a

limiting factor affecting cereal production in areas of the world where harvest is preceded by cool, damp conditions which can lead to germination before the crop is harvested. In the UK, pre-harvest sprouting is the major cause of increased alpha-amylase hydrolytic enzyme activity, which has a negative effect on wheat grain quality and leads to large economic losses (Lunn et al., 2001). Therefore, dormancy at harvest in cereals is a desirable trait to eliminate pre-harvest sprouting occurring. Additionally, mutants that display reduced levels of seed dormancy in *Arabidopsis* have been shown to have reduced seed longevity, which also has agricultural and horticultural implications (Clerkx et al., 2003).

In crop species, equal dormancy levels are desirable in a population to ensure that germination and seedling establishment occurs in synchrony. However, in the wild, it may be advantageous for dormancy levels within a population to show some variance, to reduce the amount of direct competition occurring between seeds. In the case of the grasses *Aegilops ovate* and *A. kotschyi*, the upper and lower caryopses are dispersed together, but the two grains germinate in different years due to differences in dormancy levels, and so avoid direct competition with one another (Gutterman, 1980/1981).

In contrast to wheat, the opposite problem occurs in barley grains, whereby grains are too dormant at harvest, preventing grains from germinating in the synchronous manner that is required for the malting process (Gubler et al., 2005). Barley grains that are too dormant must be after-ripened, and so extra costs associated with storage are incurred.

1.2.2 Regulation of dormancy by the seed coat

It is well established that the seed coat can contribute to dormancy in many plant species. The seed coat not only functions to protect the embryo, but also acts as a

physical barrier to embryo growth prior to germination. Additionally, the impermeability of the seed coat to water and oxygen contributes to its function to prevent germination. Seed coat thickening has been associated with increased dormancy in the past. *Chenopodium polyspermum L.* seeds from mother plants grown in long days have lower germination frequencies and thicker seed coats in comparison to seeds from mother plants grown in short days (Pourrat and Jacques, 1975). Additionally, ABA-deficient and ABA-insensitive mutants display reduced thickness of the mucilage layer surrounding the seed coat (Karssen et al., 1983).

A group of seed coat mutants called *transparent testa (tt)* has thinner seed coats which are more permeable to GA and the GA biosynthesis inhibitor Paclobutrazol (PAC) (Debeaujon et al., 2000). This decrease in seed coat thickness is due to a reduction in the accumulation of anthocyanidins, which affects the mechanical strength of the seed coat. It is the polymeric nature of proanthocyanidins coupled with their ability to bind proteins that confer the cell-cementing properties to the seed coat and allow it to act as an impermeable barrier that prevents the uptake of water (Debeaujon et al., 2000).

Additionally, the *tt* mutants also display reduced dormancy phenotypes (Debeaujon et al., 2000), suggesting that anthocyanidin accumulation is required for correct dormancy levels. *tt7-1* seeds show thermoinhibition (the repression of germination by high temperatures) resistance at both 32°C and 34°C (Tamura et al., 2006). Other *tt* mutants have been isolated from a screen for fast seed germination at 10°C (Salaita et al., 2005). Together this evidence suggests that TT may have a role in regulating dormancy and germination levels in response to both high and low temperatures.

1.3 Hormonal regulation of dormancy and germination

The two phytohormones ABA and GA are the main hormones that are involved in regulating seed dormancy and germination (Figure 1.2), although roles for additional hormones such as brassinosteroid (BR), ethylene, auxin and strigolactone have also been described. ABA acts as a positive regulator of dormancy and negatively regulates germination whilst GA has the opposite effect. It is thought that the actual ratio of ABA: GA is what regulates these processes and not the absolute levels *per se*. Alterations to the ratio of ABA: GA can lead to different dormancy levels. In many cases, changes in environmental conditions that are important for seed dormancy or germination regulation lead to alterations in the transcription of genes involved in ABA or GA metabolism. This suggests that the regulation of ABA and GA levels are central to the mechanism by which dormancy and germination are regulated.

BR is a positive regulator of seed germination and germination of BR signalling mutants are more sensitive to ABA (Steber and McCourt, 2001). Overexpression of the BR biosynthesis gene *DWARF4* (*DWF4*) overcomes inhibition of germination caused by exogenous ABA (Divi et al., 2010). Ethylene also acts a positive regulator of seed germination (KeÇpczyński and KeÇpczyńska, 1997, Beaudoin et al., 2000). The understanding of the role of auxin in germination regulation remained unclear for a number of years; although more recent studies show it does have a role (Ogawa et al., 2003). Strigolactone has been shown to act as a positive regulator of germination during thermoinhibition by reducing the ABA:GA ratio (Toh et al., 2012).

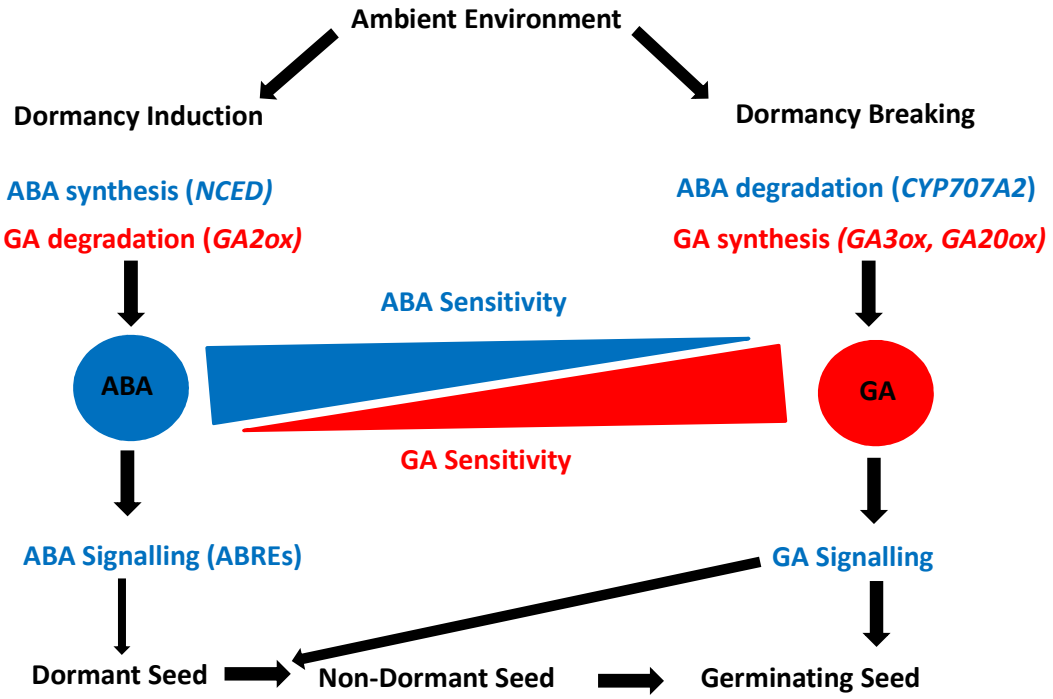

Figure 1.2: Regulation of seed dormancy and germination by ABA and GA. The ambient environment regulates dormancy induction and dormancy breaking through regulation of ABA and GA metabolism. ABA synthesis and GA catabolism promote dormancy induction whilst GA synthesis and ABA catabolism promote dormancy breaking. ABA and GA sensitivity is also regulated, with seeds becoming less sensitive to ABA and more sensitive to GA as the seed looses dormancy on the transition to germination. Adapted from Finch-Savage and Leubner Metzger (2006).

1.3.1 ABA

ABA, which is derived from epoxycarotenoid cleavage, acts as an important stress signal and is also involved in regulating a number of developmental and growth processes under non-stressful conditions. During seed development, ABA levels peak during mid-maturation and this coincides with the induction of dormancy levels (Kermode, 2005). ABA which is synthesised during seed development can be of dual origin, the embryo and/or the maternal tissues (Kucera et al., 2005). There are contrasting opinions as to whether ABA synthesised in the embryo or endosperm is

important for seed dormancy regulation. Finkelstein et al., (1994) argue that it is the ABA that is synthesised in the embryo that is required to impose lasting dormancy. ABA produced from the maternal tissues or by application of ABA (that mimics maternal ABA) during seed development does not induce dormancy however, this ABA is involved in other aspects of seed development (Finkelstein, 1994). In contrast, Lefebvre et al., (2006) show that studies using *nine-cis-epoxycarotenoid dioxygenase6* (*nced6*) and *nced9* mutants reveal that ABA synthesised in both the endosperm and embryo is important for inducing dormancy.

The ABA biosynthesis pathway is now well understood and the germination phenotypes of a large number of mutants in parts of the biosynthetic pathway have been characterised. The ABA biosynthetic pathway has been extensively reviewed in Nambara and Marion-Poll (2005) (Figure 1.3). Briefly zeaxanthin epoxidase catalyses the synthesis of violaxanthin from zeaxanthin through an intermediate called antheraxanthin. Through an unknown mechanism, violaxanthin is converted to neoxanthin. NCED enzymes cleave the *cis*-isomers of violaxanthin and neoxanthin to form xanthoxin. Xanthoxin is then converted by ABA DEFICIENT2 (ABA2) to abscisic aldehyde, which is then oxidised to form ABA by abscisic acid aldehyde oxidase (AAO3).

The germination of mutants that are deficient in ABA synthesis or signalling is generally increased, which stems from the fact that dormancy is not induced in the absence of detectable ABA. A number of *aba* mutants, which have lower levels of ABA show reduced seed dormancy. *aba1* mutants were isolated from a screen of revertents of non-germinating GA-deficient mutants (Koornneef et al., 1982). Two additional ABA-deficient mutants; *aba2* and *aba3* were isolated from a screen of an ethyl methane

sulphonate (EMS) population for seeds that germinated in the presence of PAC (which is characteristic of seeds with reduced ABA levels) (Léon-Kloosterziel et al., 1996).

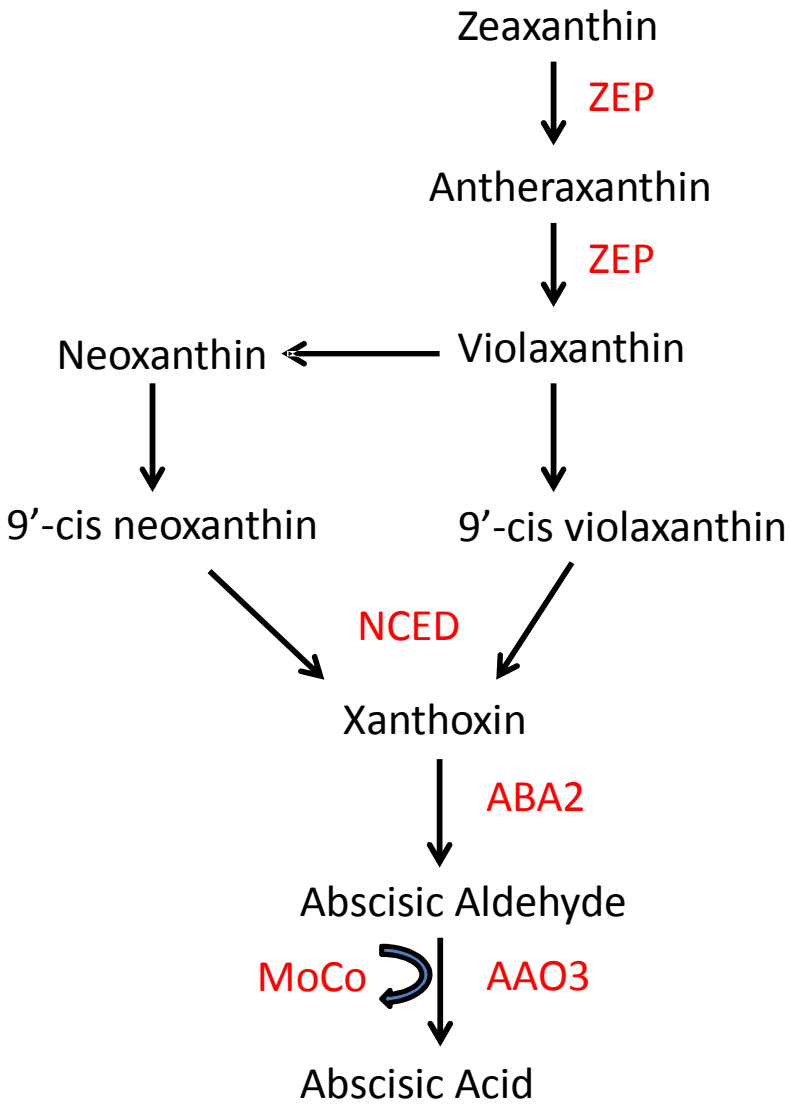

Figure 1.3: Schematic of ABA biosynthesis pathway.
The synthesis of violaxanthin is catalysed by zeaxanthin epoxidase (ZEP) through the intermediate antheraxanthin. *cis* isomers of neoxanthin and violaxanthin are cleaved by 9-*cis*-epoxycarotenoid dioxygenases (NCED) to form xanthoxin. Xanthoxin is converted to abscisic aldehyde by a short-chain alcohol dehydrogenase (ABA2). AOO3, an abscisic aldehyde oxidase cataylses the oxidation of abscisic aldehyde to abscisic acid. The AOO3 protein contains a molybdenum cofactor which is activated by MoCo sulpherase. Adapted from Nambara and Marion-Poll (2005).

During seed development the *NCED* family members *NCED5, NCED6* and *NCED9* have been shown to be important regulators of ABA synthesis and are involved in regulating dormancy (Tan et al., 2003, Seo et al., 2004, Lefebvre et al., 2006, Frey et al., 2012, Martínez-Andújar et al., 2012). *nced6nced9* double loss-of-function mutants have reduced ABA content and decreased levels of seed dormancy (Lefebvre et al., 2006). The *NCED* genes, *NCED2*, *NCED5* and *NCED9*, have been shown to contribute to the thermoinhibition of germination by high temperatures by increasing ABA levels (Toh et al., 2008). *NCED4* expression is upregulated in a *DELAY OF GERMINATION1* (*DOG1*) near isogenic line (NIL), one of a number of *DOG* NILs which were derived from crosses between the reference accession Landsberg *erecta* (L*er*) and accessions from different world regions (Bentsink et al., 2010).

In contrast to biosynthetic mutants, mutants of genes involved in ABA catabolism show increased dormancy, since ABA levels are elevated. *CYP707A1* and *CYP707A2* encode 8'- hydroxylases which is considered to be the key enzyme involved in ABA catabolism (Kushiro et al., 2004, Saito et al., 2004).

A number of *abscisic acid insensitive* (*abi*) mutants were isolated based on their ability to germinate in the presence of ABA, suggesting that they were insensitive to ABA (Koornneef et al., 1984, Finkelstein, 1994). *ABI3, ABI4* and *ABI5* encode B3, AP2, and basic leucine zipper (bZIP) domain families respectively and regulate a similar sub-set of genes during seed development (Giraudat et al., 1992, Finkelstein et al., 1998, Finkelstein and Lynch, 2000, Lopez-Molina and Chua, 2000). *abi3* mutant seeds display seed development defects including reduced seed dormancy and storage reserve proteins (Finkelstein and Somerville, 1990, Nambara et al., 1992). Embryos of *abi3* seeds are morphologically normal, although seeds remain green and do not

develop desiccation tolerance (Nambara et al., 1992). Together this suggests that ABI3 is a central regulator of the late maturation phase of seed development (Parcy et al., 1994). In contrast to *abi3* mutants, *abi4* and *abi5* mutants do not display dormancy defects but are necessary for the inhibition of germination by ABA (Brocard-Gifford et al., 2003).

1.3.2 GA

Evidence from GA-deficient mutants such as *ga1-3* and *ga2-1*, which are defective in seed germination, show that GA is absolutely required for germination, as seeds fail to germinate without exogenous GA (Koornneef and Veen, 1980). The biochemical pathway for GA biosynthesis is well characterized and the majority of genes encoding enzymes in the pathway has been identified (Sun, 2010).

The activation and deactivation of GA has been extensively reviewed by Yamaguchi (2008). Briefly, terpene synthase and P450 enzymes are involved in early stages of GA biosynthesis, which leads to the production of GA_{12}. GA_{12} is then converted to a bioactive form, GA_4 by GA 20-oxidase (GA20ox) and GA3ox. Key enzymes in the GA deactivation pathway are encoded by the *GA2ox* genes which use $C\text{-}_{19}$ and $C\text{-}_{20}$-GAs.

The DELLA family of GRAS proteins, which are characterised by two leucine rich areas flanking a VHIID motif, are negative regulators of plant growth and have been shown to have a role in seed germination (Lee et al., 2001, Tyler et al., 2004, Cao et al., 2006, Penfield et al., 2006). In *Arabidopsis* there are five DELLA proteins, RGA, GAI, RGA LIKE1 (RGL1), RGL2 and RGL3. DELLA proteins are nuclear transcriptional regulators, which repress GA signalling by causing transcriptional reprogramming (Sun, 2010). The GA signal targets DELLAs for degradation via ubiquitination and the 26S proteasome

pathway, releasing the plant from the DELLA-mediated growth constraint (Silverstone et al., 2001). This targeting of DELLAs for degradation occurs when GA binds to its soluble receptor, GA INSENSITIVE DWARF1 (GID1). The GID1-GA complex then causes the binding to DELLA and consequently degradation (Hirano et al., 2007).

The increased germination capacity of loss-of-function *della* mutants suggests that they have a role in negatively regulating germination (Cao et al., 2006, Penfield et al., 2006). A loss-of-function mutation in *RGL2* was able to rescue the germination defect in *ga1-3* mutant seeds, leading to the conclusion that *RGL2* acts as a negative regulator of GA responses in the control of seed germination (Lee et al., 2002b). Cao et al., (2006) suggest that light promotes seed germination in part by inactivating or destabilising GAI and RGA proteins and this is likely to be mediated through the regulation of GA metabolic genes.

1.3.3 ABA and GA crosstalk

In the *ga1-3* mutant, ABA biosynthetic genes are activated and ABA deactivation genes suppressed, suggesting that GA regulates ABA metabolism (Oh et al., 2006). Additionally, RGL2 is responsible for promoting ABA synthesis when GA levels are reduced. The increase in ABA levels in turn forms a positive feedback loop by promoting *RGL2* expression (Piskurewicz et al., 2008). Following transfer of imbibed seeds to 4°C and then 20°C, a decrease in ABA content occurs before germination (Ali-Rachedi et al., 2004). This decrease in ABA levels is also apparent upon imbibition at warm temperatures (Chiwocha et al., 2005), suggesting that the promotion of germination by cold stratification does not require a reduction in ABA levels.

1.4 After-Ripening

The dry storage of freshly harvested dormant seeds is known as after-ripening (Bewley, 1997). After-ripening integrates both time and environmental factors to regulate the germination potential of seeds (Carrera et al., 2007). The speed at which dormancy is lost in response to after-ripening is dependent on a number of factors which include environmental conditions during seed maturation, seed storage and germination (Donohue et al., 2005). The molecular mechanism through which after-ripening reduces dormancy levels is not well understood. After-ripening is thought to be associated with a loss of sensitivity to ABA and an increase in sensitivity to GA and ethylene (Finch-Savage and Leubner-Metzger, 2006). Increased expression of genes associated with ABA biosynthesis, such as *NCED6, NCED9* and *ABA1* is found in primary dormant seeds in comparison to after-ripened seeds (Cadman et al., 2006). The expression of *CYP707A2,* which is involved in ABA catabolism, is higher in after-ripened seeds than primary dormant seeds (Cadman et al., 2006). The regulation of GA levels also appears to be important for after-ripening since the expression of *GA3ox2*, which is involved in GA biosynthesis, is higher in after-ripened seeds in comparison to primary dormant seeds (Cadman et al., 2006).

Additionally, a microarray approach was taken to investigate genes whose expression was altered in response to after-ripening in Cape Verde Islands (Cvi) seeds (Cadman et al., 2006, Finch-Savage et al., 2007). Thirty genes were identified as being downregulated in response to after-ripening and this included the dormancy regulating gene *DOG1* (Finch-Savage et al., 2007). Although the expression of *DOG1* is reduced during after-ripening, DOG1 protein levels remain stable, but undergo an alteration to the structure of the protein which may render it non-functional (Nakabayashi et al.,

2012). Another recent transcriptome analysis revealed a specific gene set associated with the after-ripened state (Carrera et al., 2008). Although ABA was able to repress the germination of after-ripened wild-type seeds, it did not have an effect on the expression of genes associated with after-ripening (Carrera et al., 2008).

1.5 Genetics of dormancy and germination

1.5.1 DOG1

Using natural variation studies and forward genetic screens, a number of loci has been identified that have an important role in dormancy regulation, most notably *DOG1* and *REDUCED DORMANCY 4* (*RDO4*) (Leon-Kloosterziel et al., 1996, Peeters et al., 2002, Bentsink et al., 2006). *DOG1* was isolated from a natural variation study using a number of *Arabidopsis* accessions (Bentsink et al., 2006). Expression of *DOG1* is seed specific and peaks during late seed development, which coincides with the period during which seed dormancy is initiated. *DOG1* expression was found to be greater in the highly dormant accession Cvi than in the L*er* accession, which shows low levels of dormancy (Bentsink et al., 2006). *DOG1* expression has been shown to require ABA to induce dormancy (Bentsink et al., 2006) and *DOG1* expression is induced by an ABA mediated sugar signalling pathway (Teng et al., 2008).

A recent study investigated the potential of using *DOG1* expression as a marker for dormancy (Chiang et al., 2011). The results showed that *DOG1* expression in developing siliques acted as a good indicator of dormancy levels. However, in contrast *DOG1* expression does not correlate with dormancy levels in after-ripened seeds (Nakabayashi et al., 2012). Orthologues of *DOG1* have been identified in wheat and barley (Ashikawa et al., 2010). Although theses orthologues show little sequence

similarity to *Arabidopsis DOG1* and the seed specific expression is not conserved, the function does appear to be conserved. Ectopic overexpression of either of these *DOG1* orthologues leads to an increase in seed dormancy levels in *Arabidopsis*. The authors suggest that *DOG1* homologues could be important transgenes for reducing pre-harvest sprouting in wheat (Ashikawa et al., 2010).

1.5.2 RDO

Four *rdo* mutants were isolated from mutagenesis screens of L*er* seeds to determine low dormancy loci (Leon-Kloosterziel et al., 1996, Peeters et al., 2002). These four mutants all display reduced dormancy phenotypes but do not have altered levels or sensitivity to ABA and *rdo4* shows no difference in its requirement for GA (Peeters et al., 2002). The reduced dormancy phenotype of *rdo4* mutants has been described to be partly due to its reduced expression of *DOG1* and other dormancy associated genes (Liu et al., 2007).

RDO4 is a C3HC4 RING finger protein and is thought to act as the E3 ligase that is responsible for the monoubiquitination of histone H2B, which leads to changes in histone H3 methylation (Liu et al., 2007). In turn, this regulation of the ubiquitination of histone H2B is thought to lead to regulation of gene transcription efficiency. The phenotypes of *rdo4* mutants are not restricted to dormancy, as the mutant displays a number of pleiotropic phenotypes associated with plant architecture and flower morphology, suggesting that its regulation of transcription is important for the control of many developmental pathways (Liu et al., 2007).

Recent work has identified RDO2 as a homologue of Transcription Factor S-II (TFII-S) which is involved in overcoming transcription arrest by RNA- Polymerase II (Liu et al.,

2011). A role for TFII-S in the regulation of seed dormancy has been described (Grasser et al., 2009) and this involves the expression of *DOG1* (Mortensen et al., 2011). There is currently little information about the function of RDO1 and RDO3 in regulating dormancy.

1.5.3 MFT

MOTHER OF FT is a homologue of the phosphatidylethanolamine-binding proteins (PEBP) FT and TERMINAL FLOWER1 (TFL1), which have opposite roles in the promotion of flowering (Bradley et al., 1997, Kardailsky et al., 1999, Kobayashi et al., 1999). MFT has been shown to have a role in regulating the germination of after-ripened seeds, as *mft* loss-of-function seeds are hypersensitive to ABA (Xi et al., 2010). Expression of *MFT* is directly regulated by ABI3 and ABI5 and upregulated by DELLA proteins (Xi et al., 2010). An MFT homologue has been identified in wheat which is upregulated in embryos matured at 13°C in comparison to 25°C, suggesting that it is responsible for promoting or maintaining dormancy (Nakamura et al., 2011). The overexpression of *MFT* in immature embryos leads to a reduction in precocious germination that occurs in wild-type. The repression of germination by MFT could be overcome by the application of exogenous GA, suggesting that MFT may function to repress GA synthesis (Nakamura et al., 2011). High expression of *MFT* was also found to correlate with increased dormancy levels in Cvi seeds that had been buried in the soil (Footitt et al., 2011).

1.5.4 FLC

A role for the floral repressor FLC in regulating dormancy has been proposed (Chiang et al., 2009). These authors showed that dormancy levels were reduced in seeds

expressing high levels of *FLC* that were matured at 10°C. This phenotype was however reliant on imbibition also occurring at low temperature. Expression levels of *FLC* were found to positively correlate with germination levels when seeds were imbibed at low temperature. The increase in germination was linked to increased expression of *CYP707A2* and *GA20ox1* expression, suggesting that levels of ABA and GA may be altered in these seeds. Another study analysed the effect of *FLC*-deficiency on dormancy levels during after-ripening and found that *flc-101* seeds germinated to wild-type levels (Liu et al., 2011). This suggests that an alternative pathway must also be important for the regulation of dormancy in response to low temperature.

1.6 Temperature regulation of dormancy and germination

1.6.1 Temperature regulation of dormancy

Temperature signalling pathways in vegetative tissues are well understood, whereas in seeds, the knowledge is much more limited. The germination of different species of plants responds to temperature in different ways. Cold may promote germination in one species, whilst warm temperatures promote germination in other species. In many summer annual plants, dormancy is usually lost during winter and germination occurs the following spring or summer and, therefore, the exposure to cold during imbibition is required. Conversely, the germination of winter annuals often requires exposure to warm temperatures during imbibition, reflecting the loss of dormancy over summer and germination in autumn (Baskin and Baskin, 2004). In the seed bank, winter temperatures promote dormancy whilst spring temperatures alleviate it (Footitt et al., 2011). This suggests that if seeds have not germinated before the onset of winter, the

low temperatures experienced during the winter induce secondary dormancy, which delays germination until the spring.

In *Arabidopsis*, temperature is known to be an important factor in determining the levels of dormancy that are induced in the seed during maturation and this is extensively reviewed in Fenner (1991). Observations linking maturation temperature to dormancy have been made in a number of different species since the 1950s. In *Rosa,* a positive correlation was found between germination frequencies and the mean average daily temperature for the last thirty days of seed ripening (VonAbrams and Hand, 1956). *Stellaria Media* seeds which were collected from the field throughout the year displayed increased levels of dormancy when seeds were matured in the winter in comparison to the spring (van der Vegte, 1978). Additionally, links have been made between the pre-anthesis temperature and dormancy levels in both tobacco and wild oat (Thomas and Raper, 1975, Sawhney et al., 1985). This suggests that not only the environment which is directly experienced by the developing seed is important, but the parent plant is able to transmit signals that provide the seed with vegetative environmental information (Fenner, 1991).

In *Arabidopsis* low temperature during seed maturation also promotes high dormancy levels (Schmuths et al., 2006, Donohue et al., 2008, Chiang et al., 2009), but the mechanism that regulates this process is currently unknown. Elwell et al., (2011) analysed the effect of different parental environments on development throughout the plant lifecycle, showing that the parental environment had the potential to affect germination, root growth, gravitropism and the time to produce the first floral bud. The dormancy levels which are induced in seeds affect the timing at which germination occurs and this in turn affects the environmental conditions which are experienced by

the plant in the following generation. Therefore, it is important that the correct levels of dormancy are induced to ensure that environmental conditions are optimal for growth and development.

1.6.2 Low temperature regulation of germination

In *Arabidopsis*, the germination of dormant seeds is promoted during imbibition by exposure to a period of cold and in the laboratory this process is known as “cold stratification”. Exposure to cold is thought to promote germination by reducing dormancy levels, although it is difficult to determine whether factors are indeed breaking dormancy or promoting germination. Finch-Savage and Leubner-Metzger (2006) propose that any factor that widens the environmental requirements for germination should be regarded as a dormancy release factor.

The mechanism through which low temperature breaks dormancy is not well understood, although it has been shown that increases in *GA3ox1* and *GA20ox3* expression, which are both involved in the promotion of GA synthesis at 4°C in comparison to 22°C are important (Yamauchi et al., 2004). Additionally, there is also a reduction in *GA2ox2* expression, which is involved in GA catabolism in response to cold stratification (Yamauchi et al., 2004). Together these alterations to expression of genes regulating GA metabolism contribute to an increase in bioactive GA levels in cold stratified imbibed seeds. A reduction in ABA levels is apparent upon imbibition at both warm (22°C) and cold (4°C) temperatures (Chiwocha et al., 2005). This suggests that the promotion of germination by cold stratification does not require a reduction in ABA levels. There is also little difference in levels of auxin and cytokinins during imbibition at warm and cold temperatures (Chiwocha et al., 2005), so a central role for these hormones can be discarded.

A recent transcriptome analysis of *Arabidopsis* Columbia (Col) seeds at ten time points from freshly harvested seed through to post-germinated seeds is the first study to include a global analysis specifically aimed at identifying genes which are differentially regulated by cold stratification (Narsai et al., 2011). This study revealed that more than ten thousand genes are differentially expressed during forty eight hours exposure to low temperature (Narsai et al., 2011). The greatest number of differentially expressed genes which change occurs between twelve hours and forty eight hours of cold stratification. Genes encoding proteins involved in ethylene signalling including *ETHYLENE RESPONSE FACTOR1*(*ERF1*)*, ERF2* and *ERF5* are induced at the onset of cold stratification, whereas between twelve hours and forty eight hours of cold stratification, there is an over-representation of genes involved in RNA processing, protein synthesis and nucleotide metabolism. The increase in expression of ERF family members is unsurprising given that ethylene promotes seed germination through endosperm weakening and rupture in both *Lepidium sativum* and *Arabidopsis* and via an antagonistic effect on ABA signalling (Finkelstein et al., 2008, Linkies et al., 2009). *ethylene resistant 1* (*er1*) mutants show an increase in ABA sensitivity and synthesis along with an increase in seed dormancy (Beaudoin et al., 2000, Chiwocha et al., 2005). However, the mechanism by which ethylene regulates seed germination is less well understood in comparison to ABA and GA.

Additional transcriptional analyses have been carried out to try and identify key genes which are important for the promotion of germination by low temperature (Yamauchi et al., 2004, Finch-Savage et al., 2007). A comparison was made to identify overlapping genes between these two studies and this comparison revealed twenty three common genes which were upregulated and twenty common genes which were downregulated in response to low temperature (Finch-Savage et al., 2007). These studies were carried

out in two different ecotypes and the small number of common genes may reflect differences in the way in which low temperature promotes germination in different ecotypes.

1.6.3 High temperature regulation of germination

High temperatures during imbibition often suppress germination, through a process known as thermoinhibition, which appears to rely on a finite regulation of ABA and GA levels (Toh et al., 2008). Application of fluridone, an inhibitor of ABA synthesis increases the germination of dormant seeds imbibed at 27°C, but has no effect on seeds imbibed at 13°C (Ali-Rachedi et al., 2004). This suggests that in contrast to thermoinhibition, ABA is not central to the mechanism that regulates the promotion of germination by low temperature. In *Arabidopsis* higher expression of the ABA biosynthesis genes *NCED2, NCED5* and *NCED9* is observed in seeds imbibed at 34°C in comparison to 22°C, thus suggesting that they contribute to enhanced ABA biosynthesis at high temperature (Toh et al., 2008). Expression of the ABA catabolic genes *CYP707A1, CYP707A2* and *CYP707A3* is significantly reduced at 34°C. In addition to regulation of ABA in response to high temperature, GA also plays an important role. Levels of both GA_1 and GA_4, which are bioactive forms of GA, are reduced in seeds imbibed at high temperature and this is due to a reduction in expression of GA biosynthesis genes. This suppression requires ABA since *aba2-2* mutants display increased expression of these genes at high temperature. Similarly, in lettuce (*Lactuca sativa* L.), *LsNCED4* mRNA levels are increased in response to imbibition at high temperature; whereas mRNA levels of *LsGA3ox1* are decreased thus leading to higher ABA and lower GA levels (Schwember and Bradford, 2010).

1.6.4 Alternating temperature regulation of germination

In contrast to the requirement of cold for germination in *Arabidopsis*, the seed germination of some species is promoted by alternating temperatures and this process is very poorly understood. The requirement for alternating temperatures for germination was identified in studies using *Musa balbisiana* (Stotzky and Cox, 1962). An observation was made that the germination of seeds was lower in the winter when artificial heating was provided to maintain a constant temperature (Stotzky and Cox, 1962). Alternating temperatures have been shown to promote the germination of dormant *Arabidopsis* seeds (Ali-Rachedi et al., 2004)

1.7 Light regulation of germination

In addition to temperature, light is another key regulator of dormancy and germination. The photoperiod during seed maturation, like temperature is able to regulate dormancy levels, whereby short days induce high dormancy levels and long days induce low levels of dormancy ((Munir et al., 2001),Penfield group, unpublished). The control of seed germination by light has been shown to rely largely on the activity of phytochromes. Phytochromes are photochromic biliproteins which absorb red light and far red light. In *Arabidopsis* five phytochromes have been identified (PHYA-E). Phytochromes are synthesised in an inactive red-light absorbing form and then undergo photo conversion to a biologically active far-red light absorbing form. *Arabidopsis* seed germination is regulated in a reversible manner by red and far-red light and this is determined by the phytochromes (Shinomura et al., 1994, Hennig et al., 2002). In particular, it is PHYA and PHYB which have been shown to mediate the control of seed germination by light; although a role has also been suggested for PHYE (Shinomura et

al., 1996, Hennig et al., 2002). PHYB is the only phytochrome which has been shown to have a role in dark regulation of germination. *long hypocotyl3* (*hy3*) mutant seeds, which lack functional PHYB are not able to germinate in the dark (Shinomura et al., 1994).

The promotion of germination by light is associated with an increase in GA biosynthesis (Koornneef and Veen, 1980, Hilhorst and Karssen, 1988, Ogawa et al., 2003) and this is due to an increase in expression of GA biosynthetic enzymes such as GA 3β-hydroxylase (Yamaguchi et al., 1998). Light has been shown to promote germination by inhibiting the function of PHYTOCHROME INTERACTING FACTOR3- LIKE5 (PIL5), which is a negative regulator of germination (Oh et al., 2006). *pil5* mutant seeds are shown to germinate constitutively under non-inductive light conditions. The role of PIL5 in regulating germination has been shown to involve the regulation of GA and ABA through modulating expression of their metabolic genes (Penfield et al., 2005, Oh et al., 2006). Additionally, Penfield et al., (2010) have suggested that another PIF family transcription factor, PIF6 has a role in the regulation of germination in dormant seeds and it is the alternatively spliced variant of *PIF6* that confers this role. *PIF6* expression is high during seed development and, therefore, the important time for PIF6 action is during seed development and not in the imbibed seed, where PIF6 expression declines.

1.7.1 Light and temperature crosstalk

The bHLH transcription factor SPT, which has been shown to have a role in ambient temperature responses (Sidaway-Lee et al., 2010) was originally identified due to its ‘spatula’ shaped siliques and was shown to be involved in carpel development (Heisler

et al., 2001). Later, a role for SPT in seed germination was revealed. SPT acts as a light-stable repressor of *GA3ox1,* which regulates seed responses to both cold stratification and light (Penfield et al., 2005). Germination of the *spt-2* mutant is repressed in response to cold stratification, but germination is permitted in response to other stimuli such as light and after-ripening. This suggests that responses of the PIF family of transcription factors to temperature can be independent of responses to other signals and stimuli (Penfield, 2008). Interestingly, the role of phytochromes during imbibition appears to be temperature dependent (Heschel et al., 2007). PHYE contributes to germination at low temperatures, whereas PHYA is important for germination at warm temperatures. PHYB on the other hand is important for germination over a range of temperatures (Heschel et al., 2007). This therefore, suggests that the phytochromes are not only important for responding to light signals but temperature signals also.

1.8 Thesis Aims

The main aim of this thesis was to further the understanding of the role of temperature in regulating seed dormancy and germination. To meet this aim, work will be executed through two main projects. Firstly the effect of temperature during seed maturation on regulating dormancy levels will be investigated. An important aim of this project was to try and identify components of the mechanism through which temperature regulates seed dormancy. The ability of a number of mutants to alter dormancy levels in response to different maturation temperatures will be investigated. It is hoped that these experiments will reveal a gene or genes which plays a role in the pathway through which temperature regulates seed dormancy. Additionally, RNA-Seq will be carried out to investigate how maturation temperature affects the developing seed

transcriptome. Secondly, a forward genetic screen will be used to isolate mutants that show a defect in the promotion of germination by cold stratification. The characterisation of a number of selected mutants from the forward genetic screen will be carried out to reveal additional information about the low temperature promotion of germination.

Chapter 2 Materials and Methods

2.1 Seed preparation

Following dehiscence, seeds were harvested from plants. Seeds were ready to harvest from the plant when siliques readily opened when touched. Poorly filled seeds were excluded from germination trials using a 250µm sieve (Fisher Scientific). Freshly harvested seeds (used within twenty four hours from harvest) were used for germination assays. Seeds for after-ripening studies were stored in glass vials in the dark at room temperature.

2.2 Seed sterilisation

A bleach solution was prepared by dissolving six Klorsept tablets in 100ml sterile water. 1ml 100% ethanol was pipetted onto the seeds and the tubes were inverted to mix. The ethanol was removed and 1ml ethanol bleach (5% bleach in 1ml 100% ethanol) was pipetted onto the seeds. Tubes were inverted and left to stand for ten minutes. The ethanol bleach was removed and 1ml 100% ethanol was pipetted onto the seeds. This ethanol was removed and a final ethanol wash was carried out. Any remaining ethanol was removed by evaporation in a sterile flow hood.

2.3 Plant growth media

2.3.1 Water-agar medium

0.9% water-agar medium was used for dormancy assays. Medium was autoclaved before use.

2.3.2 Murashige and Skoog medium

Half strength Murashige and Skoog (MS) (Duchefa Biochemie) medium was used to germinate seeds for plant growth. The pH was altered to 5.7 using KOH and 9g/L agar was added. Medium was autoclaved before use.

2.3.3 Supplements to water-agar

ABA (Sigma Aldrich), Paclobutrazol (Greyhound Chromatography), GA_4 (Sigma Aldrich) or Norflurazon (Greyhound Chromatography) was dissolved in 100% Methanol to a concentration of 1mM. The stock solutions were then added to 50ml water-agar to produce the desired concentration. The control for experiments was produced by adding methanol to water-agar to produce the desired concentration.

2.4 Germination

2.4.1 Germination for plant growth

Sterilised seeds were cold stratified in the dark in a Sanyo MIR-154 incubator for three days. Seeds were germinated on MS plates under a twelve hour white light (80µmol.m-^{2}.s-^{1}), twelve hour dark light regime at 22°C in a Sanyo MLR growth chamber.

2.4.2 Germination for dormancy assays

Sterilised freshly harvested seeds were cold stratified in the dark in a Sanyo MIR-154 incubator for the desired length. Seeds were germinated on water-agar plates under a

twelve hour white light (80µmol.m-^{2}.s-^{1}), twelve hour dark light regime at 22°C in a Sanyo MLR growth cabinet for seven days. In one experiment seeds were germinated at four different temperatures (12°C, 17°C, 22°C and 27°C). Following this, germination was scored as the emergence of the radicle through the seed coat using a Leica MZ6 stereomicroscope. For dormancy assays, a minimum of twenty seeds from five individual seed batches, each from independent plants were used. Germination frequency (%) was calculated as the percentage of seeds germinating in each individual seed batch.

2.4.3 After-ripening assays

Seeds for after-ripening assays were stored within twenty four hours of harvest in sealed glass vials at room temperature in the dark. Seeds were germinated on water-agar plates on a weekly basis under a twelve hour white light (80µmol.m-^{2}.s-^{1}), twelve hour dark light regime at 22°C in a Sanyo MLR growth cabinet for seven days.

2.5 Plant growth

2.5.1 Plant growth for dormancy assays

Ten day old seedlings were transferred from MS plates to John Innes seed compost (Levington) in P40 trays. Plants grown for seed production for dormancy assays were in most cases grown to flowering in a Sanyo MLR growth cabinet at 20°C under a long day light regime (sixteen hours white light (70µmol.m-^{2}.s-^{1}), eight hours dark). First flowering was defined as the anthesis of the first flower. Plants were then shifted to a second Sanyo MLR growth cabinet running at the same conditions, but with the indicated seed maturation temperature. For one particular experiment, a temperature regime which included a daytime temperature of 20°C and night time temperature of

16°C with the long day light regime was used. Plants for this experiment were grown constantly under these conditions. A list of all mutant seeds used in this thesis is provided in Table 2.1.

Table 2.1: Information for all mutant lines used in this thesis.

Mutant line	Background	Mutation	Source
dog1-2	Col	γ irradiation	(Bentsink et al., 2006)
rdo1	L*er*	EMS	Marten Koornneef and Wim Soppe
rdo2	L*er*	EMS	Marten Koornneef and Wim Soppe
rdo3	L*er*	EMS	Marten Koornneef and Wim Soppe
rdo4	L*er*	EMS	Marten Koornneef and Wim Soppe
gai-t6 rga-t2 rgl2-1 rgl1-1	L*er*	Transposon	(Achard et al., 2006)
aba1-1	L*er*	EMS	NASC - N21
aba1-6	L*er*	EMS	NASC - N3772
aba2-3	Col	EMS	NASC - N3834
aba3-1	Col	EMS	NASC - N157
cyp707a2-1	Col	T-DNA	(Kushiro et al., 2004)
ago4-1	Ler	T-DNA	(Zilberman et al., 2003)
dcl3-1	Col	T-DNA	(Xie et al., 2004)
rdr2-1	Col	T-DNA	(Xie et al., 2004)
ros1-4	Col	T-DNA	(Penterman et al., 2007)
drm1-2 drm2-2	Col	T-DNA	(Agorio and Vera, 2007)
linc1-2	Col	T-DNA	NASC - N653787
cbf1 RNAi	Col	RNAi	(Novillo et al., 2007)
cbf2	Col	T-DNA	(Novillo et al., 2004)
CBF3 RNAi	Col	RNAi	(Novillo et al., 2007)
CBF1 ox	WS	CaMV 35S	(Jaglo-Ottosen et al., 1998, Gilmour et al., 2000)
CBF2 ox	WS	CaMV 35S	(Jaglo-Ottosen et al., 1998, Gilmour et al., 2000)
CBF3 ox	WS	CaMV 35S	(Jaglo-Ottosen et al., 1998, Gilmour et al., 2000)
ice1-2	Col	EMS	(Chinnusamy et al., 2003)

hos1-1	C24	EMS	(Ishitani et al., 1998)
hos1-3	Col	T-DNA	NASC - N569312
hos1-4	Col	T-DNA	NASC - N631629
hos1-5	Col	T-DNA	NASC - N552108
los4-1	C24	EMS	(Gong et al., 2005)
nup160-2	Col	T-DNA	NASC - N660612
tfl2-1	Col	Fast neutrons	NASC - N3796
tfl2-2	Col	Fast neutrons	NASC - N3797
arp6-1	Col	T-DNA	Phil Wigge
phya-211	Col	γ irradiation	(Reed et al., 1994)
phyb-9	Col	γ irradiation	(Reed et al., 1994)
cry1-301	Col	Fast neutrons	(Mockler et al., 1999)
hy1-1	L*er*	Fast neutrons	NASC - NW67
hy2-1	L*er*	EMS	NASC - N68
tt4-1	L*er*	EMS	NASC - N8605
tt5-1	L*er*	Fast neutrons	NASC - N86
tt6-1	L*er*	EMS	NASC - NW67
ft-10	Col	T-DNA	(Yoo et al., 2005)

2.5.2 Plant growth in glasshouse and growth rooms

Ten day old seedlings were transferred from MS plates to John Innes seed compost (Levington) in P40 trays. Under glasshouse conditions, natural light was supplemented with white light to maintain a long day light regime (sixteen hours white light (150µmol.m^{-2}.s^{-1}), eight hours dark). The temperature ranged from approximately 13°C to 32°C.

In the growth room, the temperature was maintained at 20°C with a long day light regime (sixteen hours white light (70µmol.m^{-2}.s^{-1}), eight hours dark).

2.6 Forward genetic screen

An EMS forward genetic screen was carried out by Steve Penfield prior to the start of this project. Briefly, EMS mutagenised freshly harvested Col seed was exposed to cold stratification (three days at 4°C, dark) and then transferred to twelve hour light, twelve

hour dark light regime at 22°C. After one week, germination was analysed and any seeds which did not germinate were taken forward to the next part of the screen where they were dried and then after-ripened for one month. Seeds that germinated following after-ripening for one month were taken through to the next generation and the resulting progeny was re-tested. Fourteen mutants which did not germinate in response to cold stratification were selected by Steve Penfield for further characterisation.

2.7 Physiology methods

2.7.1 Exogenous GA application

2.7.1.1 Spraying of plants

Sterilised seeds were sown on MS plates. Seeds were cold stratified for three days at 4°C. Seeds were germinated under a twelve hour white light (80µmol.m-^{2}.s-^{1}), twelve hour dark light regime at 22°C. Ten day old seedlings were transferred to soil and grown under long day conditions with a temperature regime of 20°C/18°C. Plants were sprayed with either 100µM GA (Sigma Aldrich) in 2% Methanol or 2% Methanol on a weekly basis. After five weeks, photographs were taken of the plants.

2.7.1.2 Seedling growth on GA medium

Sterilised seeds were germinated on water-agar plates that were supplemented with either 100µM GA (Sigma Aldrich) or 100 µM Methanol and cold stratified for three days Seeds were germinated under a twelve hour light (80µmol.m-^{2}.s-^{1}), twelve hour dark light regime at 22°C. After fourteen days photographs were taken of seedlings.

2.7.2 Flowering time

Plants were grown in a growth cabinet at 15°C under a long day light regime (70µmol.m-^{2}.s-^{1}). The number of rosette and cauline leaves that were produced were recorded as well as the number of days until first anthesis. This experiment was carried out by Natalie Palmer, an undergraduate project student.

2.7.3 Hypocotyl elongation

Sterilised seeds were sown on MS plates and cold stratified for three days at 4°C. Plates were then transferred to a growth cabinet at 22°C and exposed to two days constant white light. Plates were then exposed to continuous monochromatic red LEDs (PEAK 660nm, 10µmol.m^{-2}.s^{-1}), far-red LEDs (PEAK 756nm, 10µmol.m^{-2}.s^{-1}), blue (PEAK 439 and 455nm 10µmol.m^{-2}.s^{-1}) or white light provided by fluorescent tubes (PEAK 434, 455, 631 and 707nm 10µmol.m^{-2}.s^{-1}) for seven days. Hypocotyls of approximately twenty seedlings were then measured using digital callipers (Draper).

2.7.4 Growth rate

Plants were grown in a growth cabinet at 15°C under a long day light regime (70µmol.m-^{2}.s-^{1}). Growth rate was recorded on a weekly basis by imaging plants using a CreativeLive webcam. At the growth stages that were measured, there is very little overlap between leaves and so the measurement of leaf area can give an accurate measure of total leaf area. Total rosette area was calculated for approximately ten plants per genotype and analysed using ImageJ. This experiment was carried out by Natalie Palmer, an undergraduate project student.

2.8 Seed coat morphology analysis

Seeds were imbibed in water for approximately two hours and seed coats were dissected. Seed coats were fixed by Meg Stark (Technology facility, University of York) over-night in 2.5% glutaraldehyde, 4% formaldehyde in 100mM phosphate buffer, washed in buffer then post-fixed in 1% osmium tetroxide for an hour, washed in buffer then dehydrated through an acetone series. They were then infiltrated and embedded in Spurr resin. Sectioning was performed by Meg Stark (Technology facility, University of York) using a Leica ultramicrotome. Sections (0.5µm) were stained with 0.6% toluidine blue in 0.3% sodium carbonate. Seed coat morphology was observed using an Invent Flu + CCD light microscope using an x40 objective lens.

2.9 Embryo morphology analysis

Dry mature seeds were imbibed in water for approximately two hours. Embryos were then dissected from seeds and photographed using a Leica MZ16F stereomicroscope with a Spot RT3 CCD camera.

2.10 ABA and GA analysis

70mg of seed was flash frozen in liquid nitrogen and ground using a pestle and mortar. Samples were immediately weighed and transferred to a tube with 1.9ml extraction solvent (99:1 Isopropanol: acetic acid) and 10µl 5µg/ml internal standard (d2-GA_1, d2-GA_4 (Law Mander (ANU) and d6-ABA (ICON Isotope))). Samples were shaken in the dark at 4°C over-night. The supernatant was collected following centrifugation for five minutes at 4°C. The sample was re-extracted with 1.1ml extraction solvent for two hours on a shaker in the dark at 4°C. The supernatant was collected following centrifugation for five minutes at 4°C. The supernatant was then dried in a speed-vac.

The dried supernatant was re-suspended in 50µl methanol and 2µl injected and analysed on an ultraperformance liquid chromatography (UPLC)-Mass Spectrometry (MS) system consisting of an Acquity UPLC system (Waters) coupled to a Finnigan LTQ ion trap mass spectrometer (Thermo Electron) by Anuja Dave and Anja Hellwege. Chromatographic separation of the phytohormones was performed on a Waters Acquity UPLC BEH C18 1.7 mm, 50 3 2.1-mm column using a gradient of mobile phases water + 0.1% acetic acid and acetonitrile + 0.1% acetic acid with a flow rate of 1 mL/minute. Eluted compounds were ionised on the mass spectrometer using a HESI source, and MS data were collected in full scan mode over the mass range m/z 100 to 500 in negative ionization mode. Ions at m/z 263.1 for ABA, 269.1 for d6-ABA, 347.2 for GA_1, 349.2 for d2-GA_1, 331.2 for GA_4, and 333.2 for d2-GA_4 were used for quantification. ABA, GA_1, and GA_4 were quantified using response factors calibrated between internal standards and phytohormone standards ABA, GA_4 (Sigma Aldrich) and GA_1 (Peter Hedden, Rothamstead Research) by Anuja Dave and Anja Hellwege.

2.11 Oxylipin analysis

70mg of seed was flash frozen in liquid nitrogen and ground using a pestle and mortar. Samples were immediately weighed and transferred to a tube with 1.9ml extraction solvent (70:30 Acetone: citric acid) and 10µl 2µg/ml internal standard (Prostaglandin A1 in methanol (Sigma Aldrich)). Samples were shaken in the dark at 4°C for three hours. The acetone was then evaporated overnight. The remaining aqueous layers were extracted three times with diethyl ether. Briefly, 500µl diethyl ether was added and the ether phase was removed following centrifugation for two minutes. The extracts were dried using a speed-vac. The dried extracts were re-suspended in 50µl 60:40 methanol: water in a tapered vial. Oxylipin analysis was carried out by Anuja Dave using a LCQ

mass spectrometer (Thermo Separation Products). Separation was achieved on a LUNA 5 mm C18(2) 150 mm 32 mm column (Phenomenex) using a gradient of mobile phases water + 0.2% formic acid and methanol + 0.2% formic acid with a flow rate of 0.4 mL/minute. LC-MS data were collected in full MS scan mode over the mass range m/z 150 to 500 in positive ionisation mode. Oxylipins were then quantified using response factors calibrated between prostaglandin A1 and authentic oxylipin standards, including JA, OPDA (Larodan), and JA-Ile (gift from Paul Staswich) by Anuja Dave.

2.12 Fatty Acid Methyl Ester (FAME) analysis

FAME extractions and analyses were carried out by Anja Hellwege. 100mg of seed was flash frozen in liquid nitrogen and ground using a pestle and mortar. The ground seed sample was added to a glass vial with 10µl 15:0 TAG (5µg/ml in chloroform) added as an internal standard, 500µl 1M methanolic HCL in methanol and 200µl hexane. Vials were vortexed gently and incubated at 85°C for two hours. 250µl 0.9% KCl was added to the cooled tubes and the layers were allowed to separate. 100µl of the upper hexane layer containing the FAMEs was removed for analysis. FAME content was determined by gas-chromatography with flame ionization detection (FID) (GC Trace Ultra, Thermoquest Separation Products). A 1µL aliquot of the hexane layer was injected into a 3mm internal diameter FocusLiner containing glass wool (SGE, Milton Keynes, UK) at 230°C in programmed flow mode with H2 as carrier gas. The H2 flow program was as follows: initial: 0.3mL/min for 0.1min then ramp at 5mL/min/min to 0.5mL/min for the remainder of the run. The split ratio was maintained at 1:250 and a gas saver slow of 20 mL/min was initiated at 1.5min into the run. Separation was achieved using a narrow-bore cyanopropyl polysilphenylene-siloxane capillary column (BPX70; 10m length x 0.1mm internal diameter 0.2µm film thickness; SGE). FAMEs were separated

using the following temperature program: initial:150°C 0.1 min, then ramp at 16°C/min to 220°C, followed by cool-down to initial conditions at 120°C/min. The FID was run at 300°C with air, H2, and make-up N2 gases flowing at 350, 35, and 30 mL/min, respectively. The signal was collected and peaks detected and integrated using ChromQuest 4.2 software (Thermo Electron Corporation). FAMEs were identified and quantified relative to the Supelco 37 component FAME mix (Sigma Aldrich).

2.13 DAPI staining

Embryos were dissected from seeds that had been imbibed in water for two hours and placed on a slide. 2µl 2µg/ml 4', 6-diamidino2-phenylindole (DAPI) (New England Biolabs) in MS-glucose (4.4% MS salts, 5% MES and 475mM glucose) was added to the mount. Mounts were dried on a hot plate. A further 30µl 2µg/ml DAPI in MS-glucose was added.

2.14 Confocal microscopy

Nuclei that were stained with DAPI were visualised on a Zeiss LSM 510 meta on an Axiovert 200M using a x60 objective lens with an excitation wavelength of 358nm. Images were obtained with a constant set of microscopic and image-intensity parameters. Images were analysed using the Zeiss LSM Image Browser software.

2.15 Nuclear volume analysis

Nuclear volume was calculated using DAPI stained nuclei using the following formula: $4/3\pi r^3$ which assumes nuclei to be spherical. An average of approximately two hundred nuclei from five biological replicates was analysed.

2.16 Molecular biology methods

2.16.1 RNA extractions from seeds

RNA was extracted from approximately 10mg of dry, developing or imbibed (dry seeds placed on water-agar plates for twenty four hours) seeds. Seed material was snap frozen in liquid nitrogen and ground with 150ml of XT buffer (0.2M sodium borate, 30 mM EGTA, 1% SDS, 1% sodium deoxycholate, 2% polyvinylpyrollidone,10 mM DTT, and 1% IGEPAL [pH 9.0]) using a pestle and mortar. This was allowed to thaw and treated with 6µl of proteinase K (PCR grade, Roche) at 42°C for ninety minutes. Precipitation then followed on ice for sixty minutes with 12µl of 2M KCl. The supernatant was collected following centrifugation at 4°C for twenty minutes. RNA was precipitated from the supernatant overnight at -20°C with 54µl 8M LiCl. This RNA was collected by centrifugation at 4°C for twenty minutes. This RNA was cleaned up further using the clean-up protocol of the RNEasy Plant RNA Isolation kit (Qiagen) according to the manufacturer's instructions. RNA concentration was quantified using a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific) at 260 nm.

2.16.2 RNA Extractions from seedlings

100mg of two week old seedlings were harvested and snap frozen in liquid nitrogen. Tissue was homogenised in liquid nitrogen using a pestle and mortar to prevent thawing. RNEasy Plant RNA Isolation kit (Qiagen) was used to extract RNA from the tissue according to the manufacturer's instructions. RNA concentration was quantified using a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific) at 260 nm.

2.16.3 cDNA synthesis

First strand cDNA was synthesised from 2µg of total RNA. The RNA and 1µl 10mM oligo-dT (Invitrogen) were incubated at 70°C for ten minutes. A mix of 4µl reaction buffer, 2µl 0.1M DTT, 2µl 10µM dNTP mix and 1µl Superscript Reverse Transcriptase were added and incubated at 42°C for one hour. Samples were diluted to 200µl with sterile water.

2.16.4 Real-Time PCR

Real-Time (quantitative) PCR was used to compare the expression levels of different genes. cDNA produced from RNA was used in Real-time PCR reactions. For all reactions, the experiment was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Reaction mixes contained 2 µL of cDNA, 1 µL of primers (each primer at 10 µM), 10.5 µL of nuclease free water and 12.5 µL of Power SYBR green PCR master mix (Applied Biosystems). An exception to this was the determination of *CBF1* levels, and this used the Taqman detection system. This system was used as an alternative to the SYBR green detection system as a probe is also used in addition to primers, which increases the specificity. This reaction mix contained 5 µL of cDNA, 2 µL of primers (each primer at 10 µM), 2.5µl Taqman probe, 3 µL of nuclease free water and 12.5 µL of Taqman master mix (Applied Biosystems). A list of all primers used is provided in Table 2.2.

Reactions were carried out in a sealed ninety six well plate which were centrifuged at 5000 x g for two minutes. All Real-Time PCR reactions were carried out with the following cycle conditions; two minutes at 50 °C, ten minutes at 95 °C, followed by forty cycles of 95 °C for fifteen seconds and 60 °C for one minute. Transcript levels were

detected in two biological replicates for each sample using a standard curve derived from one reference sample with an arbitrary value set to one.

The expression of genes of interest was normalised to the expression of two control genes, and the control genes used differed with experiments. In Figures 3.16, 3.18, 4.3 and 4.8 *ACTIN2* and *AT3G06240* were used because the expression of these genes did not change with maturation temperature (Kendall et al., 2011) or developmental stage (eFP browser (Bassel et al., 2008)). In Figure 4.10, *TUBULIN9* and *AT3G06240* were used because the expression of these genes did not change with the three different developmental stages tested (eFP browser (Bassel et al., 2008)). In Figures 5.24 and 5.25 *TUBULIN9* and *AT3G06240* were also used and this was because the expression of these genes was unaffected by temperature and the *hos1* mutation (RNA-Seq results).

Table 2.2: Primers used for Real-Time PCR.
F and R after the primer names denote the forward and reverse primers respectively. All primers were ordered from Sigma Aldrich.

Primer	Sequence 5'-3'
CBF1 F	TGGCTGAAGGCATGCTTTTA
CBF1 R	ACAAAAATGGAAACGACTATCGAAT
CBF1PROBE	[6FAM]CGCCGCCGTCTGTTCAATGGA[TAM]
ACT2 F	TGAGAGATTCAGATGCCCAGAA
ACT2 R	TGGATTCCAGCAGCTTCCAT
AT3G06240 F	GCGAAGATTCACCTCGATCTG
AT3G06240 R	TTATGTGAAGACACAATGAGCTTACG
TUBULIN9 F	GCGGCGAGCACGGTATT
TUBULIN9 R	TTGATCCTTTCAAGCTGTAGATCTGT
DOG1 F	TCTCGAGTGGATGAGTTTGCA
DOG1 R	CGTGAGATCGTCGTTGAGCTAA
GA2ox6 F	CCACGCAAATCCGACAGC
GA2ox6 R	GCCAAATCTCTAACCGTGCGT
NCED4 F	GCTTCCTCCAACAGACTGTGAA
NCED4 R	CGGATGTAAGCGCCGTTAA
GA2ox3 F	ATCAACTTCTTTGCTTTGCATCAC
GA2ox3 R	TCCAATCCTTTTAGTACCGTAACCA
COR15B F	CGTTGCTCAGCGCAAGAA
COR15B R	CGAGGATGTTGCCGTCACT
FT F	GATATCCCTGCTACAACTGGAACA
FT R	GAATTCCTGCAGTGGGACTTG
GA2ox2 F (Achard et al., 2008)	GGACCAAACGGTGACGTTG
GA2ox2 R (Achard et al., 2008)	GTACTCCTCCACCGACTCACG
CYP707A2 F (Barrero et al., 2009)	AAATGGAGTGCACTCATGTC
CYP707A2 R (Barrero et al., 2009)	CCTTCTTCATCTCCAATCAC
MFT F	ATCACTAACGGCTGCGAGAT
MFT R	CGGGAATATCCACGACAATC

2.16.5 RNA-Sequencing

Wild-type green cotyledon seeds which were matured at 20°C and 15°C and *hos1-3* green cotyledon seeds which were matured at 15°C were dissected from siliques between five to seven hours after dawn. RNA was extracted from these seeds using the method outlined in 2.16.1. The RNA was tested for integrity using an Aligent 2100 bioanalyser by Celina Whalley in the Technology Facility (University of York). The bioanalyser uses a capillary electrophoresis based separation method, which separates the RNA molecules depending on their size into the three RNA components present; mRNA, tRNA and rRNA. cDNA was synthesised from the RNA samples in the Sequencing Laboratory (University of Exeter). Preliminary analyses of the data were performed by Konrad Paszkiewicz in the Sequencing Laboratory (University of Exeter). The analysis was carried out using the Tophat and Cufflinks programmes, which is based on the protocol published by Trapnall et al., (2012). Firstly, reads are mapped to the *Arabidopsis* genome. The resulting alignment files are then provided to Cufflinks to generate a transcriptome assembly which are then merged using the Cuffmerge package. Cuffmerge is able to use the merged assembly to calculate expression levels, test the statistical significance of observed changes and then identify differentially expressed genes.

Expressed genes were described as being differentially expressed between temperatures or genotypes if the expression was twofold or more different and the expression was significantly different ($P \leq 0.05$ by student's t-test). The expression of genes of interest was confirmed using Real-Time PCR.

2.17 Statistics

To determine whether the differences between means were statistically significant, a two-tailed Student's t-test was performed. Means are described as being significantly different throughout the thesis when $P \leq 0.05$. Statistical tests were not carried out on Real-Time PCR data because only two biological replicates were used.

Chapter 3 Characterisation of temperature regulation of seed dormancy

3.1 Introduction

To date the understanding of temperature signalling in seeds is extremely limited. The effect of low temperature in promoting the germination of seeds is probably the temperature signalling pathway that is best understood in seeds. In contrast, the situation in vegetative tissues is different. Cold sensing is known to take place through at least two mechanisms, the cold acclimation pathway and the vernalisation pathway. On a molecular level, both of these pathways are now well characterised, although work is still dedicated to understanding these mechanisms more thoroughly. However, mechanisms which sense temperatures in the ambient range are also present and are now starting to be characterised.

Previous studies have shown that the temperature during seed maturation can affect dormancy levels, with cool maturation temperatures leading to high dormancy levels (Schmuths et al., 2006, Donohue et al., 2008, Chiang et al., 2009). However, the mechanisms that control the temperature regulation of dormancy are not well understood. A role for the involvement of phytochromes in the temperature regulation of dormancy has been suggested (Donohue et al., 2008). Here, functional *PHYB* and *PHYD* expression is required for the germination of seeds that have been matured at low temperature. In addition, a recent transcriptomic analysis showed that expression of *PHYB* and *PHYE* are downregulated in dry seeds that have been matured at low temperature (Kendall et al., 2011). Together these data suggest that temperature is able to affect the light requirement for germination by acting directly on phytochrome levels. Additionally, a role for FLC in regulating dormancy which is induced by low

temperature has been suggested, since seeds expressing high levels of *FLC* are unable to induce strong dormancy levels when seeds are matured at 10°C (Chiang et al., 2009).

In response to cold temperatures, the plant increases endogenous levels of ABA (Chen et al., 1983) and exogenous application of ABA is sufficient to induce genes involved in cold and drought responses (Shinozaki et al., 2003). ABA is thought to play a central role in regulating the induction and maintenance of dormancy. The action of ABA is antagonised by GA and the finite balance between these two hormones is thought to be important to determine seed dormancy levels. Response to GA is also necessary for proper cold signalling as the DELLA proteins have been shown to be components of the CBF1-mediated cold stress response (Achard et al., 2008). Therefore, these two hormones are likely to play an important role in transmitting the environmental sensing to changes in seed dormancy.

The aims of this chapter were to determine the way in which maturation temperature controls dormancy, with a focus on ABA and GA. Additionally, the dormancy phenotypes of a number of mutants in response to different maturation temperatures are presented in order to start to determine a molecular mechanism.

3.2 Results

3.2.1 Development of an assay to investigate the effects of maturation temperature on seed dormancy

To try and understand the mechanism by which temperature regulates dormancy levels, a system was developed in which plants were grown during the vegetative

phase under standard laboratory conditions and then switched to either warm or cool temperatures from first flowering until the end of seed maturation (Figure 3.1). This is referred to from now onwards as the seed's 'maturation temperature'. Warm temperatures were either 20°C or 22°C. Cool temperatures included a range of temperatures from 10°C to 17°C. These different temperatures were chosen to sample the range of likely behaviours of seed set under natural conditions. Using this range avoids undue emphasis being placed on the behaviour observed only at one particular temperature. Dormancy regulates the time at which germination occurs, which consequently affects the timing of all subsequent developmental stages of a plants lifecycle and, therefore, the environmental conditions which are experienced at each stage. Consequently, different seed maturation temperatures, such as those tested here, have the potential to affect the whole lifecycle of the plant by inducing different levels of dormancy. All other variable conditions, such as photoperiod, light quality and humidity were kept constant throughout the lifecycle of the plant, regardless of the temperature at which they were growing.

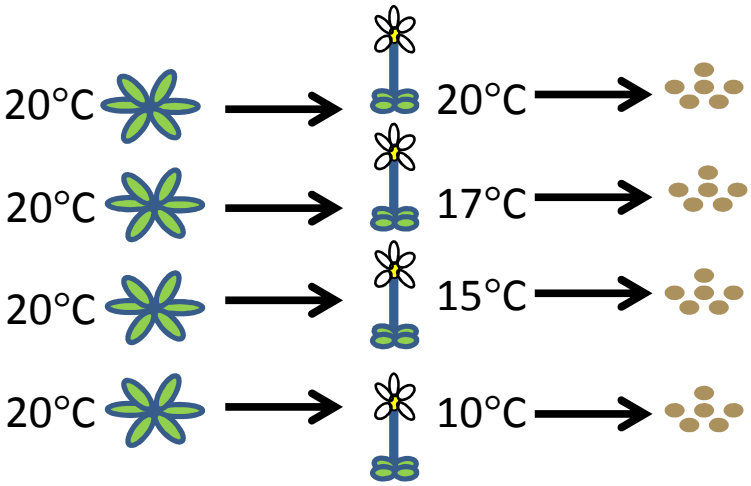

Figure 3.1: Schematic of the system used to investigate the effect of maturation temperature on dormancy.
Plants are grown at a warm temperature during the vegetative phase. At first anthesis, plants are then switched to a different temperature until the end of seed maturation. Seeds are then harvested from the plants.

3.2.2 Characterisation of the effects of maturation temperature on seed dormancy

3.2.2.1 Germination response to cold stratification

To quantify the effect of different maturation temperatures on dormancy levels, Col and L*er* seeds were matured at four different temperatures (10°C, 15°C, 17°C and 20°C). Decreases in maturation temperature led to an incremental increase in primary dormancy (referred to hereafter as dormancy) levels in Col (Figure 3.2A). In general little or no dormancy is induced when Col or L*er* seeds are matured at 20°C, suggesting that these conditions are favourable for germination and, hence, no dormancy is required to be induced in these seeds. In contrast, when seeds are matured at 10°C, strong dormancy is induced and three days of cold stratification only had a small effect on reducing dormancy levels in both ecotypes.

The results show that L*er* seeds display increased sensitivity to the decrease in maturation temperature in comparison to Col (Figure 3.2B). A reduction in temperature from 20°C to 17°C had a large effect on dormancy levels and germination was significantly reduced from approximately 100% to 10% in L*er* seeds. Germination of seeds matured at 15°C was similar to germination of seeds matured at 17°C, although they did germinate to slightly higher levels following two days of cold stratification. When matured at 10°C, cold stratification was unable to promote the germination of L*er* seeds, which remained at 0%. In contrast, germination of Col seeds matured at 10°C was promoted by cold stratification, although germination levels still remained low. These results show that by reducing maturation temperature the levels of dormancy induced are increased. It appears that the sensitivity and or response to maturation

temperature may differ between ecotypes and this is in line with previously published data (Schmuths et al., 2006, Chiang et al., 2011, Penfield and Springthorpe, 2012).

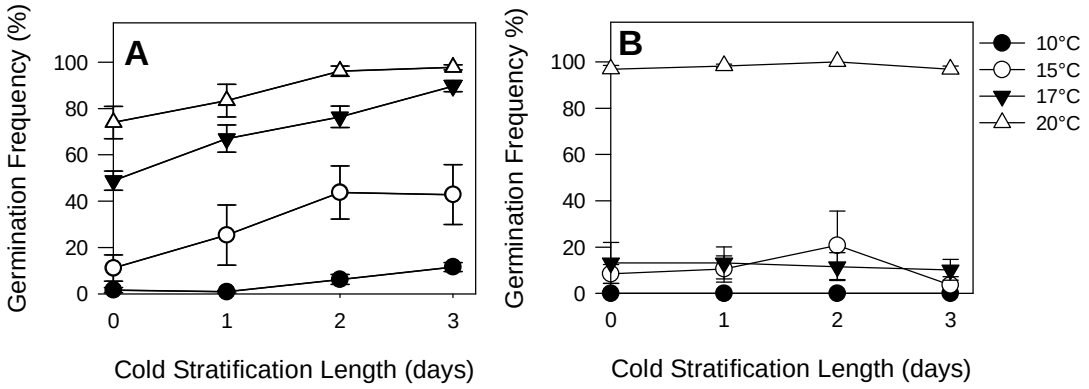

Figure 3.2: Germination of seeds matured at different temperatures in response to cold stratification.
Col (A) and L*er* (B) freshly harvested seeds matured at 10°C, 15°C, 17°C or 20°C were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

3.2.2.2 Germination response to after-ripening

In addition to characterising the dormancy of the seeds in response to cold stratification, the response to after-ripening was also analysed. This was tested using Col and L*er* seeds that were matured at either 20°C or 10°C. Freshly harvested Col and L*er* seeds that were matured at 20°C showed high germination indicative of low dormancy levels. Since germination was almost maximal in these seeds, after-ripening could not lead to a significant increase in germination (Figure 3.3). Germination of Col and L*er* seeds matured at 10°C was extremely low, meaning that, in contrast to the seeds matured at 20°C, there was potential for after-ripening to significantly increase germination. However, after-ripening had a very small effect on Col germination, whilst it had no effect on L*er* germination, which remained at 0% (Figure 3.3). This suggests

that the dormancy levels induced by low temperature (10°C) are extremely strong and cannot be broken by this standard dormancy breaking treatment.

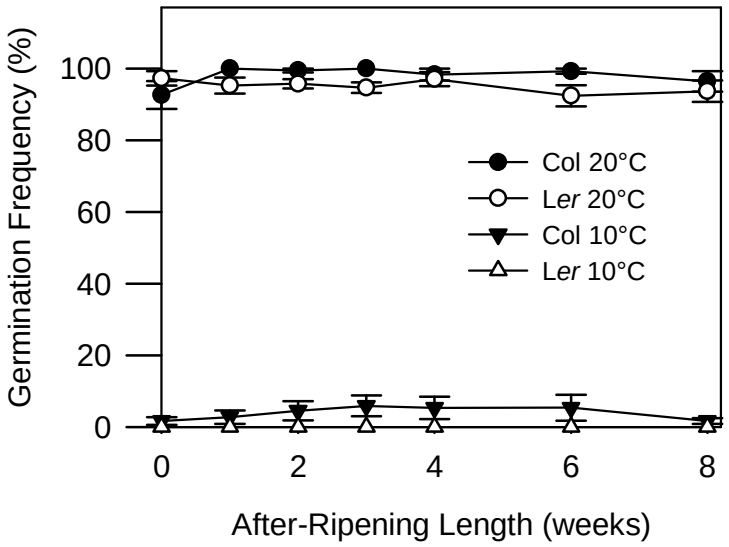

Figure 3.3: Germination of seeds matured at 20°C and 10°C in response to after-ripening.
Freshly harvested seeds were after-ripened for up to eight weeks at room temperature. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points represent the average of five individual seed batches and error bars represent standard error.

3.2.3 Characterisation of the viability of seeds matured at low temperature

As after-ripening had very little or no effect on breaking the dormancy induced by low temperature in seeds (Figure 3.3), it was possible that there could be viability issues with these seeds. To test whether the seeds were indeed viable or not, Col seeds that were matured at 10°C, 15°C and 20°C and after-ripened for approximately eight weeks were germinated on water-agar plates or MS plates. Water-agar is used as a growth medium to germinate seeds for dormancy analysis as it contains significantly lower

levels of nitrate than MS, and nitrogen is sufficient to promote germination. The germination of Col seeds matured at 20°C was approximately 90% and this was independent of the growth medium that was used. In contrast, the germination of Col seeds matured at 15°C on water-agar plates was approximately 60% following three days of cold stratification (Figure 3.4) and this was increased to approximately 90% when the seeds were germinated on MS (Figure 3.4). When the maturation temperature was reduced to 10°C, germination levels were very low when seeds were germinated on water-agar plates. However, the germination was significantly increased to approximately 90% when the seeds were germinated on MS and this reflected the germination levels of warm matured seeds. Therefore, these results suggest that the reason why seeds do not germinate following after-ripening is not due to a loss of viability.

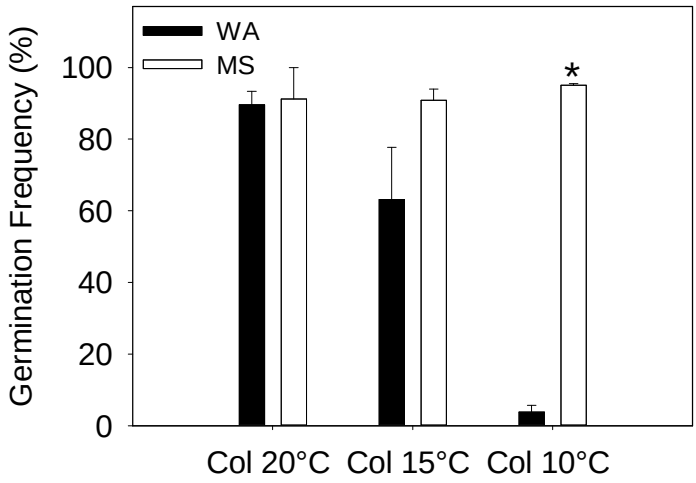

Figure 3.4: Germination of low temperature induced dormant seeds on water-agar and MS.
Col seeds matured at 20°C, 15°C or 10°C were after-ripened for approximately eight weeks and cold stratified for three days. The seeds were germinated on plates containing either water-agar or MS. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points represent the average of five individual seed batches and error bars represent standard error. * Indicates significant difference to water-agar when P≤0.05 by students t-test.

3.2.4 Characterisation of seed coat and embryo morphology

It has previously been shown that changes in seed coat morphology is linked to altered dormancy levels (Debeaujon et al., 2000). The authors show that mutants with defects in seed coat pigmentation or structure have reduced dormancy levels and positive correlations were made between seed coat thickness and dormancy levels (Debeaujon et al., 2000). Therefore, the regulation of seed coat thickness could be a possible mechanism by which temperature regulates dormancy levels. If this is the case then seeds matured at 10°C would be expected to have thicker seed coats in comparison to those matured at 20°C. To investigate this hypothesis seed coat morphology was

examined in seeds matured at 20°C and 10°C to determine if any differences were present. Cross sections of seed coats were stained with toluidine blue and then examined under a light microscope looking for changes in seed coat morphology. The results show that seeds matured at 20°C and seeds matured at 10°C have very little difference in their seed coat structure (Figure 3.5). Therefore, cool temperatures during seed maturation do not appear to be altering dormancy levels by increasing seed coat thickness.

Additionally, it was possible that the maturation temperature may have effects on embryo development which could lead to differences in dormancy levels. Therefore, embryo morphology was compared between seeds matured at 20°C and 10°C. Embryos were dissected from seeds that had been imbibed for approximately two hours and embryo morphology was examined using a light microscope. No obvious differences in morphology were observed between the embryos matured at the two temperatures (Figure 3.6). These results show that low temperature induced dormancy does not involve perturbations to seed coat or embryo morphology and, therefore, normal embryo and seed coat development is not altered by low temperature.

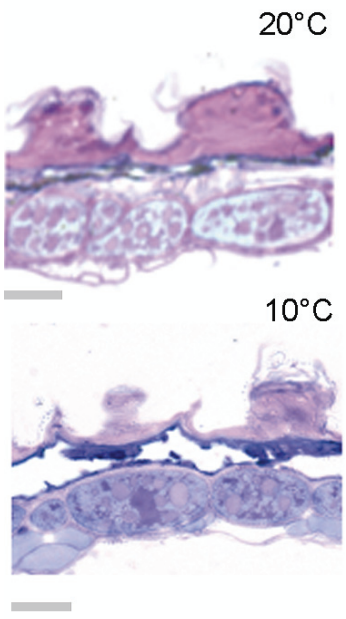

Figure 3.5: Seed coat morphology of seeds matured at 20°C and 10°C.
Seed coats were removed from freshly harvested L*er* seeds matured at 20°C and 10°C following two hours of imbibition, fixed and embedded in Spurr resin. Samples were stained with toluidine blue and visualised using an Invent Flu + CCD microscope using an x40 objective lens**.** Scale bar represents 10µM.

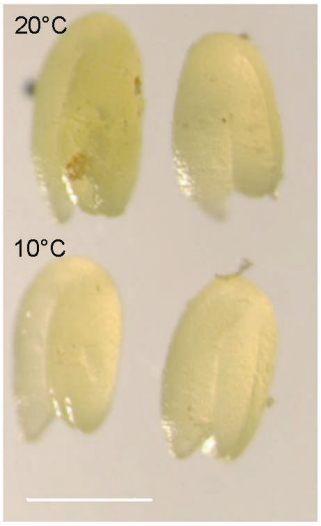

Figure 3.6: Mature embryo morphology of seeds matured at 20°C and 10°C.
Embryos were dissected from freshly harvested Col seeds matured at 20°C and 10°C which had been imbibed for two hours. Embryos were photographed using a using a Leica MZ16F stereomicroscope with a Spot RT3 CCD camera Scale bar represents 100µM.

3.2.5 Analysis of the involvement of hormones in temperature regulation of dormancy

3.2.5.1 Measurement of ABA and GA levels

A role for ABA in regulating dormancy levels is well established, however the importance of GA in inducing and maintaining dormancy is less clear (Bewley, 1997). It has been suggested that GA is simply involved in promoting germination in seeds with no dormancy (Bewley, 1997). The regulation of ABA and GA levels could be an important component of the mechanism by which temperature regulates dormancy levels. Therefore, to determine if ABA and GA levels are altered by maturation temperature and to assess whether these levels corresponded with the amount of dormancy induced in the seeds, ABA and GA levels were measured in Col and Wassilewskija (WS) freshly harvested dry seeds that were matured at either 22°C or 15°C. Col and WS seeds that were matured at low temperature showed a significant increase in ABA levels in comparison to seeds matured at 22°C (Figure 3.7A). The opposite was true for GA levels whereby levels were significantly lower in seeds

matured at 15°C in comparison to 22°C (Figure 3.7B). These results suggest that ABA and GA levels are temperature regulated in seeds and that the normal negative correlation between the two hormones is unaffected by temperature. High ABA and low GA levels correlate with high dormancy and low maturation temperature whereas warm temperature induced low dormancy levels are coupled with low ABA and high GA levels.

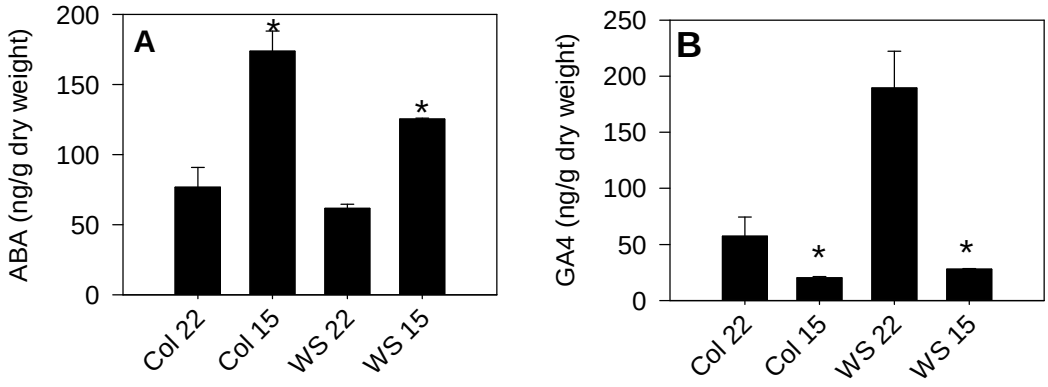

Figure 3.7: Measurement of ABA and GA levels in seeds matured at different temperatures.
ABA (A) and GA (B) measurements were made in freshly harvested dry seeds matured at 22°C and 15°C. * Indicates significant difference to 22°C when P≤0.05 by students t-test.

3.2.5.2 Measurement of OPDA levels

The Jasmonic Acid (JA) precursor 12-oxo-phytodienoic acid (OPDA) has been shown to be a key negative regulator of germination (Dave et al., 2011) and so it was hypothesised that levels of OPDA may differ with maturation temperature and hence dormancy levels. The prediction to be tested was that seeds matured at low temperature, which are highly dormant and, therefore, have low levels of germination, would contain higher levels of OPDA in comparison to seeds matured at warm

temperatures. OPDA levels were measured in Col and L*er* freshly harvested dry seeds that were matured at 12°C, 17°C and 20°C. OPDA levels were significantly lower in Col seeds that were matured at 17°C and 20°C in comparison to 12°C (Figure 3.8A). Similarly levels of OPDA were significantly lower in L*er* seeds matured at 17°C in comparison to 12°C (Figure 3.8B). In contrast to Col seeds, OPDA levels were similar for L*er* seeds matured at 20°C and 12°C. These results highlight that there is no linear correlation between OPDA levels and the maturation temperature and hence dormancy levels.

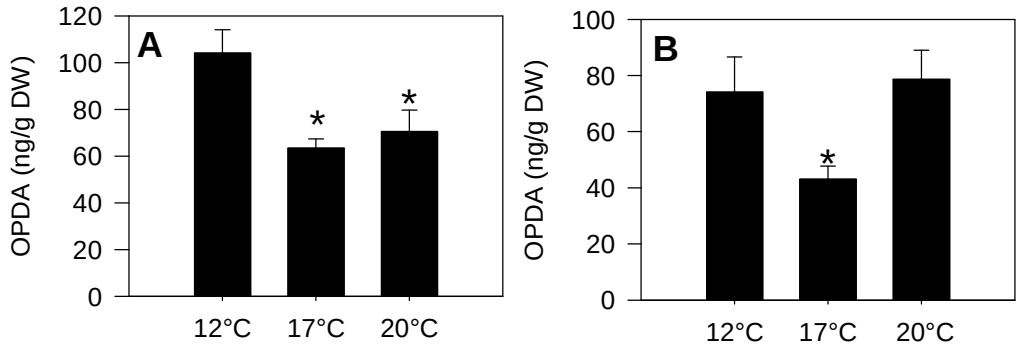

Figure 3.8: Measurement of OPDA levels in seeds matured at different temperatures.
Measurements were made in freshly harvested Col (A) and L*er* (B) dry seeds matured at 20°C, 17°C and 15°C. * Indicates significant difference to 12°C when P≤0.05 by students t-test.

3.2.5.3 Germination response to exogenous GA and NOR

High dormancy levels are induced by low temperature (Figure 3.2) and GA and ABA levels are temperature regulated in seeds (Figure 3.7). To extend the understanding of the role of ABA and GA in regulating dormancy in response to temperature, seeds matured at warm and cool temperatures were germinated in the presence of

exogenous GA or Norflurazon (NOR), an ABA biosynthesis inhibitor. Germination of seeds matured at 22°C showed low dormancy levels and, therefore, the effect of GA, NOR and GA with NOR was extremely minimal since the germination of these seeds could not be increased further (Figure 3.9). Seeds that were matured at 15°C germinated to significantly lower levels than those matured at 22°C. When these seeds were treated with NOR, there was no significant increase in germination (Figure 3.9). In contrast, the addition of GA caused a significant increase in germination to approximately 65% however; this was still lower than the germination of the warm matured seeds. When GA and NOR were added together germination of the low temperature matured seeds was the same as warm temperature matured seeds. These results suggest that an increase in GA levels has a greater capacity to increase germination in comparison to a decrease in ABA levels caused by NOR treatment, but that exogenous GA alone is insufficient to promote germination of low temperature matured seeds to its full capacity.

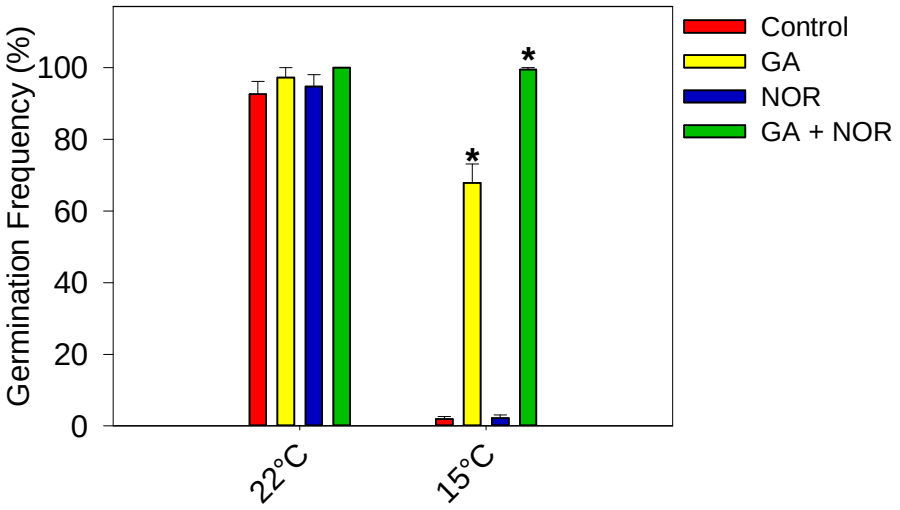

Figure 3.9: Germination of low temperature induced dormant seeds in the presence of GA or NOR.
Germination of Col seeds matured at 22°C and 15°C in response to control conditions (100µM Methanol) or in response to applied GA (100µM), NOR (50µM) or both (GA + NOR). Seeds were cold stratified for three days before being transferred to 22°C in twelve hour white light/dark cycles for seven days. Data points are the average of five individual seed batches and error bars represent standard error. * Indicates significant difference to control treatment when P≤0.05 by students t-test.

3.2.5.4 Characterisation of dormancy phenotypes of hormone synthesis and signalling mutants

To determine if dormancy levels are determined by endogenous ABA or GA levels, the dormancy levels of ABA biosynthesis mutants (*aba1-1* and *aba2-3*) and GA-signalling repressor proteins DELLA mutants (*della*) that were matured at warm and cool temperatures was analysed. These mutants have significantly reduced levels of endogenous ABA (Koornneef et al., 1982, Laby et al., 2000) and have constitutive GA-signalling (Achard et al., 2006) respectively.

When matured at 20°C these mutants, like wild-type, had low levels of dormancy (Figure 3.10, 3.11A). Since wild-type germinated to high levels it was difficult to determine whether these mutants have a role in inducing dormancy at this temperature. However, when the maturation temperature was reduced to 10°C the quadruple *della* loss-of-function mutant was compromised in low temperature induced dormancy and germination was significantly higher than wild-type (Figure 3.10). Similarly, the *aba1-1* mutant (in the L*er* background) also had significantly lower levels of dormancy in comparison to wild-type (Figure 3.11B). However, when the dormancy of the *aba2-3* mutant, which is in the Col background, was tested, the results showed that dormancy could be induced in response to low maturation temperature and the response to cold stratification was not dissimilar to wild-type (Figure 3.11B). The induction of dormancy in this mutant may indicate that this mutant is not a complete null or that ABA is less important for inducing dormancy in Col in comparison to L*er*. These results suggest that DELLAs play an important role in maintaining high dormancy levels in response to low temperature. However, the situation with ABA appears to be more complex, with contrasting phenotypes being found for the different alleles analysed here.

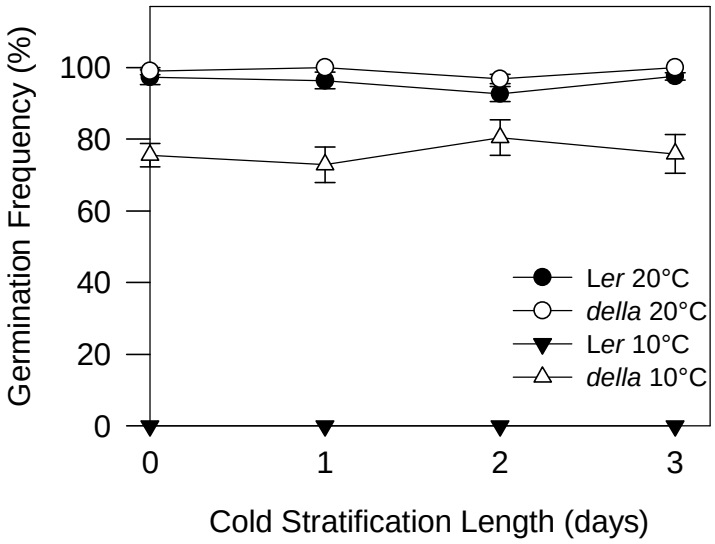

Figure 3.10: Germination of *della* seeds.
Freshly harvested seeds matured at 15°C or 20°C were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

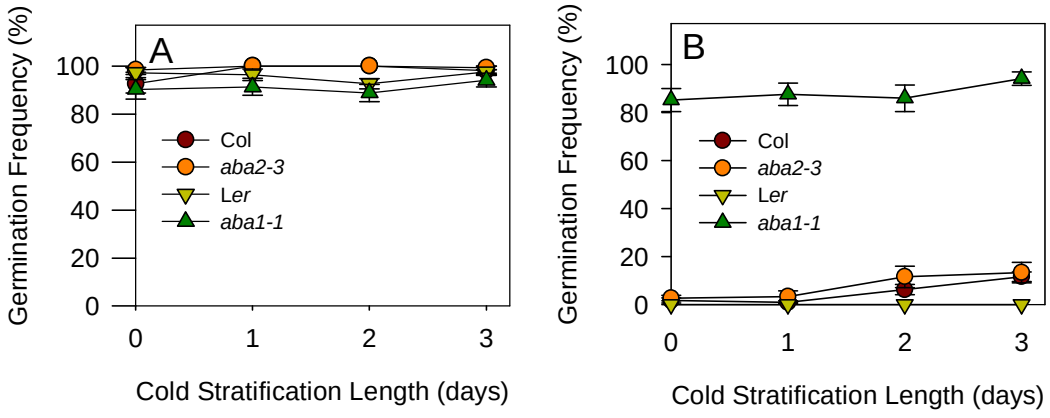

Figure 3.11: Germination of *aba* seeds.
Freshly harvested seeds matured at 20°C (A) or 10°C (B) were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

To confirm the role of ABA in low temperature regulation of dormancy, the dormancy of additional ABA-deficient mutants in the Col background was analysed. The results showed that like wild-type, low dormancy levels were induced in *aba1-6* and *aba3-1* seeds matured at warm temperatures (Figure 3.12). In contrast to wild-type seeds which were highly dormant in response to maturation at 15°C, both *aba1-6* and *aba3-1* seeds displayed significantly lower levels of dormancy when matured at this temperature (Figure 3.12). Therefore, wild-type levels of endogenous ABA are required to enter into the dormant state promoted by maturation at cool temperatures. Together these results suggest that the regulation of ABA and GA levels is central to the mechanisms regulating the induction of dormancy in response to low temperature with endogenous ABA and GA-signalling being required to promote dormancy.

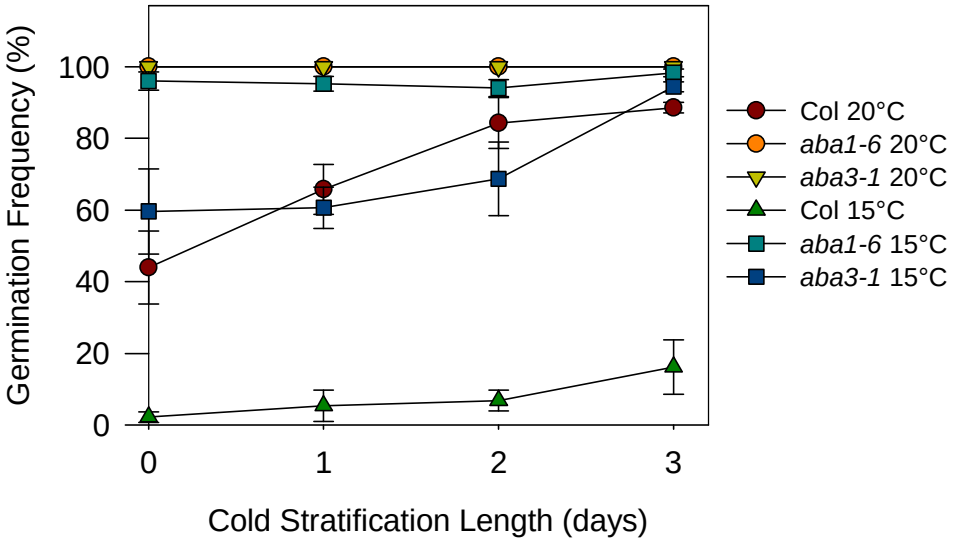

Figure 3.12: Germination of additional *aba* seeds.
Freshly harvested seeds matured at 15°C or 20°C were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

The *CYP707A2* gene encodes an enzyme involved in ABA catabolism (Kushiro et al., 2004). Therefore, to determine whether CYP707A2 has a role in regulating dormancy in response to temperature, the dormancy phenotype of the *cyp707a2-1* loss-of-function mutant was tested in response to maturation at 20°C, 17°C and 15°C. When matured at 20°C, the germination of *cyp707a2-1* seeds was very similar to wild-type (Figure 3.13). However, when the maturation temperature was reduced to 17°C, the germination of *cyp707a2-1* seeds was reduced in comparison to wild-type, although this difference was only significant following two days of cold stratification. When the maturation temperature was reduced further to 15°C, germination of *cyp707a2-1* seeds was significantly reduced in comparison to wild-type following three days of cold stratification. Therefore, when matured at low temperature, dormancy levels are increased in the *cyp707a2-1* seeds, suggesting that CYP707A2 is a negative regulator of dormancy in response to low temperature.

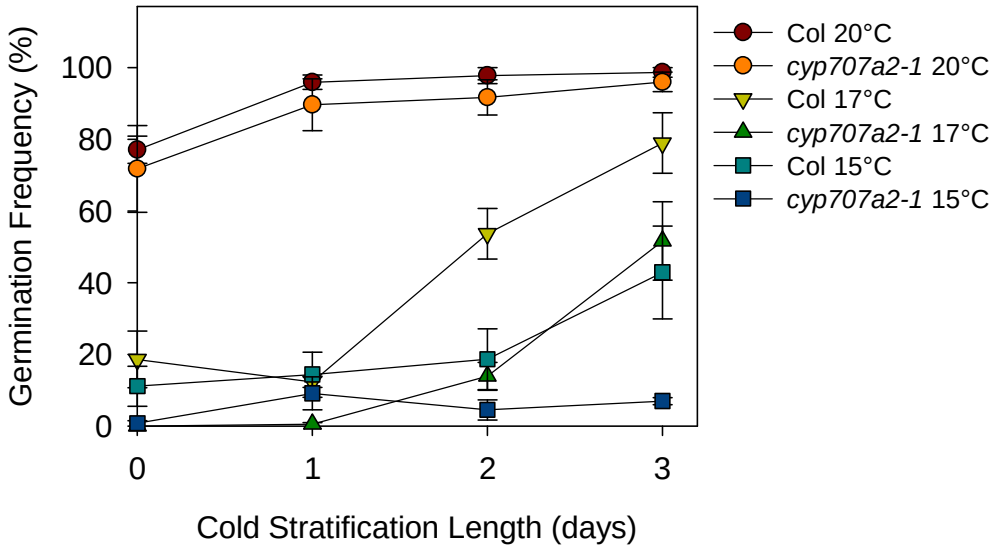

Figure 3.13: Germination of *cyp707a2-1* seeds.
Freshly harvested seeds matured at 15°C, 17°C or 20°C were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

3.2.6 Characterisation of dormancy phenotypes of additional mutants

Previous mutant screens and natural variation studies to identify genes required for the induction of dormancy have identified several genes necessary for this response (Leon-Kloosterziel et al., 1996, Peeters et al., 2002, Bentsink et al., 2006). To determine if these genes also play a role in dormancy induction in response to low temperature, the dormancy phenotypes of *dog1-2* (Bentsink et al., 2006) and the four *rdo* mutants (Leon-Kloosterziel et al., 1996, Peeters et al., 2002) was analysed in response to different maturation temperatures. These mutants were all selected on the basis that they have reduced dormancy phenotypes. The dormancy levels that were induced during maturation at 20°C were low for all seeds tested (Figure 3.14, 3.15A).

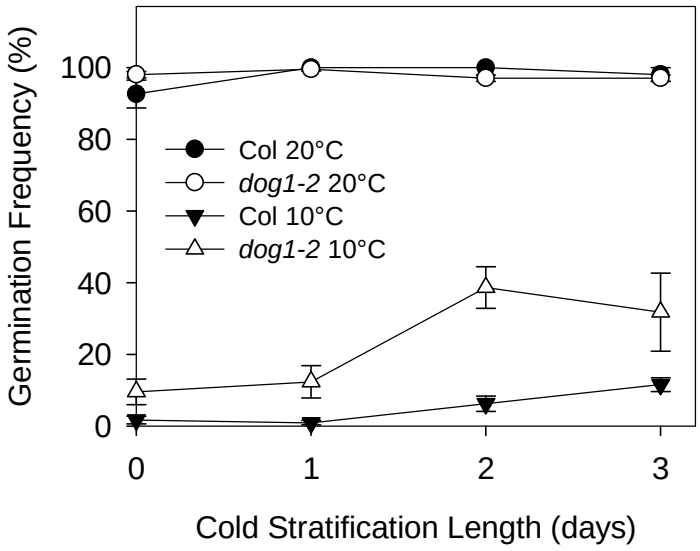

Figure 3.14: Germination of *dog1-2* seeds.
Freshly harvested seeds matured at 20°C or 10°C were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

When matured at 10°C, high dormancy levels were induced in *dog1-2* seeds, suggesting that the low temperature promoted dormancy through a DOG1-independent mechanism (Figure 3.14). However, after two days of cold stratification the dormancy levels were significantly reduced in *dog1-2* seeds in comparison to wild-type. Thus, this result suggests that DOG1 may be required for high dormancy levels in response to low temperature. The effect of cold stratification on releasing dormancy in *dog1-2* suggests that the initial dormancy levels that were induced in these seeds could have been lower than wild-type.

When the maturation temperature was reduced to 10°C, high levels of dormancy were induced in *rdo2* and *rdo3* seeds like what was seen with wild-type (Figure 3.15B). In contrast, the *rdo1* and *rdo4* mutants were compromised in their ability to induce high

dormancy levels in response to low maturation temperature (Figure 3.15B). This was especially prominent for *rdo4* mutant seeds, where approximately 60% of the seeds germinated following three days of cold stratification, which was significantly higher than wild-type. The germination of *rdo1* seeds did not increase in response to cold stratification, but germination was significantly higher than wild-type. Therefore, these results suggest that DOG1 and RDO4 are important for high dormancy levels in response to low temperature and to a lesser extent; RDO1 may also have a role. In contrast, RDO2 and RDO3 do not appear to be involved in the regulation of dormancy by low temperature.

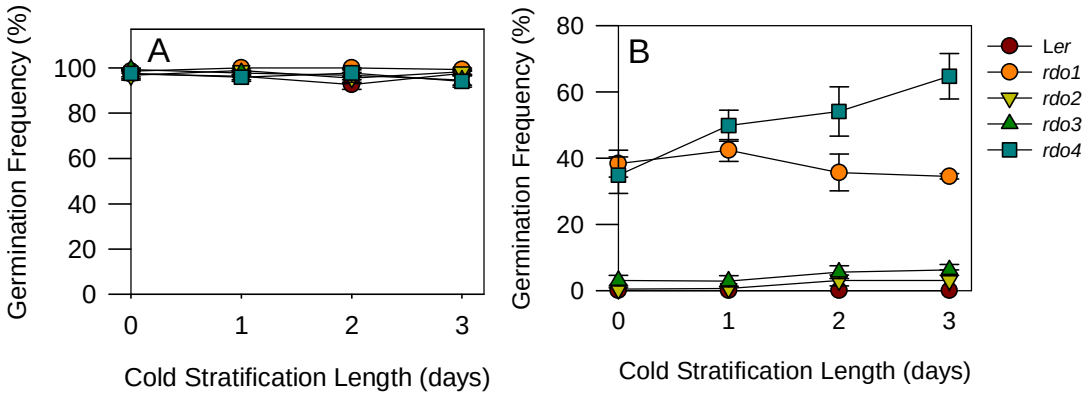

Figure 3.15: Germination of *rdo* seeds.
Freshly harvested seeds matured at 20°C (A) or 10°C (B) were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

3.2.7 Transcriptional analysis of key dormancy regulating genes

3.2.7.1 Analysis in wild-type seeds

Previous results in this chapter have indicated important roles for ABA, GA and DOG1 in regulating dormancy levels in response to low temperature. A transcriptional approach was then taken to investigate how expression of GA and ABA metabolic genes differed with maturation temperature. Since dormancy levels are initiated during seed development, a developmental time course was sampled, which included torpedo, walking stick, green cotyledon and dry seed stage Col and WS seeds. Seeds were sampled based on embryo morphology as opposed to time since there are differences in developmental rates between the seeds matured at the two temperatures.

The expression of *DOG1* was highly upregulated in dry seeds that had been matured at low temperature (Figure 3.16A). In warm matured seeds, the expression of *DOG1* decreases from the green cotyledon to dry seed stage, whereas when seeds are matured at low temperature, this expression either shows a slight increase (Col) or a large increase (WS). The persistence of *DOG1* expression in the dry seed may be important for the maintenance of high dormancy levels in these seeds.

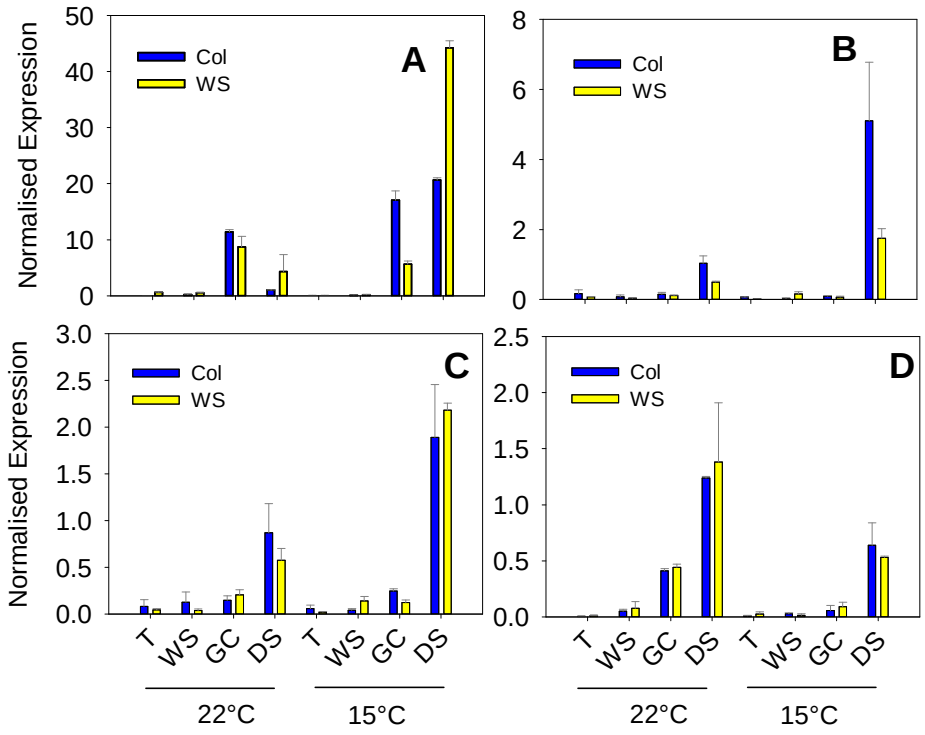

Figure 3.16: Expression of dormancy regulating genes in wild-type seeds. Expression of *DOG1* (A), *GA2ox6* (B), *NCED4* (C) and *CYP707A2* (D) in freshly harvested Col and WS seeds. Developmental stages are Torpedo (T), Walking Stick (WS), Green Cotyledon (GC) and Dry Seed (DS). Data points are the average of two biological replicates and expression is normalised to the average of two control genes *ACTIN2* and *AT3G06240*. Error bars represent standard error.

Expression of *GA2ox6* and *NCED4* was highest in dry seeds and expression was considerably higher in the seeds matured at 15°C in comparison to 22°C (Figure 3.16B, C). At earlier developmental stages there was little difference in expression between the two temperatures. The expression of *CYP707A2* was also analysed and the results show that *CYP707A2* was expressed to higher levels in warm matured seeds than low temperature matured seeds (Figure 3.16D). Again, the expression of this gene was highest in dry seeds, although expression was consistently higher in warm matured walking stick and green cotyledon stage seeds. These results suggest that the

regulation of genes involved in ABA and GA metabolism by low temperature are an important part of the mechanism regulating dormancy. The regulation of *GA2ox6, NCED4* and *CYP707A2* expression in response to temperature probably contributes to the differences in hormone levels that are observed (Figure 3.7).

3.2.7.2 Analysis in mutant seeds

As shown in Figure 3.16, the transcription levels of important dormancy regulating genes are altered by temperature during seed maturation. To further understand the roles of DOG1, ABA and GA in the regulatory network that leads to high dormancy levels in response to low maturation temperatures the expression of *DOG1, NCED4, CYP707A2* and *GA2ox6* was analysed in *dog1-2, aba2-3* and *della* dry mutant seeds that were matured at 17°C. The dormancy levels of these seeds can be observed in Figure 3.17. *aba2-3* and *della* mutants show approximately 50% fold decreases in *DOG1* expression, showing that endogenous ABA and GA are required for correct *DOG1* expression (Figure 3.18A). *dog1-2* mutants showed a tenfold decrease in expression of *GA2ox6* in comparison to wild-type, indicating that one role of DOG1 is the promotion of GA catabolism (Figure 3.18B). There appeared to be no striking differences in the expression of *NCED4* and *CYP707A2* in *dog1-2, aba2-3* and *della* mutants seeds (3.17C, D). These results show that the temperature regulation of ABA metabolism is largely independent of both DOG1 and DELLAs.

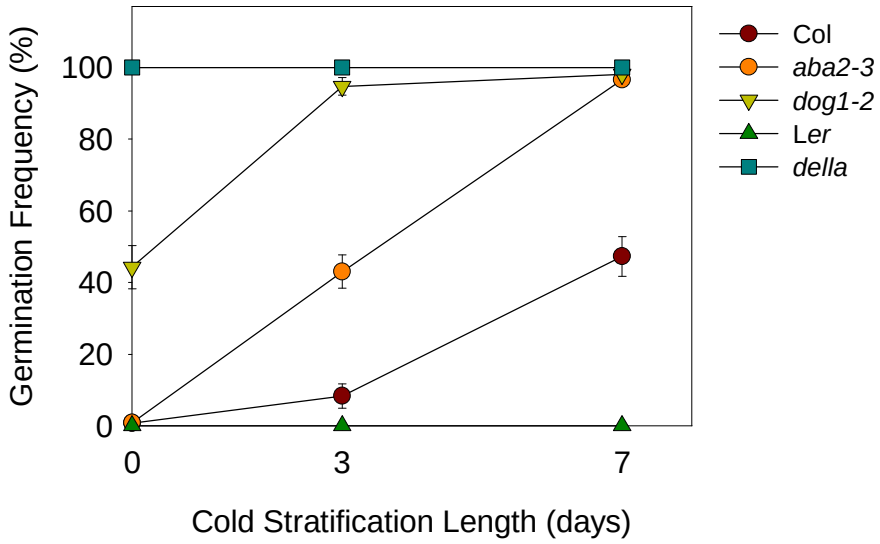

Figure 3.17: Germination of mutants matured at 17°C.
Freshly harvested seeds matured at 17°C were cold stratified for up to seven days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

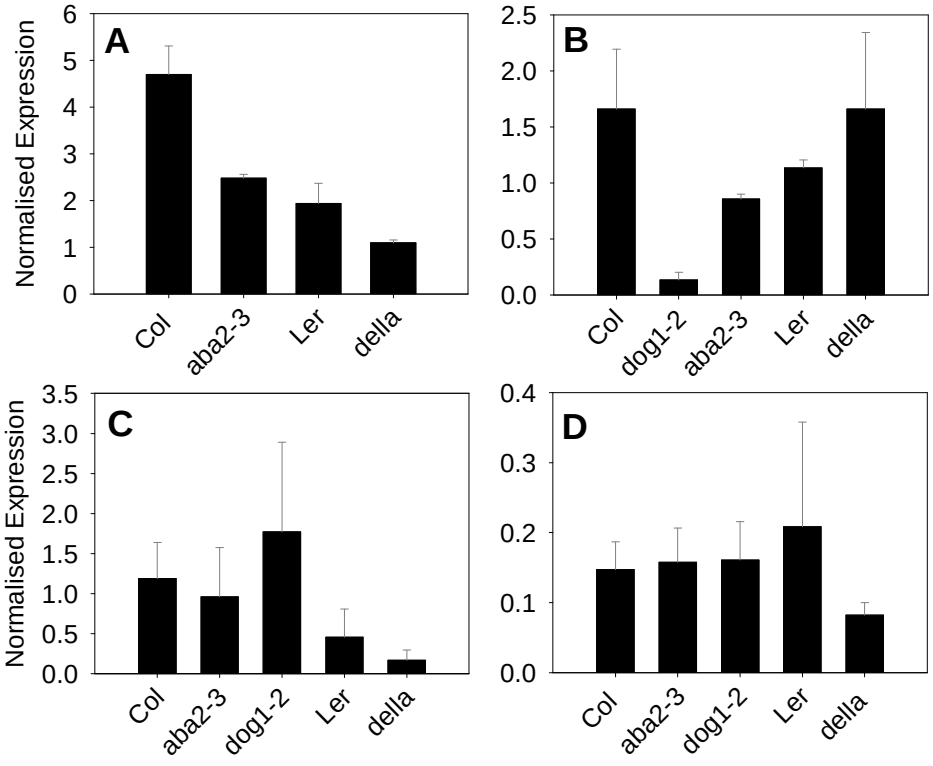

Figure 3.18: Expression of dormancy regulating genes in mutant seeds.
Expression of *DOG1* (A), *GA2ox6* (B), *NCED4* (C) and *CYP707A2* (D) in freshly harvested dry seeds that were matured at 17°C. Data points are the average of two biological replicates and expression is normalised to the average of two control genes *ACTIN2* and *AT3G06240*. Error bars represent standard error.

3.2.8 Analysis of GA and ABA levels in *dog1-2* seeds

Since functional expression of *DOG1* is required for normal levels of *GA2ox6* expression in seeds matured at low temperature (Figure 3.18B), which is important for GA catabolism, it was important to determine if this led to an increase in bioactive GA levels. GA and ABA levels were measured in *dog1-2 and* wild-type dry seeds that were matured at 17°C. Surprisingly, GA levels in *dog1-2* seeds were significantly lower than wild-type (Figure 3.19), suggesting that promotion of *GA2ox6* expression is not the only

way in which DOG1 regulates GA levels in the seed. In contrast, ABA levels were found to be the same as wild-type in *dog1-2* seeds, suggesting that DOG1 is not involved in contributing to the regulation of ABA. Therefore, it appears that the regulation of hormone levels in the *dog1 -2* mutant seeds do not correlate with its reduced dormancy, since GA levels are decreased and there is no change to ABA levels.

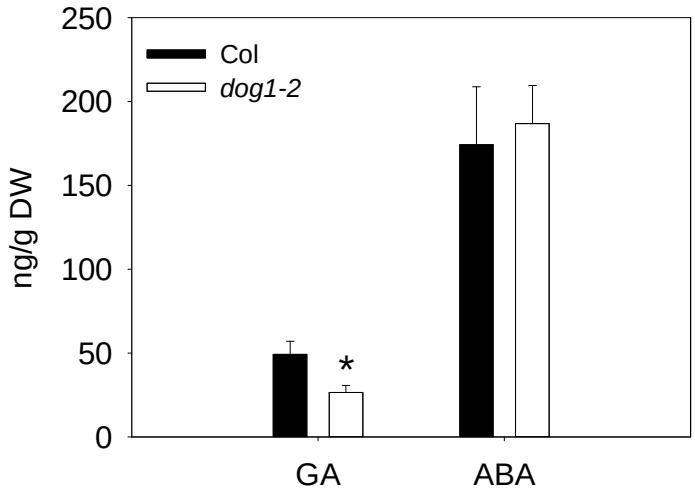

Figure 3.19: Measurement of GA and ABA levels in *dog1-2* seeds.
Measurements were made in freshly harvested dry seeds matured at 17°C. Data points represent the mean measurements in seeds from five individual plants and error bars represent standard error. * Indicates significant difference to wild-type when P≤0.05 by students t-test.

3.2.9 Comparison of temperature and DNA methylation transcriptomes

Data from a microarray study carried out on seeds matured at different temperatures provide a list of temperature regulated genes in dry L*er* seeds (Kendall et al., 2011). This list was compared with a list of genes that were differentially regulated in the DNA methylation loss-of-function mutants *dicer like3-1* (*dcl3-1*), *argonaut4-1* (*ago4-1*) and

rna-dependent rna polymerase2-1 (*rdr2-1*) (Jones Lab, unpublished) and a number of overlapping genes was found (Figure 3.20). The largest number of overlapping genes was identified when genes that were upregulated by low maturation temperature were compared to those upregulated in *ago4-1* mutant seedlings. This prompted an investigation to determine whether DNA methylation could have an important role in regulating dormancy in response to temperature.

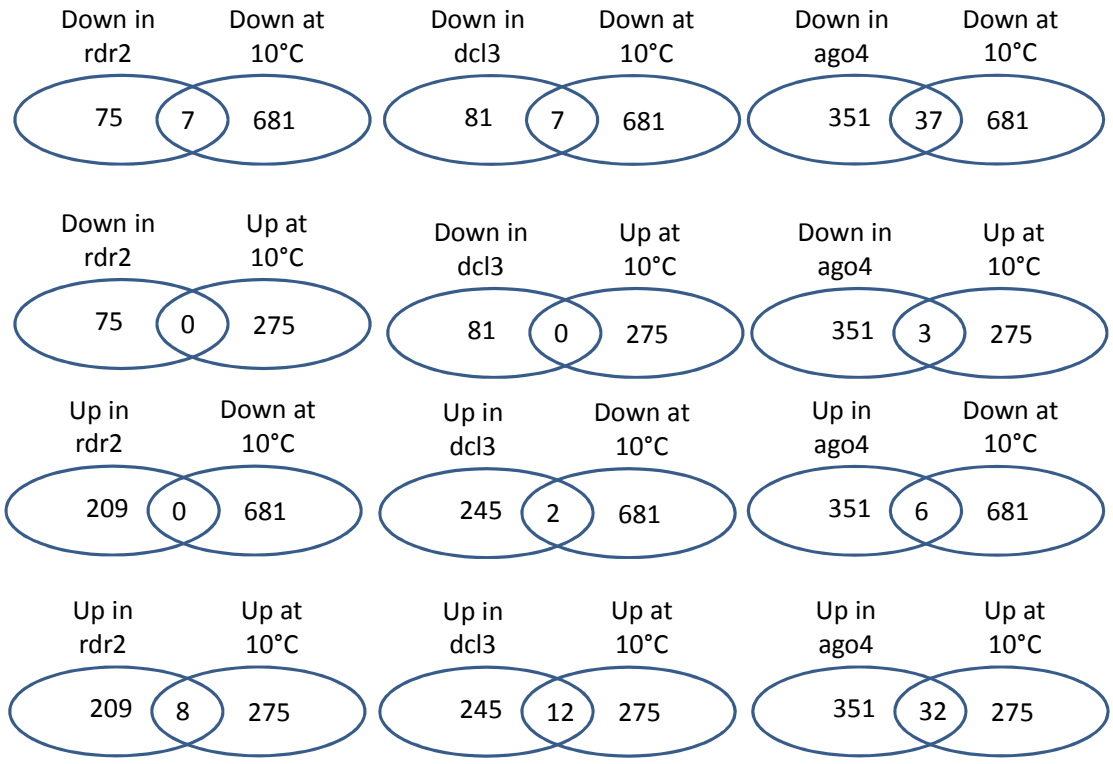

Figure 3.20: Comparison of the temperature regulated transcriptome in seeds and the transcriptomes of three DNA methylation mutants.
Transcriptome analysis in seeds from Kendall *et al*., 2011. Number of genes represents those genes that are differentially regulated by three-fold or more. Note the large overlap between genes differentially regulated by temperature and *ago4*.

3.2.10 Characterisation of dormancy phenotypes of DNA methylation mutants

To identify if DNA methylation has a role in regulating dormancy in response to temperature, a number of DNA methylation mutants which included *rdr2-1, dcl3-1, repressor of silencing1-4* (*ros1-4*), *domains rearranged methylase1-2 (*d*rm1-2*) *drm2-2-* and *ago4-1* was matured at warm and low temperatures and the dormancy levels that were induced were analysed. The *rdr2-1, dcl3-1, ros1-4* and d*rm1-2drm2-2* mutants were in the Col background, whilst *ago4-1* was in the L*er* background. In this experiment, when Col was matured at 20°C, dormancy was induced in these seeds, and this was in contrast to the dormancy levels that had been obtained for Col matured at warm temperatures in previous experiments (Figure 3.21A). In contrast to Col, the dormancy levels induced in the L*er* seeds were extremely low and this was consistent with other experiments. Therefore, this should be taken into account when analysing the mutant phenotypes. When *ago4-1* was matured at warm temperature, significantly higher dormancy levels were induced in comparison to the dormancy levels present in wild-type (Figure 3.21A). The dormancy levels of the remaining DNA methylation mutants did show some altered dormancy phenotypes, but these were generally quite subtle. One exception to this was the germination of *dcl3-1,* which showed a significantly larger increase in germination in response to cold stratification in comparison to wild-type. However, since Col germinated to lower levels than has previously been shown in this experiment, the germination of *dcl3-1* may not really be different to wild-type.

When the temperature during maturation was reduced to 15°C, the germination of *ago4-1* seeds was higher than wild-type, following three days of cold stratification but the difference was not significant (Figure 3.21B). However, the germination response of

dcl3-1, ros1-4, rdr2-1 and *drm1-2drm2-2* to cold stratification was not significantly different to wild-type.

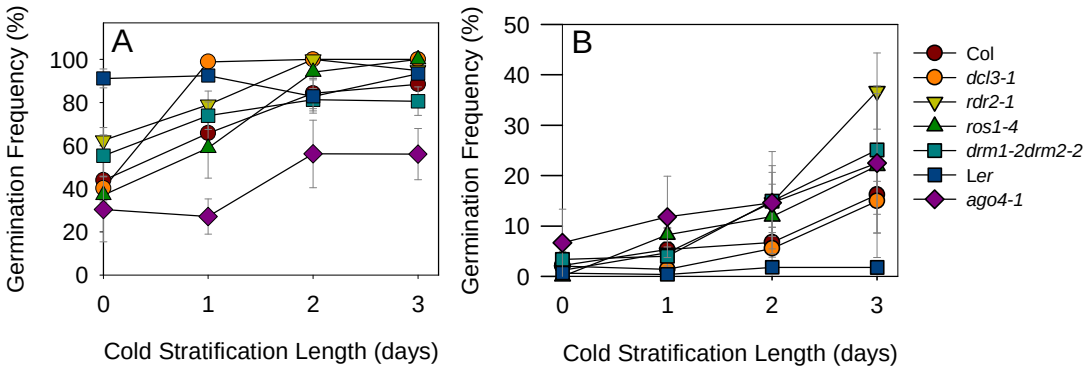

Figure 3.21: Germination of DNA methylation mutants.
Freshly harvested seeds matured at 20°C (A) or 15°C (B) were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

Based on these results it was decided to analyse the dormancy phenotype of *ago4-1* mutant seeds further. *ago4-1* and wild-type seeds were matured at 20°C, 17°C and 15°C. The germination of *ago4-1* was reduced when the seeds were matured at 20°C, although this phenotype was greatly reduced in comparison to what was observed previously (Figure 3.21A). When the seeds were matured at 17°C and 15°C, no differences between the germination of wild-type and *ago4-1* were observed (Figure 3.22). Based on these findings, it can be concluded that none of the DNA methylation mutants that was analysed showed consistent dormancy phenotypes and, therefore, it is unlikely that changes in DNA methylation are required for the induction of dormancy in response to low maturation temperature.

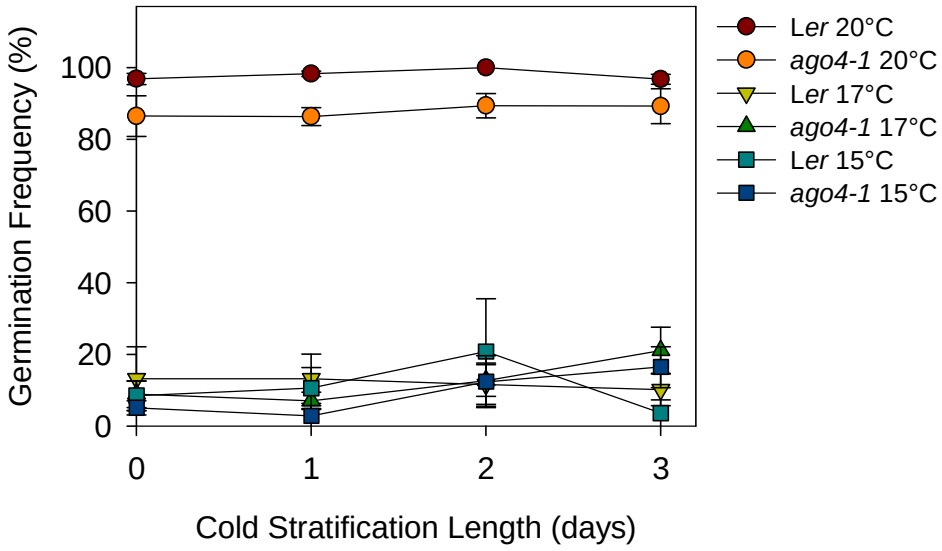

Figure 3.22: Germination of *ago4-1* seeds.
Freshly harvested seeds matured at 20°C, 17°C and 15°C were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

3.3 Discussion

The experiments presented in this chapter were designed to provide a more thorough understanding of the way in which low temperature promotes seed dormancy. Previous work has shown that lowering the temperature during seed maturation leads to an increase in seed dormancy (Schmuths et al., 2006, Donohue et al., 2008, Chiang et al., 2009) but a mechanism for this process is yet to be described. Therefore, this chapter focussed on investigating whether ABA or GA played a role in how temperature promotes seed dormancy. The results show that levels of ABA and GA are altered (Figure 3.7) and this corresponds with the temperature regulation of genes involved in ABA and GA metabolism (Figure 3.16).

3.3.1 Low temperature matured seeds are viable

The results show that low temperature during seed maturation promotes high dormancy levels and as the temperature is reduced, the induced dormancy levels increase (Figure 3.2). In general, the maturation of seeds at 20°C or 22°C leads to the induction of very low levels or dormancy. However, in one experiment only 40% of freshly harvested Col seed germinated (Figure 3.21A). Although in the same experiment L*er* seeds germinated to high levels and so it is unlikely that the environmental conditions were promoting the higher dormancy levels in Col. It is possible that these seeds were harvested too early and this could account for the increase in dormancy that was observed.

A number of treatments are able to break dormancy, although results presented in this chapter show that they are less effective when seeds have been matured at low temperatures. The effect of cold stratification on breaking dormancy decreases as

maturation temperature is reduced from 20°C to 10°C. Even three days of cold stratification had little effect on the germination of seeds that had been matured at 10°C in both Col and L*er* (Figure 3.2). It is possible that these seeds required an increased length of cold stratification to reduce the dormancy levels of seeds matured at low temperature. It has recently been shown that by increasing cold stratification to two weeks the germination of dormant seeds can be increased (Penfield and Springthorpe, 2012). However, prolonged cold stratification also has the potential to promote entry of a seed into secondary dormancy (Finch-Savage et al., 2007, Penfield and Springthorpe, 2012). Therefore, it is difficult to predict how seeds matured at low temperatures will respond to increased length of cold stratification. The amount of cold stratification required to break dormancy and induce secondary dormancy appears to be dependent on maturation temperature and the effect is accession specific (Penfield and Springthorpe, 2012). The imbibed seed must be able to determine how many 'cold days' have accumulated, much like what is required during vernalisation. The mechanism by which seeds can determine this accumulation of cold will be an interesting question to answer in the future.

The high dormancy levels that were induced by low temperature were not broken by two months of after-ripening (Figure 3.3). This analysis was only carried out using seeds that were matured at either 20°C or 10°C. After-ripening may have had a dormancy breaking effect if seeds matured under other temperatures (15°C or 17°C). It is known that strongly dormant ecotypes such as Cvi require extensive lengths of after-ripening to break dormancy (Alonso-Blanco et al., 2003, Ali-Rachedi et al., 2004) and, therefore, a response to after-ripening may have been observed if the after-ripening time had been extended.

The germination of low temperature matured seeds could be greatly increased when germinated on MS instead of water-agar (Figure 3.4). This highlighted that the reduced germination that is observed in freshly harvested seeds matured at low temperature does not represent a loss of viability and is instead an active process initiated by the plant to keep seeds from germinating. It is possible that the 10% of seeds that did not germinate in this experiment were inviable and this could be tested by scarifying the seed coats or by germinating in the presence of tetrazolium. Taken together, it can, therefore, be confirmed that the reduced germination observed in low temperature matured seeds is in fact a dormancy phenomenon (i.e. these seeds have the capacity to germinate but are programmed not to).

3.3.2 ABA and GA metabolism is important for dormancy regulation in response to temperature

The potential roles for the two phytohormones, ABA and GA, in the regulation of temperature effects on dormancy have been investigated in this chapter. In the past a role for GA in dormancy regulation has been contended, with some believing that GA only has a role in the promotion of germination in non-dormant seeds (Bewley, 1997). Penfield et al.,(2006) provided evidence to contest this hypothesis, where they showed that *della* mutants fail to enter dormancy when grown under glasshouse conditions even in the absence of GA synthesis. The data that are presented in this chapter clearly show that GA is an important regulator of dormancy in response to low temperature and, therefore, helps to settle this debate. Levels of GA decrease when the maturation temperature is reduced and this corresponds with alterations to expression of *GA2ox6* and a corresponding increase in dormancy levels (Figure 3.7B , 3.16B). *della* mutants have also been shown to be compromised in their ability to enter the highly dormant state when matured at low temperature (Figure 3.10). The lack of

dormancy in the *della* mutants matured at low temperature suggests that the repression of GA levels through targeted degradation via the ubiquitin-proteasome pathway is important for the mechanism regulating the promotion of dormancy. Additionally, expression of *GA2ox6* is greatly reduced in *dog1-2* dry seeds which also show reduced dormancy levels in response to low temperature, although a decrease in GA levels in *dog1-2* seeds was not observed (Figure 3.18B, 3.19). Expression levels of GA biosynthetic genes have been shown to be upregulated in imbibed *dog1-1* seeds which contributes to elevated levels of GA in these seeds (Nakabayashi et al., 2012). Therefore, it is possible that although GA levels are not elevated in *dog1-2* dry seeds which were matured at low temperature, they could be elevated during seed development or imbibition.

Although it was beyond the scope of this work, it will be important to test the dormancy phenotypes of *ga2ox6* and *nced4* loss-of-function mutants in response to low maturation temperature to understand the contribution of ABA and GA to inducing dormancy further. One would expect that these mutants would be compromised in their ability to induce dormancy in response to low maturation temperature because of their negative and positive regulation of GA and ABA levels respectively. Although it is possible that redundancy may be present between other members of the *GA2ox* and *NCED* group of genes and as a consequence low dormancy phenotypes may not be seen. For example *GA2ox2* is also upregulated by low temperature, but to a lesser extent (Kendall et al., 2011) and, therefore, obvious phenotypes may be absent.

Both ABA and GA are involved in regulation of the *DOG1* transcript, since *della* and *aba2-3* mutants show a 50% reduction in *DOG1* expression in comparison to wild-type (Figure 3.18A). The fact that *DOG1* expression is reduced to similar levels in both

mutants suggests that ABA and GA may regulate *DOG1* through a common intermediary. There is no difference in *NCED4* or *CYP707A2* expression levels in *dog1-2* dry seeds in comparison to wild-type and this corresponds with wild-type levels of ABA (Figure 3.18C, D). However, in contrast a recent study has investigated the expression profile of imbibed *dog1-1* seeds which were matured at a warm temperature and shows that expression of *NCED2* and *NCED5* is downregulated and there was a corresponding decrease in ABA levels in both dry and imbibed seeds (Nakabayashi et al., 2012). The effect of temperature may influence the target genes which are regulated by DOG1 to control dormancy and may explain why differences in expression of *NCED* genes and ABA levels are found.

DOG1 represents an important component of the mechanism that regulates dormancy in response to low temperature since dormancy is alleviated in *dog1-2* seeds following cold stratification. However, there must also be DOG1-independent mechanisms involved in the regulation, since the *dog1-2* mutant does not display a complete lack of dormancy (Figure 3.14).

The analysis of ABA biosynthetic mutant seeds that were matured at low temperature shows that ABA1 and ABA3 are necessary for high dormancy levels whereas the situation with ABA2 is less clear (Figure 3.11, 3.12). The *aba2-3* mutant shows wild-type levels of dormancy, although it is possible that this mutant is not a complete null and this may explain the phenotype. The analysis of the dormancy phenotype of the *cyp707a2-1* mutant showed that CYP707A2 is a negative regulator of low temperature induced dormancy since dormancy levels were increased in this mutant (Figure 3.13).

When applied together, exogenous GA and NOR were capable of increasing the germination levels of seeds matured at low temperature to match the germination of seeds matured at warm temperatures, suggesting a synergistic effect (Figure 3.2). However, exogenous GA was much more effective at increasing germination than NOR. Therefore, together this leads to the suggestion that GA may be more important in regulating dormancy in response to low temperature than ABA.

In contrast to ABA and GA, the results show that temperature did not regulate OPDA levels in dry seeds, suggesting that this is not an important component of the mechanism (Figure 3.8). However, measurements of OPDA levels were made using dry seeds and so if measurements had been made during seed development have a relationship between maturation temperature and OPDA levels may have been observed.

3.3.3 RDO4 and DNA methylation are not involved in the regulation of dormancy by temperature

The analysis of dormancy levels of the four *rdo* mutants revealed that *rdo4* and to a lesser extent *rdo1* mutants were unable to induce high levels of dormancy in response to maturation at low temperature (Figure 3.15). However, strong dormancy could be induced in both *rdo2* and *rdo3* mutant seeds in response to low temperature (Figure 3.15). A recent analysis of the *rdo4* and *rdo2* transcriptomes in mature siliques revealed a common gene set and *DOG1* was found to be downregulated in both *rdo4* and *rdo2* seeds (Liu et al., 2011). Since it is likely that RDO4 and RDO2 are both involved in regulating transcription efficiency (Liu et al., 2007, Liu et al., 2011), it was surprising to find that strong dormancy was induced in *rdo2* mutants and not *rdo4* mutants (Figure 3.15). This suggests that the regulation of *DOG1* transcript levels by RDO4 cannot be

completely responsible for the low dormancy phenotype since *rdo2* seeds would be expected to also contain lower *DOG1* expression levels.

A recent transcriptome study revealed that *RDO4* expression was upregulated in seeds matured at warm temperature (Kendall et al., 2011). However, this increase in expression of *RDO4* with increasing temperature does not correspond with the fact that *rdo4* mutants matured at low temperature have low levels of dormancy (Figure 3.15). Thus, it is likely that RDO4 may have a role in regulating dormancy through a general mechanism and is not involved in the temperature aspect of the regulation. It would be useful to determine whether other environmental factors known to increase dormancy levels during maturation such as short day photoperiods also fail to induce dormancy in *rdo4.* In contrast to the understanding of how RDO4 and RDO2 effect dormancy regulation, the role of *RDO1* in regulating dormancy is not well characterised.

RDO4 is responsible for the monoubiquitination of histone H2B, which leads to changes in histone H3 methylation (Liu et al., 2007). A role for chromatin remodelling in regulating dormancy has also been suggested by an analysis of the *kryptonite-2* (*kyp-2*) mutant which shows increased seed dormancy that is linked to ABA sensitivity (Zheng et al., 2012). KYP is required for histone H3 lysine 9 methylation (Jackson et al., 2002). The increased seed dormancy that is observed in the *kyp-2* mutant has been linked to increases in *DOG1*, *ABI3* and *ABI4* expression (Zheng et al., 2012).

Another example of the importance of chromatin remodelling in seed dormancy control comes from a study using the *histone deacetylase19* (*hda19*) mutant and *HDA6* RNAi seeds. Germination of *hda19* and *HDA6* RNAi seeds was hypersensitive to ABA and this is linked to alterations in expression of ABA responsive genes (Chen et al., 2010,

Chen and Wu, 2010). Taken together, there is a growing body of evidence to suggest that chromatin remodelling could be an important part of the mechanism regulating dormancy. However, evidence is lacking to support a role for chromatin remodelling in the temperature regulation of dormancy.

The comparison of genes regulated by temperature and DNA methylation suggests that DNA methylation may act as a negative regulator of a number of genes that are upregulated by low temperature (Figure 3.20). Although making comparisons between the transcript data sets of maturation temperature and DNA methylation mutants revealed some interesting relationships, it is noteworthy that the DNA methylation mutant microarray was carried out using genetic material collected from seedlings and, therefore, it is possible that the number of overlapping genes may have been higher if the samples had been made from seeds. The largest overlap between genes differentially regulated by temperature and DNA methylation mutants was found in *ago4-1* mutants, and this could be due to the fact that *ago4-1* is in the L*er* background, whereas the other mutants were in the Col background. The temperature regulated gene list was also generated in L*er.*

The results show that in one experiment, the DNA methylation mutant *ago4-1* was compromised in its ability to induce dormancy in response to low temperature during maturation (Figure 3.21A, B). However, when this experiment was repeated, the results suggested that normal dormancy levels were induced in the *ago4-1* seeds matured at 15°C and 17°C (Figure 3.22). Interestingly, *ago4-1* mutants displayed the opposite phenotype when matured at warm temperatures, whereby higher levels of dormancy were induced in comparison to wild-type. Therefore, it appears that this experiment must be repeated to determine whether or not *ago4-1* mutants do have a role in

regulating dormancy. Based on the current evidence available, it appears that DNA methylation does not appear to be the mechanism used in *Arabidopsis* for inducing dormancy in response to low temperature.

AGO4 is required for the initiation of CG methylation and gene silencing (Chan et al., 2004), and so levels of methylation are reduced in *ago4* mutants. A reduction in DNA methylation has been linked to dormancy loss in potato (*Solanum tuberosum* L.); whereby transient decreases in methylation at 5-CCGG-3 sequences during progression of tubers through dormancy precede the resumption of sprout growth (Law and Suttle, 2003). This decrease in DNA methylation may contribute to the permanent increase in transcription rates that occurs after fifty days of post-harvest storage, although other mechanisms must be involved in the regulation of transcription (Law and Suttle, 2003).

The Polycomb repressive complex 2 (PRC2) catalyses trimethylation of lysine twenty seven on histone H3 (H3k27me3). A mutation to the PRC2 component *FIE*, leads to enhanced dormancy and this could be due an upregulation of *DOG1* expression (Bouyer et al., 2011). *DOG1* was identified as an H3k27me3 target in this study, suggesting that its expression may be regulated by this repressive chromatin mark (Bouyer et al., 2011). As transcription of *DOG1* decreases from the transition from dormancy to germination, there is a dynamic switch from the predominant presence of trimethylation of lysine four on histone H3 (H3k4me3) to the repressive H3k27me3 modification (Muller et al., 2012).

Therefore, evidence is present in the literature to suggest that chromatin remodelling and DNA methylation could be important regulators of dormancy induction and

maintenance in response to temperature. It would be interesting to determine whether the levels of H3k4me3 and H3k27me3 at DOG1 chromatin differ with maturation temperature. It may be hypothesised that low maturation temperatures may decrease the levels of H3k27me3, thus leading to an increase in transcription of *DOG1*. Of course the deposition of H3k27me3 is unlikely to be the only histone modification that is involved in regulating *DOG1* transcription. Therefore, it would be useful to analyse other chromatin markers during seed dormancy and germination.

3.3.4 Conclusions

Taken together the results in this chapter have provided a thorough characterisation of the effect of maturation temperature on the dormancy levels induced. ABA and GA levels are temperature regulated in dry seeds and this correlates to changes in expression of genes associated with metabolism of these hormones. Functional expression of *DOG1, DELLA* and *RDO4* has been shown to be required for the induction of high dormancy levels in response to low temperature. A role for chromatin remodelling and DNA methylation in regulating dormancy in response to temperature has been investigated, and the evidence presented in this chapter suggests that the involvement is limited.

Chapter 4 The role of CBFs in temperature regulation of seed dormancy

4.1 Introduction

The results presented in chapter three show that reducing seed maturation temperature leads to an increase in the dormancy levels that are induced (Figure 3.2). The results showed that the regulation of ABA and GA metabolism is an important part of the mechanism that regulates the promotion of dormancy by low temperature (Figure 3.16). To establish additional components of the pathway by which low temperature promotes dormancy during seed maturation the analysis of dormancy phenotypes was extended further.

Expression of the three *C-REPEAT BINDING FACTORS* (*CBFs*) is promoted in a transient manner in response to low temperature in vegetative tissues (Gilmour et al., 1998) and, therefore, it is possible that CBFs may also have a role in temperature signalling in the seed. Similarities between the way in which cold acclimation and seed dormancy are regulated prompted the investigation as to whether CBF-dependent pathways have a role in low temperature regulation of dormancy levels. Firstly, the expression of the three *CBFs* is inducible by ABA (Knight et al., 2004), which is a key regulator of dormancy and germination. Additionally, the CBFs are also involved in the regulation of GA metabolism, and overexpression of *CBF1* leads to an increase in *GA2ox3* expression and a decrease in bioactive GA levels (Achard et al., 2008). Finally, both dormancy and the expression of the *CBFs* are regulated by the circadian clock (Fowler et al., 2005, Penfield and Hall, 2009).

The analysis of available microarray data using the eFP browser (Bassel et al., 2008) revealed that CBFs are expressed to relatively high levels during seed maturation (Figure 4.1) and so potentially could be involved in regulating dormancy induction.

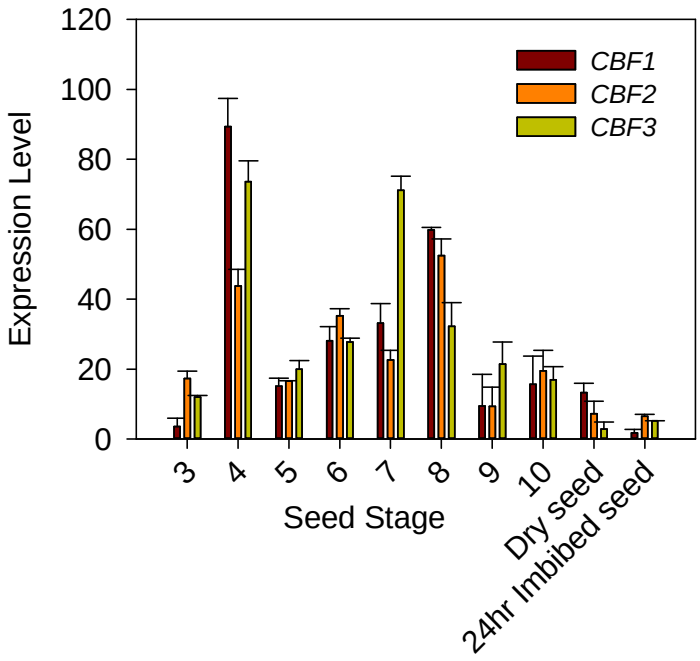

Figure 4.1: Expression of *CBFs* during seed development.
Created using publicly available data from the eFP browser (Bassel et al., 2008). Seed stage numbers represent torpedo (3) through to green cotyledon (10) stage seeds.

4.2 Results

4.2.1 Characterisation of dormancy phenotypes of *CBF*-deficient seeds

The results presented in chapter three showed that by decreasing the temperature during seed maturation the dormancy levels that are induced increase (Figure 3.2). To investigate whether CBF-dependent pathways are involved in regulating the dormancy response to temperature, loss-of-function lines were subjected to different seed maturation temperatures. The *CBF* loss-of-function lines that were used to investigate this were *CBF1* and *CBF3* RNAi lines and a *cbf2* mutant (Novillo et al., 2004, Novillo et al., 2007). When matured at 20°C, no differences in dormancy levels were apparent between the *CBF* loss-of-function and wild-type seeds (Figure 4.2A). However, when the maturation temperature was reduced to 15°C, high primary dormancy levels were initially present in all *CBF*-deficient lines, which was similar to wild-type (Figure 4.2B). When seeds were treated with cold stratification, a significant increase in germination was observed in the *CBF*-deficient lines; whereas cold stratification had little effect on the germination of wild-type. This suggests that lower levels of dormancy were induced in the *CBF*-deficient seeds. When the maturation temperature was reduced further to 10°C, strong primary dormancy was induced in both wild-type and the *CBF* loss-of-function seeds, and cold stratification had little effect on increasing the germination (Figure 4.2C). Together these results show that CBFs are required for the induction of normal levels of dormancy (i.e. the dormancy levels required when seeds are matured at 15°C); however a CBF-independent mechanism must be required for the induction of stronger levels of dormancy, which are induced by lower temperatures as the *CBF*-deficient seeds showed similar levels of dormancy to wild-type.

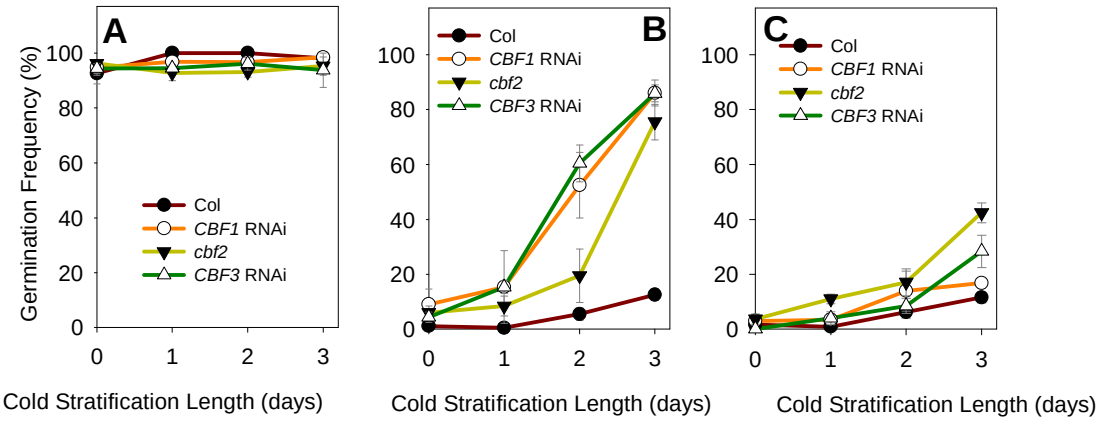

Figure 4.2: Germination of *CBF*-deficient seeds.
Freshly harvested seeds matured at 20°C (A), 15°C (B) and 10°C (C) were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

4.2.2 Transcriptional analysis in *CBF*-deficient seeds

To investigate whether the inability of *CBF*-deficient seeds to enter dormancy when matured at low temperature was coupled with changes in the expression of genes shown to be important for dormancy regulation, Real-Time PCR was used. Gene expression was analysed in wild-type seeds matured at 20 °C or 10°C and *CBF*-deficient seeds that were matured at 10°C. Previous results (Figure 3.16) showed that the expression of *GA2ox6, DOG1* and *NCED4* was upregulated and the expression of *CYP707A2* was downregulated in wild-type seeds matured at low temperature. These alterations to the expression of genes associated with hormone metabolism by maturation temperature in wild-type seeds could be confirmed here (Figure 4.3).

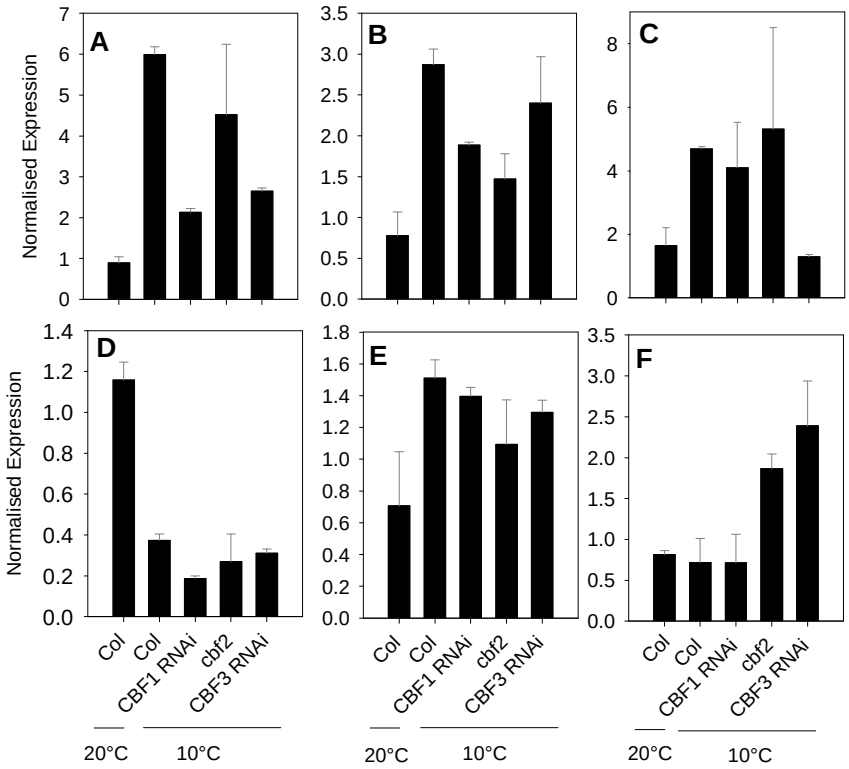

Figure 4.3: Expression of dormancy regulating genes in *CBF*-deficient seeds. Expression was measured in freshly harvested dry seeds matured at 20°C and 10°C. Expression of *DOG1* (A), *GA2ox6* (B), *GA2ox2* (C), *CYP707A2* (D), *NCED4* (E) and *GA2ox3* (F) is shown. Data points are the average of two biological replicates and expression is normalised to the average of two control genes *ACTIN2* and *AT3G06240*. Error bars represent standard error.

The expression of *DOG1* was downregulated in the *CBF* RNAi seeds in comparison to wild-type matured at 10°C (Figure 4.3A); however expression was unaltered in *cbf2* seeds. Expression of *GA2ox6* was downregulated in the all the CBF-deficient seeds (Figure 4.3B). The expression of *GA2ox2* was also analysed, and expression was shown to increase as the maturation temperature was reduced in wild-type seeds (Figure 4.3C). However the change in expression of *GA2ox2* was variable among the

CBF-deficient seeds. There was a slight decrease in expression of *CYP707A2* and *NCED4* in the *CBF*-deficient seeds in comparison to wild-type (Figure 4.3D, E). The expression of *GA2ox3* was analysed as this had been shown to be upregulated in seedlings overexpressing *CBF1* (Achard *et al*., 2008). Expression of *GA2ox3* was not altered by maturation temperature in wild-type however, there appeared to be increased levels of expression of *GA2ox3* in *cbf2* and *CBF3* RNAi seeds in comparison to wild-type (Figure 4.3F). Therefore, the decreased expression of *DOG1* and *GA2ox6* could be important for the reduced dormancy phenotype of the *CBF*-deficient seeds. Conversely alterations in the expression of genes associated with ABA metabolism are much smaller in the *CBF*-deficient seeds, suggesting that alterations to ABA levels may be less important.

4.2.3 Measurement of ABA and GA levels in *CBF*-deficient seeds

The expression of *GA2ox6* was found to be decreased in the *CBF*-deficient seeds (Figure 4.3B). Therefore, to determine whether the altered expression of *GA2ox6* resulted in different levels of GA, comparisons of endogenous GA levels were made between *CBF*-deficient and wild-type seeds. Results presented in chapter three have shown that ABA and GA levels are altered by maturation temperature and the ratio between these hormones correlates with dormancy levels (i.e. high ABA and low GA in seeds with low dormancy) (Figure 3.7). ABA levels were measured in the *CBF*-deficient seeds and the results show that ABA levels were elevated in the three *CBF*-deficient lines in comparison to wild-type. Specifically, *CBF1* RNAi and *CBF3* RNAi seeds contained approximately three times the amount of ABA in comparison to wild-type, which was significantly higher, whereas ABA levels were approximately double in *cbf2* mutant seeds in comparison to wild-type (Figure 4.4A). When the *CBF*-deficient seeds

were matured at 10°C, GA levels in the *CBF1* RNAi and *CBF3* RNAi seeds were very similar to wild-type, whilst *cbf2* mutant seeds contained significantly higher levels of GA (Figure 4.4B). Therefore, the results show that ABA levels are increased in *CBF1* and *CBF3* RNAi seeds, whilst GA levels are unaltered. In contrast, both ABA and GA levels are increased in the *cbf2* mutant. It is of course possible that levels of ABA and GA may be different in *CBF*-deficient developing seeds in comparison to dry seeds, which could be important if the CBFs are acting during seed development to regulate dormancy.

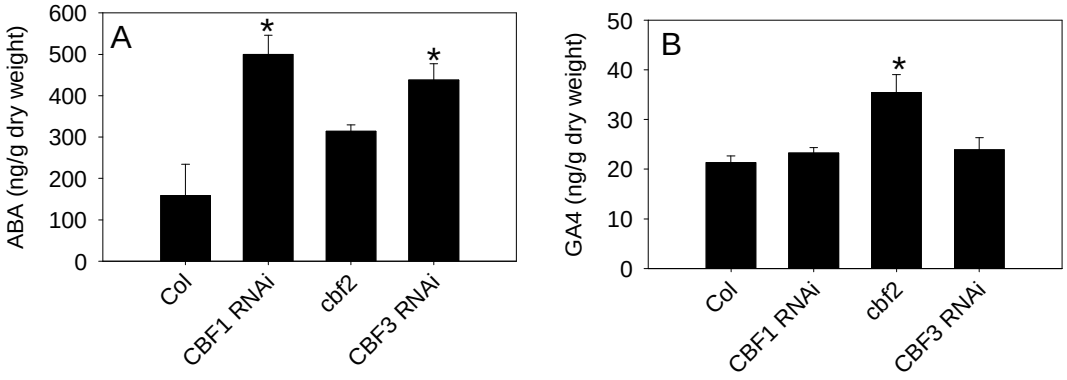

Figure 4.4: Measurements of ABA and GA levels in *CBF*-deficient seeds.
Measurements of ABA (A) and GA (B) were made in freshly harvested seeds matured at 10°C. Data points represent the mean of measurements from five seed batches from individual plants and error bars represent standard error. * Indicates significant difference to wild-type when P≤0.05 by students t-test.

4.2.4 Characterisation of dormancy phenotypes of *CBF*-overexpressing seeds

To analyse the role of the CBFs in regulating the effect of temperature on seed dormancy further, the dormancy of *CBF*-overexpressing seeds was analysed. Seeds overexpressing each of the *CBFs* in the WS background under the control of the CaMV 35S promoter were used for this analysis (Jaglo-Ottosen et al., 1998, Gilmour et al.,

2000). The overexpression of the *CBFs* led to an increase in dormancy levels in comparison to wild-type when seeds were matured at 20°C and this dormancy phenotype persisted following cold stratification (Figure 4.5). The effect of cold stratification on the promotion of germination was considerably stronger for *CBF1*-overexpressing seeds than for *CBF2* or *CBF3*-overexpressing seeds, which showed significantly higher levels of dormancy than WS (Figure 4.5).

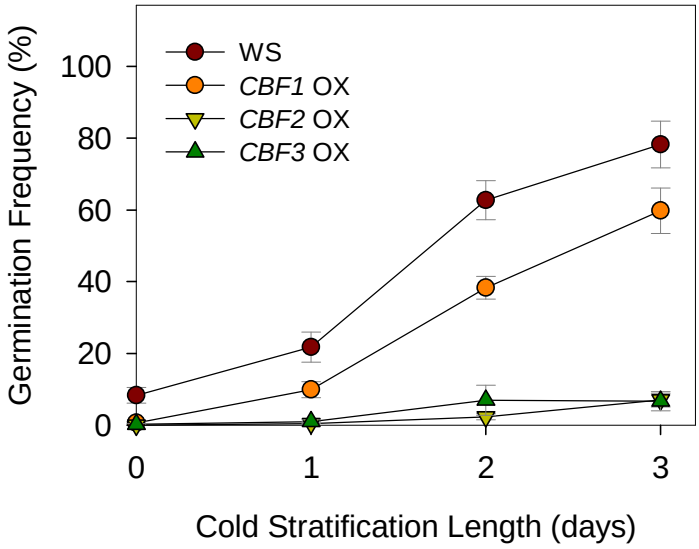

Figure 4.5: Germination of *CBF*-overexpressing seeds in response to cold stratification.
Freshly harvested seeds matured at 20°C were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

After-ripening had very little effect on increasing the germination of the *CBF*-overexpressing seeds, although it also had little effect on wild-type (Figure 4.6). The small effect of after-ripening on WS germination has been observed in a previous study (Penfield and Hall, 2009). The dormancy of *CBF*-overexpressing seeds matured at low

temperature could not be tested since plants grew very poorly and produced very little seed under these conditions. These results show that the seeds overexpressing the *CBFs* display the opposite dormancy phenotype to the *CBF*-deficient seeds and that *CBF* transcripts are necessary for and sufficient to induce increased dormancy.

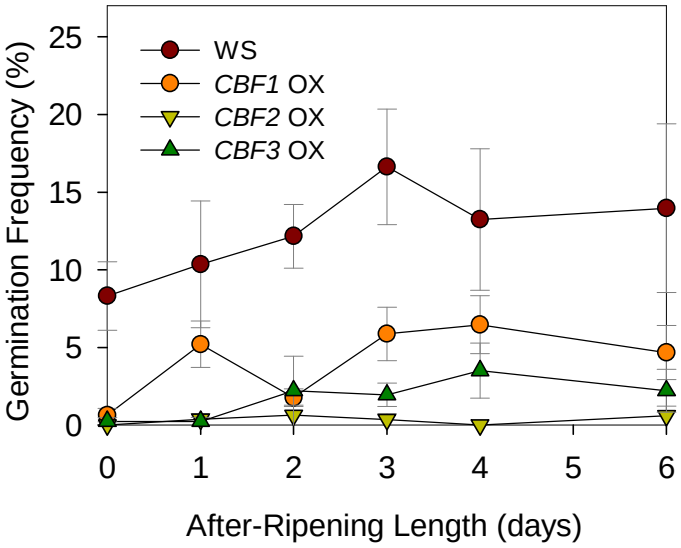

Figure 4.6: Germination of *CBF*-overexpressing seeds in response to after-ripening.
Seeds matured at 20°C were after-ripened for up to six weeks. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

4.2.5 Germination response of *CBF*-overexpressing seeds to exogenous GA and NOR

The exogenous application of GA is able to rescue the dwarfed phenotype of *CBF1*-overexpressing plants (Achard et al., 2008). Therefore, to investigate whether GA is capable of reducing the increased dormancy levels of *CBF*-overexpressing seeds to wild-type levels the germination of the *CBF*-overexpressing seeds, which were matured

at 20°C, was analysed in response to exogenous GA, NOR, GA and NOR or a control (methanol). Germination of seeds overexpressing the three *CBF*s was low in comparison to wild-type when germinated on control plates (Figure 4.7). The increased dormancy of the *CBF*-overexpressing seeds could be rescued by the addition of exogenous GA, but not by the addition of NOR, an ABA biosynthesis inhibitor. An exception to this is the germination of *CBF1*-overexpressing seeds, which require both exogenous GA and NOR to obtain wild-type levels of germination. *CBF1*-overexpressing seeds respond differently to cold stratification in comparison to *CBF2* and *CBF3*-overexpressing seeds (Figure 4.5) and this may explain the different response to GA. This result suggests that GA is sufficient to increase the germination of the *CBF*-overexpressing seeds. Since NOR alone was unable to significantly increase the germination, it seems that the misregulation of GA levels is likely to be the predominant cause of the high dormancy phenotype.

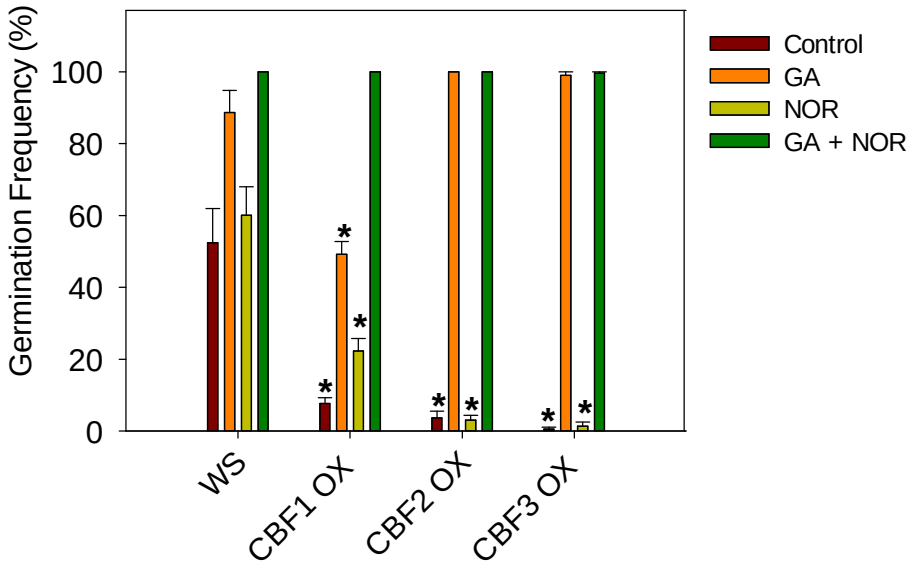

Figure 4.7: Germination of *CBF*-overexpressing seeds in response to exogenous GA and NOR.
Germination of seeds matured at 20°C in response to control conditions (100µM methanol) or applied GA (100µM), NOR (50µM) or both (GA + NOR). Seeds were cold stratified for three days before being transferred to 22°C in twelve hour white light/dark cycles for seven days. Data points are the average of five individual seed batches and error bars represent standard error. * Indicates significant difference to wild-type when P≤0.05 by students t-test.

4.2.6 Transcriptional analysis in *CBF*-overexpressing seeds

To further establish the reason why *CBF*-overexpressing seeds displayed increased dormancy levels, an analysis of gene expression was performed in freshly harvested dry seeds that were matured at 20°C using Real-Time PCR. This analysis focussed on genes found to be misregulated in the *CBF* loss-of-function seeds and those known to be important for the regulation of dormancy in response to temperature. Since *CBF*-deficient seeds show a decrease in expression of *DOG1* and *GA2ox6* (Figure 4.3A,B), this experiment tested the hypothesis that *CBF*-overexpressing seeds are more dormant than wild-type due to upregulation of *DOG1* and *GA2ox6* expression.

However, the results show that generally no increases in *DOG1* and *GA2ox6* expression were observed in the *CBF*-overexpressing seeds, which is the opposite to what is observed in *CBF*-deficient seeds and, therefore, does not support the original hypothesis (Figure 4.8A,B). There is an increase in *DOG1* expression in *CBF1*-overexpressing seeds (Figure 4.8A). However, a decrease in *DOG1* expression is present in *CBF2* and *CBF3*-overexpressing seeds (Figure 4.8A). Seeds overexpressing each of the three *CBFs* show a decrease in expression of *GA2ox6* (Figure 4.6B). However, there was an increase in expression of *GA2ox2* in *CBF*-overexpressing seeds (Figure 4.6C). In terms of expression of genes associated with ABA metabolism, the expression of *CYP707A2* appeared to be higher in *CBF1*-overexpressing seeds in comparison to wild-type, whereas expression was lower in *CBF2* and *CBF3*-overexpressing seeds when compared to wild-type (Figure 4.8D). The expression levels of *NCED4* in *CBF*-overexpressing seeds was found to be very similar to wild-type (Figure 4.8E). The upregulation of *GA2ox3* expression that is observed in *CBF1* overexpressing vegetative tissue (Achard et al., 2008) is also seen in dry seeds (Figure 4.8F). In contrast to *CBF*-overexpressing seeds, a decrease in expression of *GA2ox3* in the *CBF*-deficient seeds was not observed (Figure 4.3). Since the expression of *DOG1, GA2OX6, GA2OX2, CYP707A2* and *NCED4* shows a large amount of variability between the different *CBF*-overexpressing seeds, it is unlikely that their expression levels are important for the increased dormancy phenotype. However, *GA2OX3* is upregulated in all *CBF*-overexpressing seeds, and so may be important for the increased dormancy phenotype.

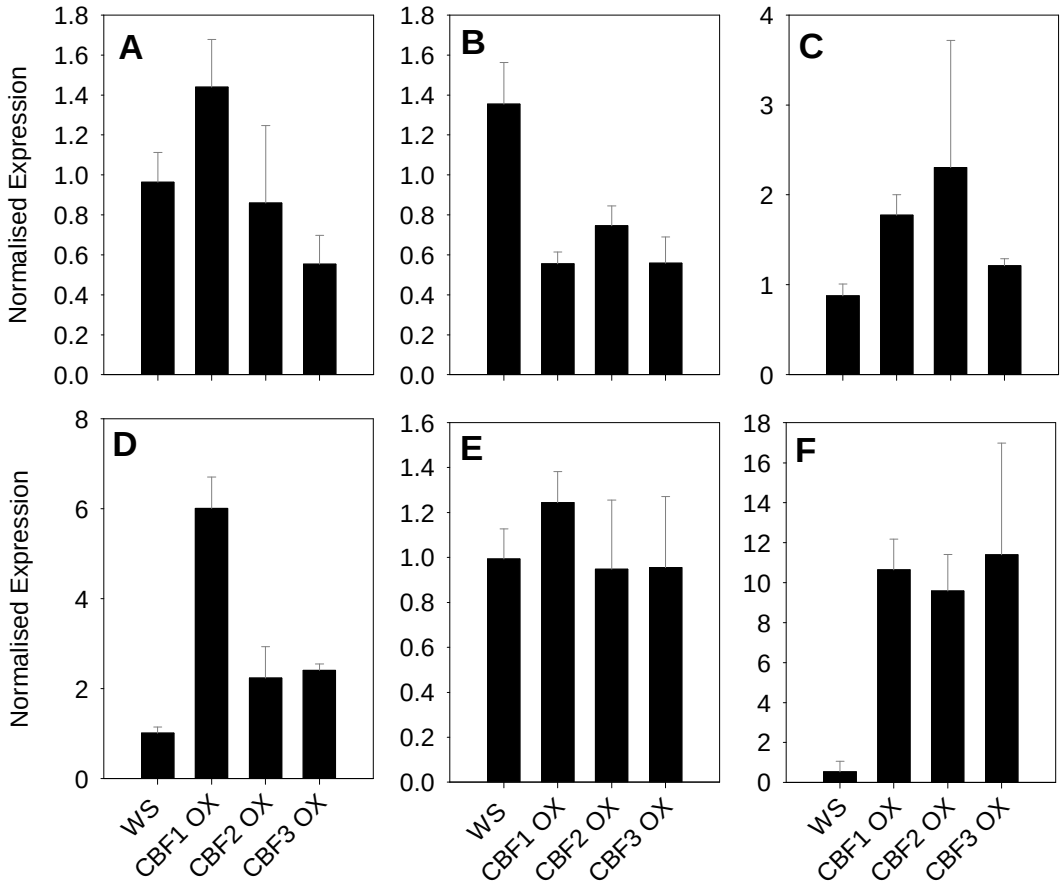

Figure 4.8: Expression of dormancy regulating genes in *CBF*-overexpressing seeds.
Expression was measured in freshly harvested dry seeds matured at 20°C. Expression of *DOG1* (A), *GA2ox6* (B), *GA2ox2* (C), *CYP707A2* (D), *NCED4* (E) and *GA2ox3* (F) is shown. Data points are the average of two biological replicates and expression is normalised to the average of two control genes *ACTIN2* and *AT3G06240*. Error bars represent standard error.

There were no common genes that show opposite misregulation in both *CBF*-deficient and *CBF*-overexpressing seeds identified in this analysis. Therefore, it seems that GA and ABA biosynthesis and catabolism pathways are not regulated in a correlated fashion by the CBF proteins as loss-and gain-of-function mutations do not have opposite outcomes. Although opposite dormancy phenotypes are present in the *CBF*

loss-and gain-of-function seeds it appears that dormancy is affected through two distinct pathways.

4.2.7 Measurement of GA and ABA levels in *CBF*-overexpressing seeds

Bioactive GA levels in vegetative tissues have been shown to be lower in plants overexpressing *CBF1* in comparison to wild-type (Achard et al., 2008). Since the increased dormancy phenotype of *CBF*-overexpressing seeds can be rescued by exogenous application of GA (Figure 4.7), levels of ABA and GA were measured to determine whether or not levels of these hormones were altered in comparison to wild-type. Levels of ABA were found to be generally similar to wild-type for *CBF2* and *CBF3*-overexpressing seeds (Figure 4.9A). In contrast, *CBF1*-overexpressing seeds contain significantly higher levels of ABA when compared to wild-type. Surprising, very little difference in levels of GA was found between wild-type and the *CBF1* and *CBF2*-overexpressing seeds (Figure 4.9B). Levels of GA were higher in *CBF3*-overexpressing seeds, although this difference was not significant (Figure 4.9B). This was surprising since expression of *GA2ox3* is upregulated in *CBF*-overexpressing seeds (Figure 4.8F), suggesting that other pathways in addition to the regulation by GA2ox3 must be involved in controlling GA levels in these seeds. Therefore, these results show that the high dormancy phenotype of the *CBF*-overexpressing seeds cannot be attributed to altered endogenous levels of GA and ABA in dry seeds because GA is either not different or backwards from what was predicted and ABA levels were not different or not different enough to be responsible for the altered dormancy levels.

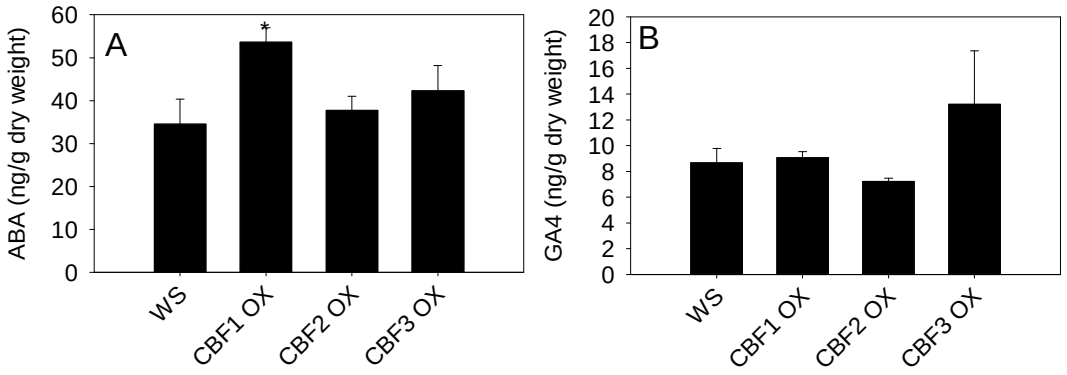

Figure 4.9: Measurements of ABA and GA levels in *CBF*-overexpressing seeds. Measurements of ABA (A) and GA (B) were made in freshly harvested dry seeds matured at 20°C. Data points represent the mean of measurements from five seed batches from individual plants and error bars represent standard error. * Indicates significant difference to wild-type when P≤0.05 by students t-test.

4.2.8 *CBF1* and *COR15b* expression is not temperature regulated in seeds

CBF expression has been shown to be upregulated transiently by exposure to low temperature in vegetative tissues (Gilmour et al., 1998). To understand if *CBF* expression is temperature regulated in seeds, *CBF1* expression was determined using a time-course experiment in which developing seeds (green cotyledon stage), twenty four hour imbibed seeds and ten day old seedlings were transferred from 22°C to 4°C (five hours after dawn) and then sampled every hour for three hours. *CBF1* expression was analysed since an analysis of previously published *CBF* primers (e.g. Franklin and Whitelam, 2007) revealed that their specificity for any one isoform was questionable, and only for *CBF1* could a specific Taqman assay be reliably developed. *CBF1* expression was initially higher in developing seeds than in imbibed seeds or seedlings (Figure 4.10A). However, the expression of *CBF1* did not increase with the shift to low temperature in developing seeds, whereas expression increased following one hour at

4°C in seedlings (Figure 4.10A). Expression of *CBF1* was extremely low in imbibed seeds and cold had no effect on promoting transcript abundance.

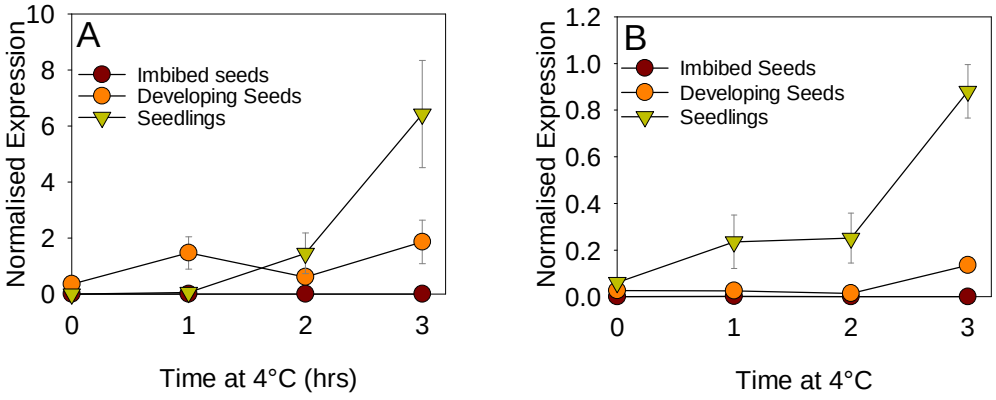

Figure 4.10: Expression of *CBF1* and *COR15b* in response to low temperature. Expression of *CBF1* (A) and *COR15b* (B) in response to a sudden cold shock at 4°C in seedlings, imbibed seeds and developing seeds. Data points are the average of two biological replicates and expression is normalised to the average of two control genes *TUBULIN9* and *AT3G06240*. Error bars represent standard error.

The expression of *COR15b,* a CBF target gene, was also analysed and the increase in expression that was observed in seedlings was similar to the expression pattern of *CBF1* (Figure 4.10B). Low temperature did not increase expression of *COR15b* in imbibed seeds and this is consistent with the low levels of expression of *CBF1* (Figure 4.10B). There was a small increase in *COR15b* expression following three hours at 4°C in developing seeds, but again levels were much lower than those observed in seedlings (Figure 4.10B). Therefore, the results show that expression of *CBF1* and *COR15b* is not temperature regulated in imbibed or developing seeds.

4.2.9 Measurement of fatty acid levels

It is well documented that changes in temperature can lead to alterations to the fatty acid composition within the plant (Wallis and Browse, 2002, Falcone et al., 2004).

Higher temperatures lead to increases in saturated and monounsaturated fatty acids, whilst low temperature promotes the increase in polyunsaturated fatty acids (Wallis and Browse, 2002, Falcone et al., 2004). Therefore, to investigate the potential effect of temperature during seed maturation on the fatty acid composition of dry seeds, the fatty acid profiles of Col and WS, along with *CBF3*-overexpressing and *CBF1* RNAi seeds that were matured at 22°C and 15°C, were analysed using ultraperformance liquid chromatography (UPLC)–mass spectrometry. For all of the genotypes tested, the total fatty acid levels were higher in seeds matured at 15°C in comparison to 22°C, however this difference was insignificant in the *CBF3*-overexpressing seeds (Figure 4.11).

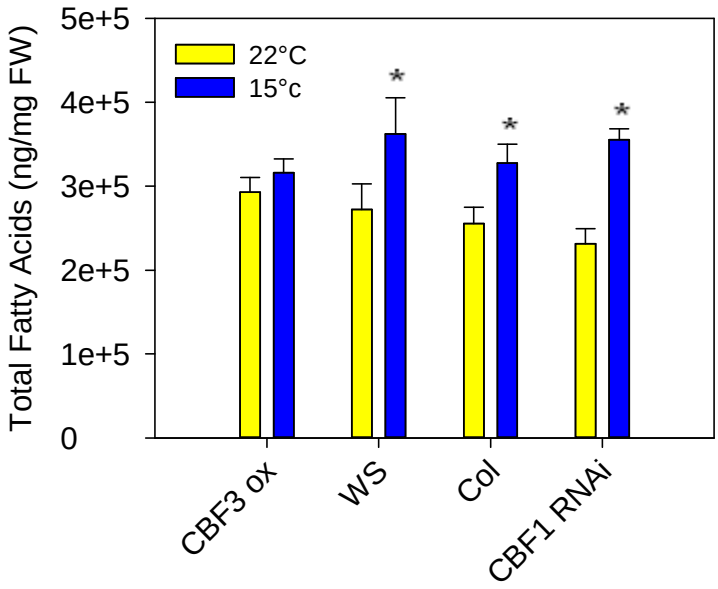

Figure 4.11: Measurement of total fatty acid levels.
Measurements were made in freshly harvested dry seeds matured at 22°C or 15°C. Data points represent the mean of measurements from five seed batches from individual plants and error bars represent standard error. * Indicates significant difference to 22°C when P≤0.05 by students t-test.

To understand the nature of these increases in fatty acid content in low temperature matured seeds the fatty acid composition profiles were analysed in more detail. The fatty acid profiles for WS (Figure 4.12A) and Col (Figure 4.12C) seeds which were matured at 22°C and 15°C were very similar. Significant increases 18:2n6c, 18:3n3 and 20:1n9 were present in wild-type seeds matured at low temperature in comparison to seeds matured at warm temperature (Figure 4.12A, C). These major changes were also present in *CBF1* RNAi seeds (Figure 4.12D), whereas the increases in 18:2n6c, 18:3n3 and 20:1n9 were smaller in *CBF3*-overexpressing seeds (Figure 4.12B).

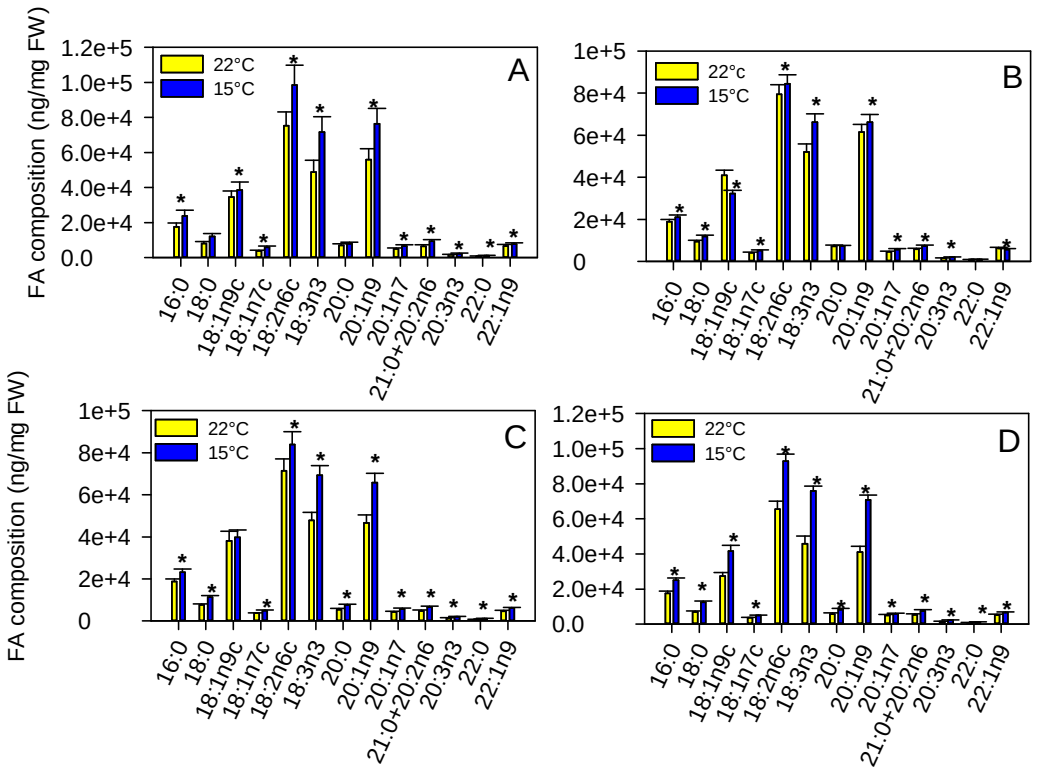

Figure 4.12: Fatty Acid compositions of seeds.
Measurements were made in freshly harvested dry WS (A), *CBF3*-overexpressing (B), Col (C) and *CBF1* RNAi (D) seeds matured at 22°C or 15°C. Data points represent the mean of measurements from five seed batches from individual plants and error bars represent standard error. * Indicates significant difference to 22°C when P≤0.05 by students t-test.

Regulation of levels of 18:1n9c appears to be temperature independent in Col and WS, whereas a significant increase in levels in *CBF1* RNAi seeds and a significant decrease in *CBF3*-overexpressing seeds at the cool maturation temperature is observed (Figure 4.13). These changes stem from *CBF3*-overexpressing and *CBF1* RNAi seeds having significantly higher and lower levels of 18:1n9c respectively when matured at 22°C compared to the wild-type. This suggests that the CBF1 and CBF3 could be important regulators of 18:1n9c levels in seeds.

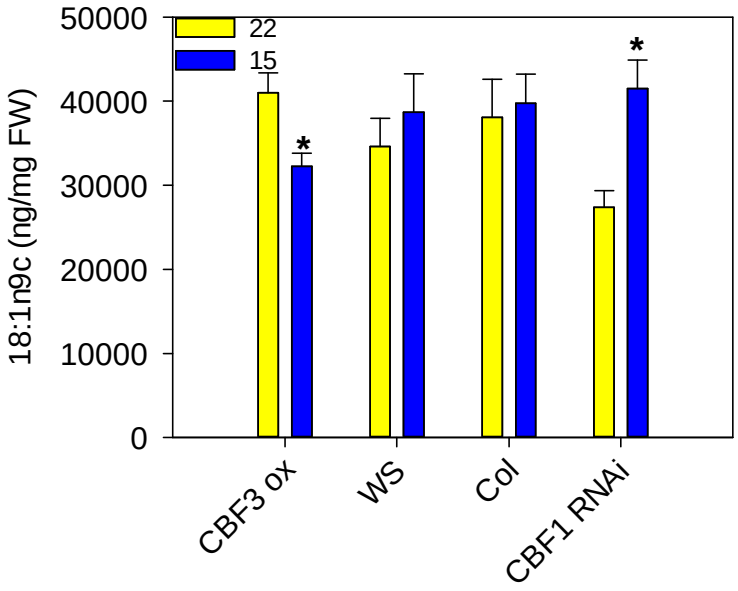

Figure 4.13: Measurement of 18:1n9c levels.
Measurements were made in freshly harvested dry seeds matured at 22°C or 15°C. Data points represent the mean of measurements from five seed batches from individual plants and error bars represent standard error. * Indicates significant difference to 22°C when P≤0.05 by students t-test.

4.2.10 Characterisation of the dormancy phenotype of *ice1-2* seeds

Since CBFs are part of a large transcriptional cascade that leads to cold acclimation it is possible that other components of this pathway may also play a role in regulating dormancy. The dormancy phenotype of the *ice1-2* mutant was tested using seeds matured at 20°C and 10°C. ICE1 is a transcription factor that is involved in promoting the expression of *CBF3* in response to low temperature (Gilmour et al., 1998, Chinnusamy et al., 2003). The *ice1-2* mutation leads to a decrease in expression of *CBF3* as well as other downstream target genes (Chinnusamy et al., 2003). As with the majority of mutants tested, low dormancy levels were induced in *ice1-2* seeds which

were matured at 20°C (Figure 4.14). When seeds were matured at 10°C, dormancy was induced in the *ice1-2* seeds, although this dormancy was alleviated significantly faster than wild-type by cold stratification. The dormancy phenotype of *ice1-2* seeds matured at 10°C was similar to that displayed by the *CBF*-deficient seeds (Figure 4.2). This result suggests that ICE1 could play a role in regulating dormancy levels, which could be independent of the CBFs.

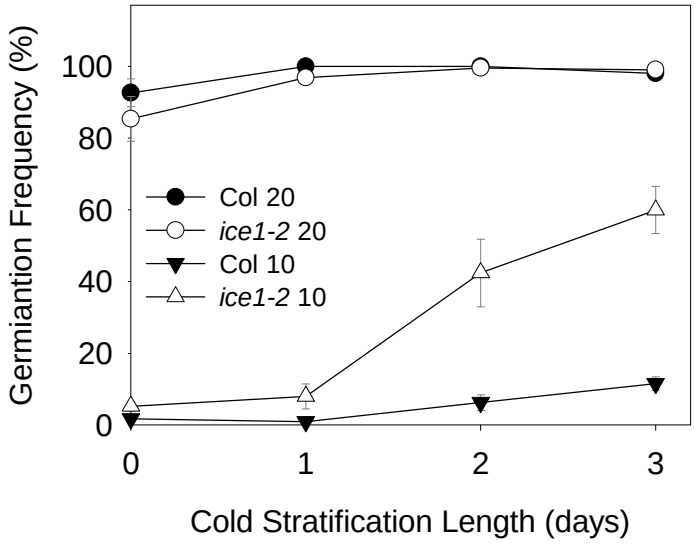

Figure 4.14: Germination of *ice1-2* seeds.
Freshly harvested seeds matured at 20°C or 10°C were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

4.3 Discussion

4.3.1 CBFs are not involved in temperature regulation of dormancy

In this chapter the dormancy phenotypes of *CBF* loss-and gain-of-function seeds and *ice1-2* mutant seeds have been analysed. The results show that there is a requirement for *CBF* expression to induce high dormancy levels in response to low temperature (15°C) (Figure 4.2). However, when this temperature was reduced to 10°C, high dormancy levels could be induced in *CBF*-deficient lines, suggesting *CBF*-independent mechanisms are also involved. Additionally, no increase in expression of *CBF1* was observed in response to low temperature in developing seeds (Figure 4.10A).

As described in section 1.1.2, post-translational regulation is important for correct employment of the cold acclimation pathway. Although not characterised in vegetative tissues, it is possible that mechanisms of post-transcriptional regulation such as alternative splicing, RNA processing and RNA silencing and post-translational control based on phosphorylation, ubiquitination and sumoylation which modify activity, sub cellular localisation and stability of proteins may be important for controlling the effect of CBFs in seeds. Therefore, although *CBF* expression is unaltered by temperature in seeds, temperature may still be involved in regulating CBFs in seeds.

The dormancy phenotype of *ice1-2* mutant seeds which were matured at 10°C was analysed and this mutant showed a dormancy phenotype which was similar to the *CBF*-deficient dormancy phenotype of seeds matured at 15°C (Figure 4.14). This result suggests that ICE1 may have a role in regulating dormancy that is independent of the CBFs. The fact that the three CBFs could be acting redundantly cannot be ruled out and analysis of the triple mutant would be required to determine this. The creation of

this triple mutant would be difficult given that the three *CBFs* are tandemly linked in a 10kb region of chromosome four.

There is a large number of additional components of the cold acclimation pathway (MYB15, SIZ1, ZAT10, ZAT12 and LOS2) (Section 1.1.3) whose dormancy levels have not been analysed but could potentially be important regulators of this pathway. An analysis of microarray data using the eFP browser (Bassel et al., 2008) shows that *ZAT10, ZAT12* and *LOS2* are expressed during seed development and expression of *ZAT10* peaks during seed maturation (the time at which dormancy is being induced) (Figure 4.15). In contrast *SIZ1* and *MYB15* are not expressed to high levels during seed development, although it is noteworthy that *SIZ1* expression does peak in dry seeds. The plants for these experiments were grown at 23°C and so it is possible that the expression patterns could be very different when plants are grown at lower temperatures. However, the results presented in this chapter show that the expression of *CBF1* is not upregulated in developing seeds matured at low temperature, thus suggesting prolonged cool temperatures do not lead to an increase in *CBF* transcripts. Therefore, it is also possible that these cold acclimation components may not be regulated by temperature in seeds.

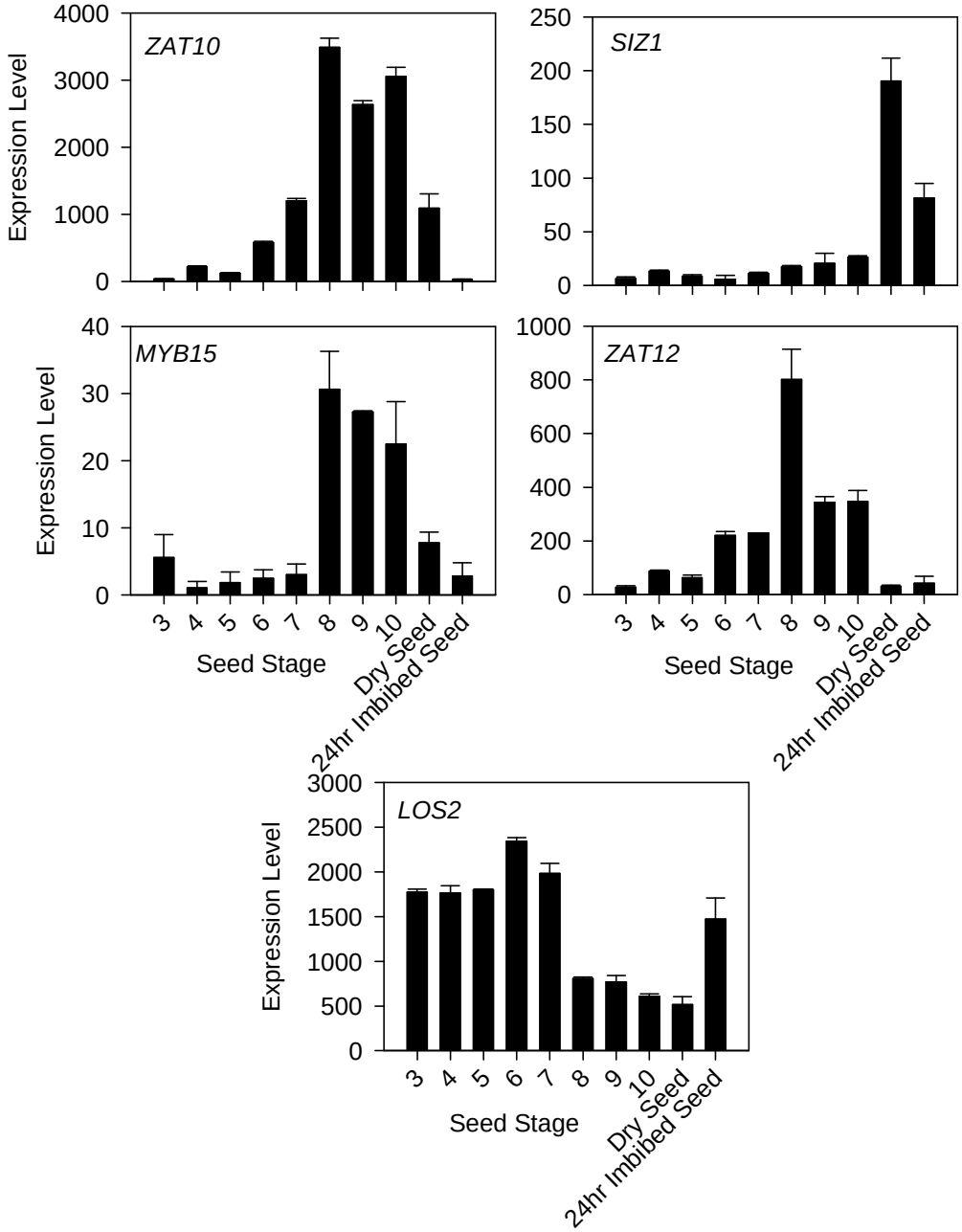

Figure 4.15: Expression of genes involved in cold acclimation in developing seeds.
Expression of *MYB15, SIZ1, ZAT10, ZAT12* and *LOS2.* Created using publicly available data from the eFP browser (Bassel et al., 2008). Seed stage numbers represent torpedo (3) through to green cotyledon (10) stage seeds.

Additionally, the dormancy phenotypes of *cor* mutants in response to different maturation temperatures could also be tested to determine if they had a role in regulating dormancy in response to temperature. Expression of *COR15a, COR15b* and *COR47* in seeds is not high in comparison to other areas of the plant (Figure 4.16). In contrast, expression of *COR78* is relatively high in walking stick stage seeds. Therefore, there are a number of cold acclimation pathway mutants whose dormancy phenotypes could be analysed in the future to potentially increase the understanding of the way in which temperature regulates dormancy.

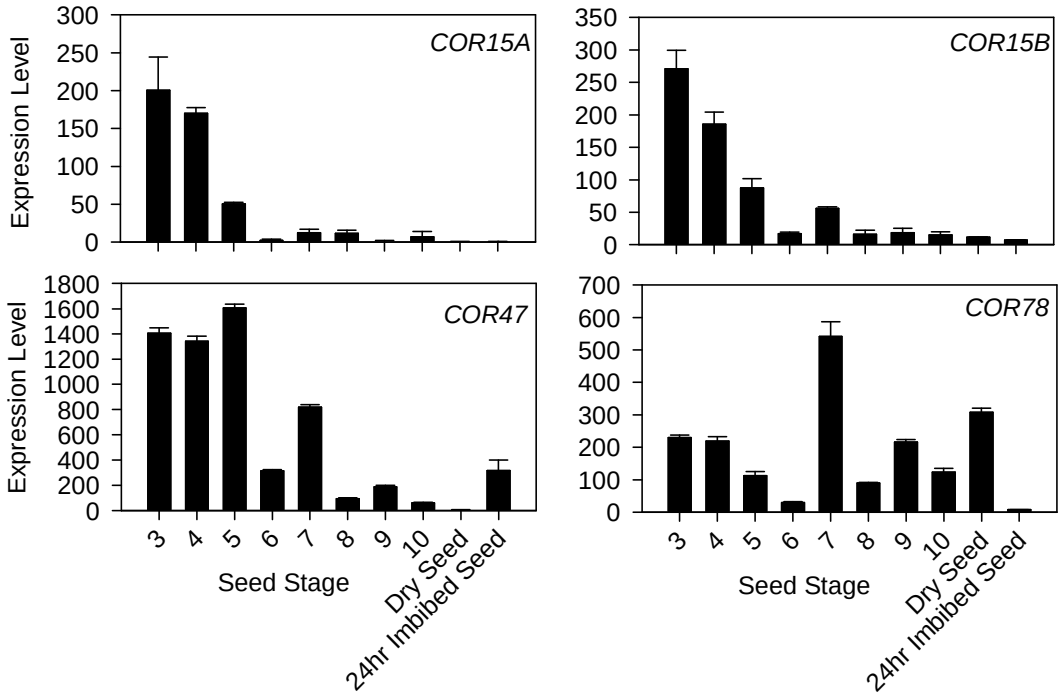

Figure 4.16: Expression of *COR* genes in developing seeds.
Expression of *COR15A, COR15B, COR47* and *COR78.* Created using publicly available data from the eFP browser (Bassel et al., 2008). Seed stage numbers represent torpedo (3) through to green cotyledon (10) stage seeds.

4.3.2 *CBF* expression is not promoted by low temperature in imbibed seeds

CBF expression has been shown to increase transiently in response to low temperature in vegetative tissues (Gilmour et al., 1998, Liu et al., 1998). Exposure of imbibed seeds to cold stratification breaks dormancy and, therefore, promotes germination in *Arabidopsis*. Given that *CBF*-overexpression leads to an increase in dormancy levels (Figure 4.5), it was not surprising that the expression of *CBF1* in imbibed seeds did not increase in response to low temperature. For this reason, the upregulation of *CBF1* expression in response to cold must be different in seedlings than the regulation that occurs in seeds. Although it has been shown that overexpression of *CBF1* in vegetative tissues leads to a decrease in bioactive GA levels (Achard et al., 2008), no decrease in GA levels was observed in freshly harvested dry seeds (Figure 4.9B). However, it could be possible that the overexpression of *CBFs* could lead to a decrease in GA levels in imbibed seeds, which was not measured in this study.

Therefore, it may be hypothesised that the repression of *CBF* expression in imbibed seeds is required for the promotion of germination by cold stratification, which requires an increase in GA levels (Yamauchi et al., 2004). This leads to the proposal that a seed specific repressor must be involved in preventing *CBF* expression in response to low temperature during seed imbibition. Perhaps the partial repression of *CBF* expression in response to cold during seed development represents the initial steps of this mechanism.

In contrast to *Arabidopsis,* the germination and establishment of seeds of some species, such as maize and soybean is chilling sensitive (Guan et al., 2009). Homologues of the *Arabidopsis CBFs* have been isolated in maize. The expression of

ZmDREB1A, ZmCBF3 and *ZmDBP3* is upregulated in response to low temperature (Qin et al., 2004, Wang et al., 2008, Wang and Dong, 2009). *ZmDREB1A* and *ZmCBF3* encode the same protein however they have different 3' Untranslated Regions (UTRs) (Wang et al., 2008). *ZmCBF3,* like the *Arabidopsis CBFs* is expressed throughout seed development, peaking at twenty three days post pollination (Wang et al., 2008). Additionally, *CBF* homologues have been isolated in soybean and their expression is also induced by low temperature (Li et al., 2005, Chen et al., 2009). It would be interesting to determine whether expression of these *CBF* homologues in response to low temperature differs in maize seeds from *Arabidopsis* seeds*;* i.e. are the *CBF* homologues upregulated in response to low temperature in imbibed seeds and could this upregulation explain why the germination is chilling sensitive in these seeds? Understanding how low temperature represses germination in these species will be important for future improvements in crop establishment.

4.3.3 ABA and GA levels do not correlate with dormancy levels in *CBF*-deficient and overexpressing seeds

GA and ABA levels were measured in *CBF*-deficient seeds and the results show that GA levels are unchanged in the *CBF1* and *CBF3* RNAi seeds, whilst levels are elevated in *cbf2* mutant seeds (Figure 4.4B, 4.9B). The increased level of GA in *cbf2* seeds is coupled with reduced expression of *GA2ox6*, a GA catabolic enzyme, in comparison to wild-type (Figure 4.3B). However, expression of *GA2ox6* and *GA2ox2* was also reduced in *CBF1* and *CBF3* RNAi seeds in comparison to wild-type (Figure 4.3B, C). Therefore, another level of regulation must be important for controlling GA levels in addition to *GA2ox* expression and subsequent catabolism of GA. It is possible that there is a decrease in DELLA protein levels via the ubiquitin-proteasome pathway

in response to the increased GA levels in the *cbf2* mutant, and so this may in turn effect the expression of additional GA metabolic genes which are regulated by DELLAs. It would be useful to characterise expression of the *DELLA* genes and determine DELLA protein levels in the *cbf* mutant seeds.

ABA levels are also elevated in the *CBF*-deficient seeds (Figure 4.4A), which is surprising given their reduced dormancy phenotype and the fact that there are only small changes to expression of *CYP707A2* and *NCED4* in these seeds (Figure 4.3D, E). It is interesting that the expression of both *NCED4* and *CYP707A2* is slightly downregulated in the *CBF*-deficient seeds, since these genes contribute to opposite regulation of ABA levels. *NCED4* promotes ABA synthesis, whilst *CYP707A2* promotes ABA catabolism.

However, as mentioned earlier, the dormancy phenotype of the *CBF*-deficient seeds that were matured at 10°C was not as strong as when the seeds were matured at 15°C (Figure 4.2). The dormancy phenotypes at 15°C and 10°C were observed in three independent experiments and are therefore, robust phenotypes, accurately representing the biology. The transcript and hormone measurements were carried out on seeds matured at 10°C and as a consequence these experiments may have been more informative if the seeds had been matured at 15°C. When seeds were matured at 10°C, only *cbf2* mutant seeds germinated to higher levels than wild-type, and so it may be unsurprising that levels of ABA are elevated in comparison to wild-type (Figure 4.4A). One would predict that if this analysis was repeated with seeds that were matured at 15°C, ABA levels would be reduced in comparison to what is observed in *CBF*-deficient seeds matured at 10°C. This may also explain why differences in transcription of genes known to be involved in dormancy are not observed in the *CBF*-

deficient seeds that were matured at 10°C. It is also possible that the dry seed stage which was selected for the hormone measurements and transcriptional analysis was the incorrect stage and so, if the analysis was repeated on developing seeds, more information may be gained.

ABA and GA levels were also measured in the *CBF*-overexpressing seeds, and although these seeds display the opposite dormancy phenotype to the *CBF*-deficient seeds (Figure 4.5), the opposite levels of these hormones was not observed (Figure 4.9). However, it is possible that if ABA and GA measurements had been made in imbibed seeds then levels may have correlated with the dormancy levels that are observed. *CBF*-overexpressing seeds also displayed elevated levels of ABA and this was surprisingly coupled with an increase in expression of *CYP707A2* (Figure 4.8D) and no change in expression of *NCED4* (Figure 4.8E). Therefore, the alteration in expression of *CYP707A2* in the *CBF*-overexpressing seeds would be predicted to lead to a decrease in ABA levels, which is the opposite to what is observed.

The expression of *NCED4* and *CYP707A2* does not appear to correlate with the dormancy levels which are induced in *CBF*-overexpressing seeds and the increase in ABA levels. However, it is possible that a range of post-transcriptional and post-translational mechanisms could also be important for modulating NCED4 and CYP707A2 protein abundance and function. The regulation of ABA metabolism has been studied mostly at the transcriptional level (Nambara and Marion-Poll 2005), although mechanisms of post-transcriptional and post-translational regulation have been identified. Genetic analysis of the *supersensitive to ABA and drought1* (*sad1*) mutant indicated that ABA biosynthesis is controlled at the level of mRNA stability (Xiong et al., 2001, Nambara and Marion-Poll 2005). *SAD1* encodes a peptide which is

required for mRNA processing (Xiong et al., 2001). At the post-translational level, ABI5 protein accumulation, phosphorylation, stability and activity is regulated by ABA during germination (Lopez-Molina et al., 2001).

Levels of *GA2ox3* expression were upregulated in *CBF*-overexpressing seeds, although there was no difference in GA levels for *CBF1* and *CBF2*-overexpressing seeds and an increase in GA levels in *CBF3*-overexpressing seeds (Figure 4.8F, 4.9B). Therefore, the regulation of *GA2ox3* expression is not important for the regulation of GA levels in *CBF*-overexpressing dry seeds. The expression of *GA2ox6* was reduced in *CBF*-overexpressing seeds in comparison to wild-type; whilst the expression of *GA2ox2* was slightly upregulated in the *CBF*-overexpressing seeds in comparison to wild-type (Figure 48B, 4.8C). Again, the post-translational regulation of DELLA proteins by the ubiquitin-proteasome pathway could play a role in controlling GA metabolic gene expression, and alterations to some of the genes tested here could be a result of altered DELLA protein levels.

Therefore, it appears that the regulation of ABA and GA levels in the *CBF*-deficient and overexpressing seeds by genes involved in hormone metabolism is complex, and suggests that post-transcriptional and post-translational mechanisms could also be important. The levels of ABA and GA in the *CBF*-deficient and overexpressing seeds do not correlate with the dormancy levels, suggesting that hormone independent pathways will also be important for the regulation of dormancy.

4.3.4 Fatty acid composition is altered by maturation temperature

The results presented in this chapter have shown that the fatty acid composition of seeds is altered when the maturation temperature is reduced from 22°C to 15°C (Figure 4.12, 4.13). There were a number of changes to the amounts of individual fatty acids in response to maturation temperature in wild-type seeds (Figure 4.13A, C). However, a number of these changes to individual fatty acid levels were altered in the *CBF3*-overexpressing and *CBF1* RNAi seeds (Figure 4.13B, D). Although levels of 18:1n9C remained similar between the two maturation temperatures for wild-type seeds, levels of this fatty acid were altered in the *CBF* loss-and-gain-of-function seeds (Figure 4.13). A decrease in oleic acid (18.1) in response to a reduction in temperature has been described in soybean (Lanna et al., 2005). Increases in polyunsaturated fatty acids (PUFAs) have been linked with decreases in temperature in soybean, rape and sunflower (Werteker et al., 2010). In the Werteker et al (2010) study, a link is made between changes in linolenic acid (18.3) levels and temperature and this link is stronger in rape than in soybean, which they suggest explains why rape is better adapted to cold climates than soybean. In contrast, the results presented in this chapter show the opposite, in that levels of linolenic acid increase with a reduction in temperature.

It is possible that seeds which have higher dormancy levels may require increased storage reserves and so this may explain why total fatty acid levels increase in seeds matured at low temperature (Figure 4.11). Long chain fatty acids have been shown to be inducible by ABA in *Brassica napus* (Finkelstein and Somerville, 1989). Results shown in chapter three demonstrate that ABA levels are higher in seeds matured at low temperature (Figure 3.7A) and, therefore, this could represent an important mechanism for increasing fatty acid levels in the seed.

Microarray studies investigating the low temperature transcriptome in seeds showed that *FATTY ACID DESATURASE3* (*FAD3*) is upregulated in seeds matured at low temperature in comparison to warm temperature (Kendall et al., 2011). FAD3 is a desaturase that converts linoleic acid to linolenic acid by inserting a double bond at the ω-3 position (Zhang et al., 2012). Results in this chapter show that levels of linolenic acid (18:3n3) are higher in low temperature matured seeds, but this was not coupled with a decrease in the levels of the precursor, linoleic acid (18.2n6c) (Figure 4.12). As a consequence, it may be that the increase in *FAD3* expression at low temperatures is responsible for the increased conversion of linoleic acid to linolenic acid observed and that the increased linolenic acid is important for the altered dormancy. It would be interesting to test this hypothesis by analysing the dormancy levels of *fad3* loss-of-function mutants. Levels of eicosenoic acid (20:1n9), which is the major storage form of triglyceride, were higher in wild-type seeds which were matured at low temperature (Figure 4.12). Interestingly, *abi3* mutants have reduced levels of eicosenoic acid (Finkelstein and Somerville, 1990), yet *ABI3* is downregulated in low temperature matured seeds (Kendall et al., 2011), thus suggesting that ABI3 cannot be responsible for the increased in eicosenoic acid levels observed here.

4.3.5 Conclusions

The results discussed in this chapter contribute to the understanding of how low temperature during seed maturation contributes to increasing dormancy levels. Functional expression of the *CBFs* is required for the induction of normal dormancy levels, but when the maturation temperature is dropped considerably, a CBF-independent mechanism is also involved. The transcriptional analysis in *CBF*-loss and gain-of function seeds suggests that although they have opposite dormancy

phenotypes, the processes that are controlling these differences are caused by different pathways. The results also suggest that an increase in storage reserves may be another consequence of reduced maturation temperature, and that the CBFs may have a role in regulating the fatty acid composition of the seed.

Chapter 5 The role of HOS1 in temperature regulation of seed dormancy

5.1 Introduction

Strong dormancy levels could be induced in *CBF*-deficient seeds matured at 10°C (Figure 4.2), and so the search for cold acclimation pathway components that have a role in the regulation of dormancy by temperature was extended. This chapter will focus on the characterisation of the dormancy phenotype of *hos1* mutants in response to different maturation temperatures and experiments designed to understand the mechanism by which HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1 (HOS1) regulates dormancy. The *hos1* mutant was selected for investigation based on its involvement in temperature signalling and the regulation of flowering time. The role of HOS1 in these processes and additional evidence suggesting that HOS1 may act within the nuclear pore complex (NPC) will be discussed in this introduction.

HOS1 is an E3 ligase that targets ICE1, the positive regulator of *CBF3* expression for ubiquitination and is consequently, a negative regulator or cold acclimation (Figure 5.1) (Lee et al., 2001, Chinnusamy et al., 2003, Dong et al., 2006a). *hos1* mutants show elevated levels of *CBF* expression and as a consequence also display elevated levels of expression of CBF target genes (Lee et al., 2001). Although HOS1 was originally identified as a regulator of cold acclimation, the mutant also displays a flowering time phenotype which has recently been investigated in more detail (Ishitani et al., 1998, Lee et al., 2001, Lazaro et al., 2012).

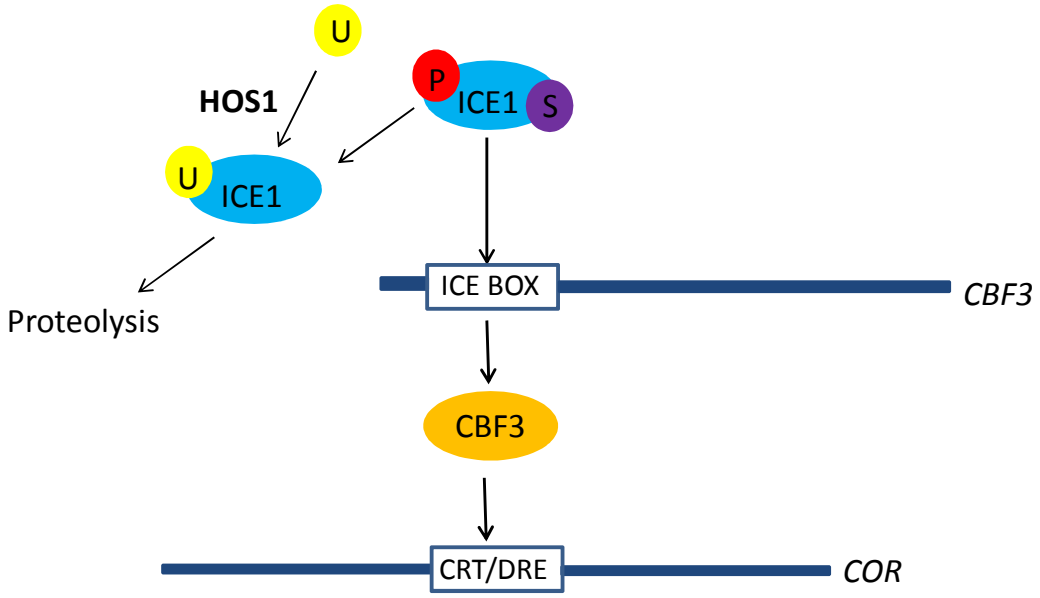

Figure 5.1: Schematic of HOS1 post-translational regulation of ICE1.
Phosphorylated and sumoylated ICE1 is targeted for proteolysis following ubiquitination by the E3 ligase HOS1. The consequence of this is that ICE1 can no longer promote expression of *CBF3*, which therefore, prevents expression of the *COR* genes. P, phosphorylation; U, ubiquitination; S, sumoylation. Adapted from Chinnusamy et al., (2007).

Lazaro et al., (2012) propose a role for HOS1 in the integration of temperature and photoperiod signals to regulate flowering time. The early flowering phenotype of *hos1* plants has been linked to decreased expression of *FLC* (Lee et al., 2001, Lazaro et al., 2012). However, *hos1-2* mutant plants do flower earlier in an *flc*-deficient background, suggesting that a FLC-independent mechanism must also be important (Lazaro et al., 2012). The early flowering phenotype of *hos1-2* requires functional *CONSTANS* (*CO*), *SOC1* and *FT* expression (Lazaro et al., 2012).

The effect of the *hos1-2* mutation on the expression of the flowering time genes *FT* and *CO* revealed that HOS1 is required to repress morning expression of *FT* during long days, whereas HOS1 does not appear to be an important regulator of *CO* expression

(Lazaro et al., 2012). HOS1 interacts with CO *in vivo* and is thought to regulate *FT* expression through its modulation of CO protein levels (Lazaro et al., 2012).

Another recent study has proposed a role for HOS1 as a component of the NPC (Tamura et al., 2010). The NPC is a large protein complex, comprising at least thirty nucleoporins, which spans the nuclear envelope and is responsible for mediating the traffic of molecules in and out of the nucleus (Eckardt, 2010). In the Tamura et al., (2010) study, a proteomic analysis using GFP-tagged nucleoporins showed that HOS1 immunoprecipitated with two nucleoporin constructs; RNA EXPORT FACTOR1 (RAE1)-GFP and Nup43-GFP (Tamura et al., 2010). RAE1-GFP and Nup43-GFP coimmunoprecipitate with at least thirteen nucleoporins each, indicating that both of these proteins are components of the *Arabidopsis* NPC (Tamura et al., 2010).

The HOS1 protein contains a region with homology to the vertebrate nucleoporin EMBRYONIC LARGE MOLECULE DERIVED FROM YOLK SAC (Elys) (Tamura et al., 2010), which is involved in initiating and targeting nuclear pore assembly to the chromatin (Rasala et al., 2006). ELYS depletion in *Xenopus* egg extracts leads to a strong reduction in chromatin binding by a large number of nucleoporins (Davis and Blobel, 1987, Meier et al., 1995, Walther et al., 2002). This suggests that ELYS is required for the association of chromatin with the Nup107-160 complex and for correct NPC formation (Gillespie et al., 2007). ELYS depleted extracts contained smaller nuclei than the control extract and DNA replication is abolished (Gillespie et al., 2007). ELYS contains an AT hook DNA binding domain which is known to mediate a protein's direct interaction with DNA or chromatin (Reeves and Nissen, 1990, Metcalf and Wassarman, 2006). This AT hook domain allows ELYS to directly interact with chromatin (Gillespie et al., 2007, Rasala et al., 2008), however a second chromatin binding domain is also

present (Rasala et al., 2008). This AT hook domain is not present in the HOS1 protein, yet it is possible that HOS1 could share homology with the so far uncharacterised second chromatin binding domain of the ELYS protein.

5.2 Results

5.2.1 Characterisation of dormancy phenotypes of *hos1* mutants

5.2.1.1 Characterisation of mutant alleles in the Col background

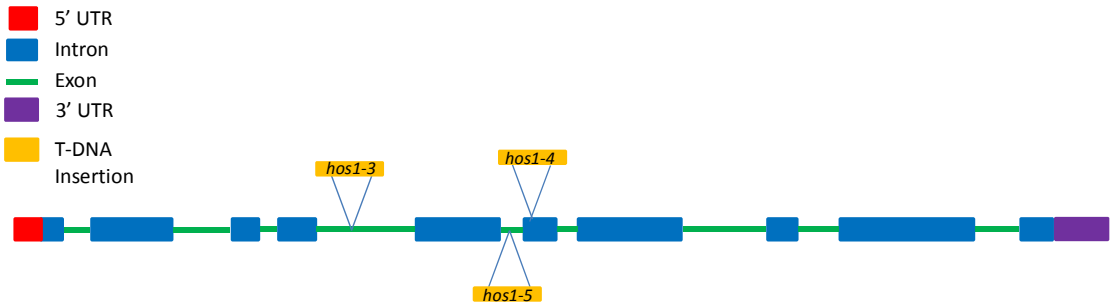

Figure 5.2: Location of the three T-DNA inserts in HOS1.
Location of the T-DNA inserts in *hos1-3, hos1-4* and *hos1-5* as hypothesised using Seqview.

To investigate whether HOS1 has a role in regulating dormancy in response to low temperature, three T-DNA insertion mutants, which were named *hos1-3* (SALK_069312), *hos1-4* (SALK_131629) and *hos1-5* (SALK_052108) (Figure 5.2), were exposed to maturation temperatures of 20°C, 17°C and 15°C. Dormancy levels were low in seeds matured at 20°C for both wild-type and the three *hos1* mutant alleles (Figure 5.3A). When the maturation temperature was reduced to 17°C, dormancy was induced in wild-type seeds however; no dormancy was induced in *hos1* seeds (Figure 5.3B). When seeds were matured at 15°C, significantly lower dormancy levels were induced in *hos1* seeds in comparison to wild-type (Figure 5.3C). Even when the maturation temperature was reduced to 10°C a complete lack of dormancy was still observed in *hos1-3* seeds (Figure 5.4). Therefore, seeds lacking functional *HOS1* gene expression are incapable of inducing dormancy levels in response to low temperature.

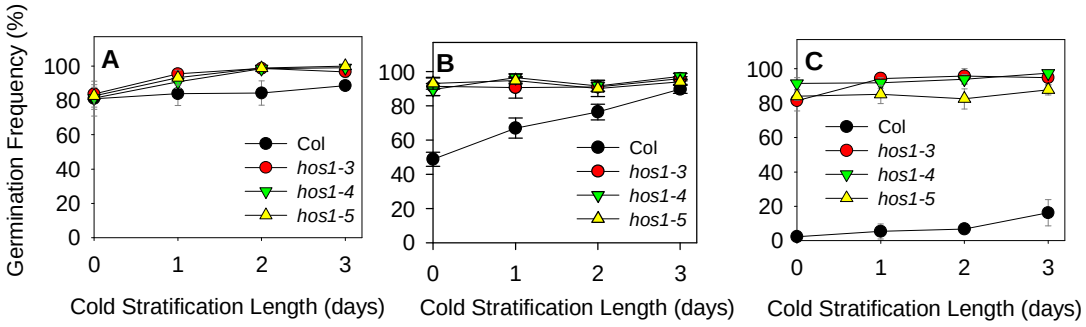

Figure 5.3: Germination of *hos1* seeds.
Freshly harvested seeds matured at 20°C (A), 17°C (B) or 15°C (C) were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

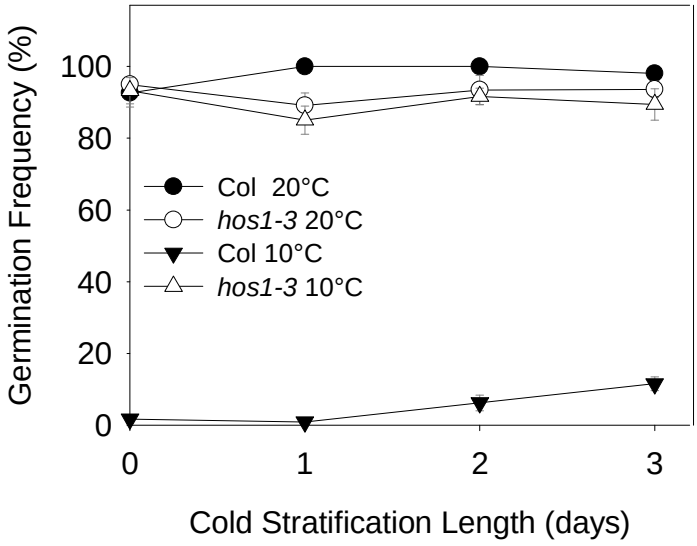

Figure 5.4: Germination of *hos1-3* seeds.
Freshly harvested seeds matured at 20°C and 10°C were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

5.2.1.2 Characterisation of mutant allele in the C24 background

It was possible that the reduced dormancy phenotype of *hos1* mutants was not a specific response to temperature and instead a more general effect on dormancy. To investigate this, the dormancy levels of *hos1-1* mutant seeds, which are in the C24 background was analysed. Unlike Col and L*er* seeds, dormancy is induced when C24 seeds are matured at warm temperatures and so *hos1-1* and C24 seeds were matured at 20°. High dormancy levels were induced in C24 and this dormancy was alleviated when the seeds were cold stratified (Figure 5.5). In contrast, significantly lower dormancy levels were induced in *hos1-1* seeds. This result suggests that HOS1 must be involved in a general mechanism regulating seed dormancy and not specifically in response to temperature.

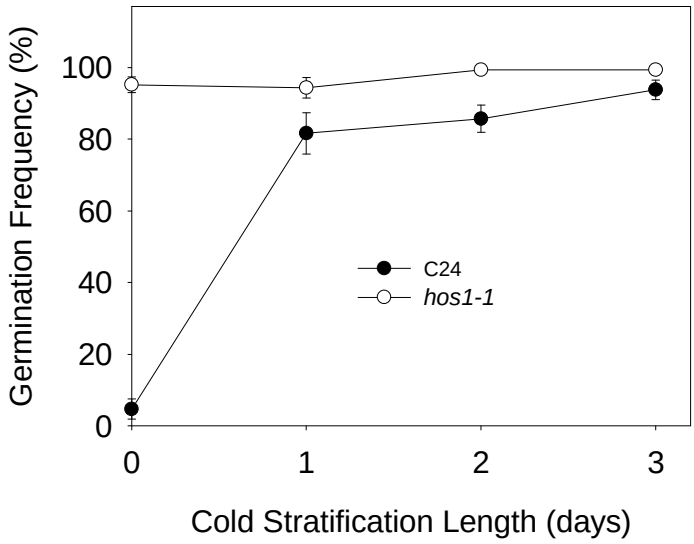

Figure 5.5: Germination of *hos1-1* seeds.
Freshly harvested seeds matured at 20°C were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

5.2.2 Expression of *HOS1* in response to different maturation temperatures

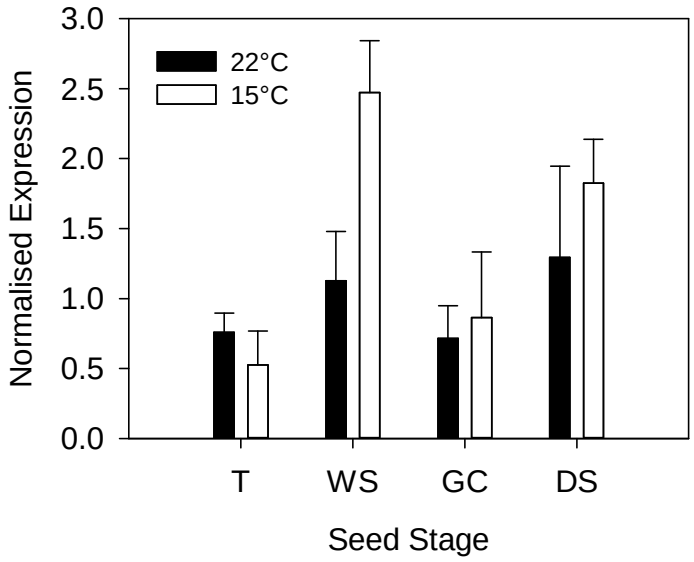

Figure 5.6: Expression of *HOS1* in developing seeds.
Seeds were matured at 22°C and 15°C. Developmental stages are Torpedo (T), Walking Stick (WS), Green Cotyledon (GC) and Dry Seed (DS). Data points are the average of two biological replicates and expression is normalised to the average of two control genes *ACTIN2* and *AT3G06240*. Error bars represent standard error.

The expression of *CBF1* does not increase in response to low temperature in developing seeds (Figure 4.10A). To test whether *HOS1* expression is temperature regulated in developing seeds, Real-Time PCR was used to analyse *HOS1* expression at two maturation temperatures (22°C and 15°C) in Col torpedo, walking stick, green cotyledon and dry stage seeds, which were harvested five hours after dawn. *HOS1* expression appears to be higher in walking stick stage seeds matured at 15°C in comparison to 22°C (Figure 5.6). Seeds sampled at other stages did not show large differences in *HOS1* expression between the two temperatures. This suggests that *HOS1* expression at walking stick stage is altered by temperature, but since the low

dormancy phenotype is not specific to low temperatures (Figure 5.5) it is unlikely that this is important in the dormancy regulation.

5.2.3 Characterisation of dormancy phenotypes of seeds overexpressing *HOS1*

To understand the role of HOS1 in regulating dormancy further, the effect of overexpression of *HOS1* on dormancy levels was investigated. Using a number of lines overexpressing *HOS1* with a translational fusion to Cyan Fluorescent Protein (CFP) in a *hos1-3* background (Dana MacGregor and Susannah Bird, Penfield Lab), an analysis was carried out to determine if the mutant dormancy phenotype could be rescued. When the seeds were matured at 20°C, dormancy levels were low in all of the overexpressing lines and wild-type (Figure 5.7A). Line 3.2 did show slightly higher dormancy levels than the other lines, but the difference was not significant following cold stratification. When the seeds were matured at 15°C, the low dormancy phenotype of *hos1-3* could be confirmed, whereas high dormancy levels were induced in wild-type (Figure 5.7B). In contrast, seeds overexpressing *HOS1* had intermediate dormancy levels. Following cold stratification, lines 4.5, 4.6 and 5.1 and 5.5 germinated to significantly higher levels than wild-type, whereas germination of 3.2 and 5.6 were not significantly different to wild-type. This result suggests that the overexpression of *HOS1* in the *hos1-3* background leads to partial complementation of the dormancy phenotype. The fact that there was only partial complementation could be due to an indirect effect of the CFP tag that is also present in this line.

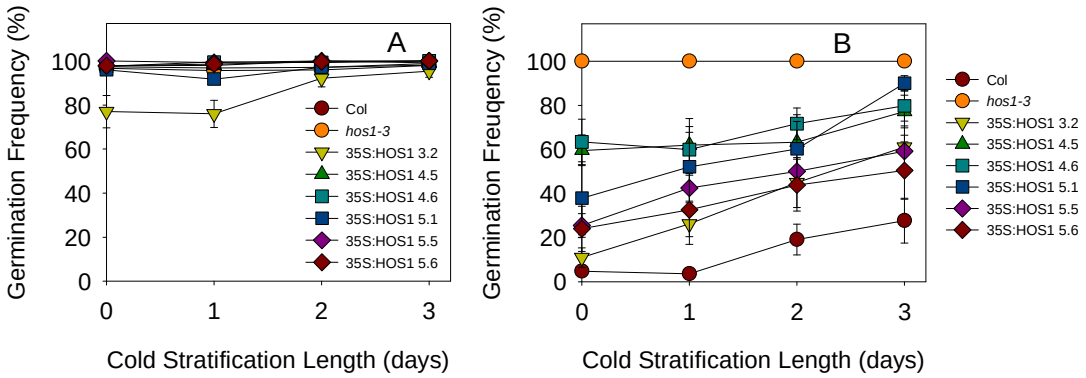

Figure 5.7: Germination of *HOS1*-overexpressing seeds.
Freshly harvested seeds matured at 20°C (A) or 15°C (B) were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

5.2.4 Characterisation of the maternal effect of HOS1 on dormancy

The *Arabidopsis* seed is made up of both maternal and zygotic structures and, therefore, it is possible that the effect of HOS1 on dormancy could be through a maternal or zygotic pathway. Maternal signals are known to control dormancy levels, for example the photoperiod experienced by plants during the vegetative phase has an effect on the levels of dormancy that are induced (Penfield lab, unpublished) and in this example, the maternal signal must come from a vegetative tissue and not a seed tissue.

To test whether HOS1 is acting through a maternal pathway, reciprocal crosses were made between *hos1-3* and Col and the F_1 seeds were matured at 15°C. The dormancy of freshly harvested F_1 seeds was then analysed. When *hos1-3* was crossed with Col pollen, the germination was significantly higher than wild-type, suggesting that the

hos1-3 mutation could be acting in a dominant or maternal manner (Figure 5.8). However, when Col was crossed with *hos1-3* pollen, dormancy levels were high and this was similar to what was observed for homozygous Col seeds (Figure 5.8), thus suggesting that the effect of the *hos1-3* mutation was recessive. Therefore, the results from the analysis of the dormancy of the seeds from the reciprocal crosses suggest that HOS1 effects seed dormancy through a maternal pathway.

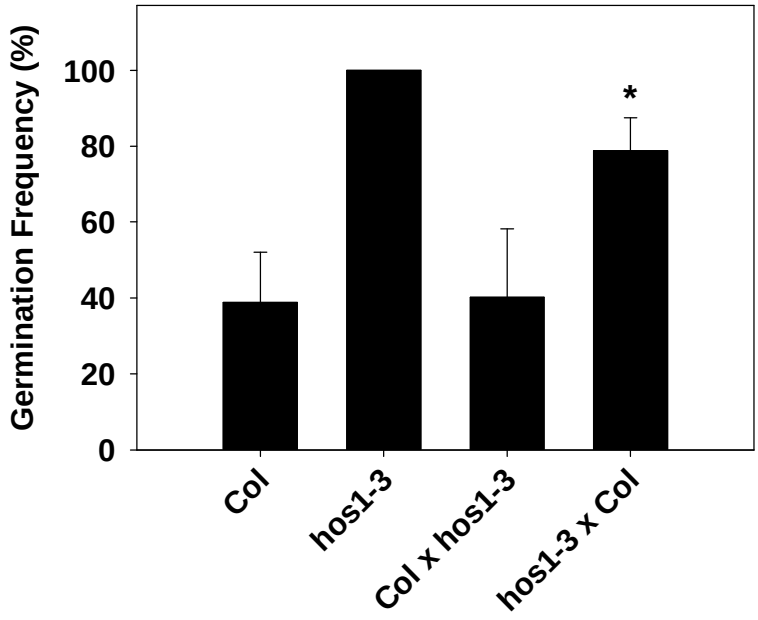

Figure 5.8: Germination of seeds from F_1 reciprocal crosses between *hos1-3* and Col.
Freshly harvested F_1 seeds matured at 15°C were germination for seven days at 22°C in twelve hour white light/dark cycles. Germination was scored as radical protrusion. Data points are the average of seeds from five independent crosses and error bars represent standard error. * Indicates significant difference to wild-type when P≤0.05 by students t-test.

5.2.5 Characterisation of dormancy phenotypes of HOS1 interacting proteins

A yeast two-hybrid screen identified potential proteins that interact with HOS1 (Dana MacGregor, unpublished). These include ABRE BINDING FACTOR1 (ABF1), ABF3 and an unknown protein which was named HOS1 INTERACTING PROTEIN1 (HIP1). ABF1 and ABF3 share overlapping effects to ABI5 and these three proteins antagonistically regulate each other's expression (Finkelstein et al., 2005). To investigate whether any of these proteins that interact with HOS1 are involved with HOS1 in the regulation of dormancy, mutants for these proteins were included in the low seed maturation temperature screen. The hypothesis that was being tested was that if HOS1 regulates dormancy through these proteins then they too would display dormancy phenotypes.

When the seeds were matured at 20°C, some dormancy was induced in wild-type, but this was quickly reduced when the seeds were cold stratified (Figure 5.9A). A number of the mutants showed higher initial dormancy levels, but these differences were not significant when compared to wild-type and, again cold stratification led to a large increase in germination. When the seeds were matured at 15°C, initial results following short cold stratification lengths revealed no apparent phenotype for the mutant seeds, with dormancy levels appearing similar for all the *hip* and *abf* mutant lines (Figure 5.9B). This cold stratification time course was extended to four weeks to see whether any phenotypes were revealed for *hip1-1, hip1-2, hip1-3, hip1-4, abf1-1* and *abf3-1* during this prolonged cold. The responses to this extended cold stratification were more varied, with *hip1-1* entering secondary dormancy significantly quicker than wild-type (Figure 5.9B). The three other *hip1* alleles showed high secondary dormancy following

twenty eight days of cold stratification, but this was only significantly different to wild-type for *hip1-2* and *hip1-4*. The secondary dormancy levels in the *abf1-1* and *abf3-1* mutants showed no significant difference to wild-type. Although differences are apparent in response to extended periods of cold, there is no real apparent difference in primary dormancy levels, thus suggesting that HOS1 does not act through HIPs and ABFs to regulate dormancy in response to low temperature.

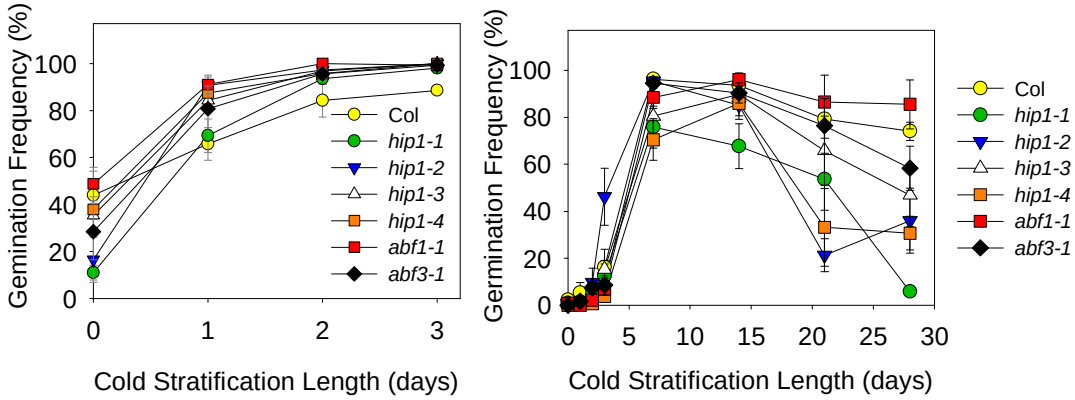

Figure 5.9: Germination of *hip1*, *abf1* and *abf3* seeds.
Freshly harvested seeds matured at 20°C (A) or 15°C (B) were cold stratified for up to three days (A) or twenty eight days (B). Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

5.2.6 Measurement of ABA and GA levels

Results presented in chapter three showed that ABA and GA levels were temperature regulated in seeds (Figure 3.7) and these levels were positively and negatively correlated with dormancy levels respectively. Therefore, it was hypothesised that *hos1* mutant seeds may contain reduced levels of ABA and increased levels of GA, which would contribute to the low dormancy phenotype. To investigate whether ABA or GA levels are altered in *hos1* seeds levels were measured in freshly harvested dry seeds.

Levels of GA were significantly higher in *hos1* seeds in comparison to wild-type (Figure 5.10A). *hos1-5* seeds showed the lowest levels of GA out of the three mutant lines. ABA levels were found to be slightly higher in *hos1-3* and *hos1-4* seeds than wild-type and levels were significantly higher in *hos1-5* seeds (Figure 5.10B).

The increase in GA levels that is observed in *hos1* seeds correlates with low dormancy levels (Figure 5.10A, 5.3). In contrast, the increases in ABA levels were not expected since increased ABA levels do not correlate with low dormancy, although this may reflect a feedback loop between the two hormones. Together it seems that the low dormancy phenotype of *hos1* mutant seeds could be due to alterations in GA metabolism.

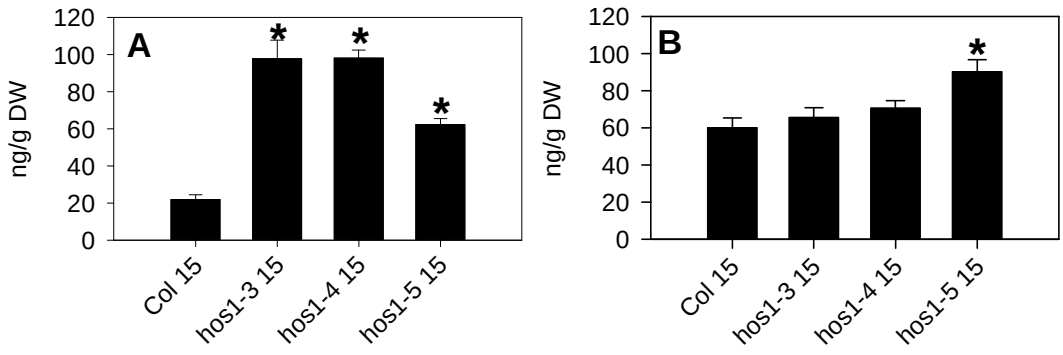

Figure 5.10: Measurement of ABA and GA levels in hos1 seeds.
GA (A) and ABA (B) measurements were made in freshly harvested dry seeds matured at 15°C. Data points are the average of five individual seed batches and error bars represent standard error. * Indicates significant difference to wild-type when P≤0.05 by students t-test.

5.2.7 Characterisation of sensitivity of *hos1* seeds to ABA and PAC

A number of low dormancy mutants, such as *dog1-2,* shows defects in ABA or GA sensitivity or signalling (Bentsink et al., 2006). Reduced sensitivity to ABA or increased sensitivity to GA could explain the low dormancy phenotype of *hos1* seeds. Therefore, to determine whether ABA and GA sensitivity is altered in *hos1* mutant seeds, seeds were germinated in the presence of increasing concentrations of ABA and PAC, the GA biosynthesis inhibitor. Germination of all three *hos1* mutant alleles in response to PAC was very similar to wild-type (Figure 5.11A). Similarly, the response of *hos1* seeds to ABA was comparable to wild-type, with the only exception being in response to 1µM ABA, where *hos1* seeds were mildly hyposensitive (Figure 5.11B). Therefore, it appears that defective ABA and GA sensitivity is not responsible for the lack of dormancy in *hos1* seeds.

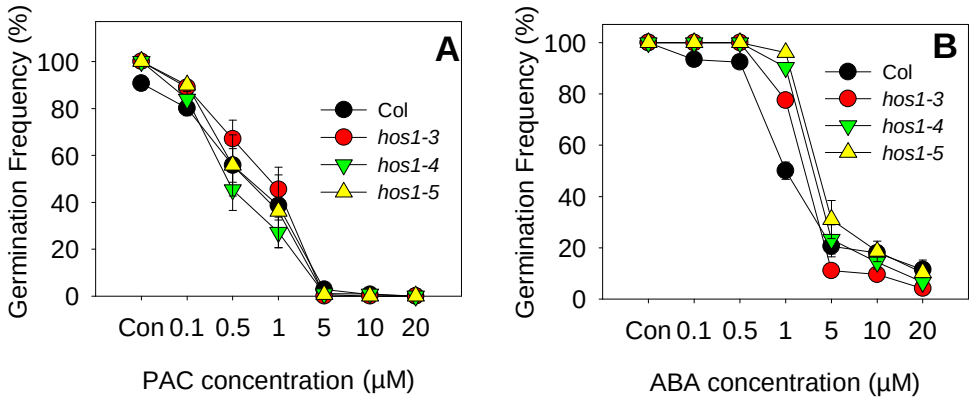

Figure 5.11: PAC and ABA sensitivity of *hos1* seeds.
Seeds matured at 20°C were after-ripened for approximately two months. Cold stratified seeds were germinated in the presence of increasing concentrations of PAC (A) and ABA (B) and germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

5.2.8 Characterisation of dormancy phenotypes of additional early flowering mutants

The dormancy process is believed to be an adaptive event which occurred late in evolution and, therefore, genes which are involved in other phase transitions, such a reproduction, may have been co-opted to regulate dormancy (Bassel et al., 2011). Flowering time and dormancy both require precise environmental sensing and responses to multiple seasonal cues so that developmental timing can be accurately matched to seasonal conditions (Chiang et al., 2009). A number of mutants which display flowering time phenotypes also exhibit altered dormancy phenotypes (Kurup et al., 2000, Gómez-Mena et al., 2001, Chiang et al., 2009, Penfield and Hall, 2009). For example, the *late elongated hypocotyl* (*lhy*) *circadian clock associated1* (*cca1*) double mutant has an early flowering phenotype and seeds display reduced dormancy (Penfield and Hall, 2009). Since *hos1* mutants display such a strong low dormancy phenotype (Figure 5.3) and are early flowering (Ishitani et al., 1998, Lee et al., 2001, Lazaro et al., 2012), an experiment was carried out to determine whether other early flowering mutants may also display a similar low dormancy phenotype.

To investigate this, the dormancy phenotypes of *arp6-1* and two *tfl2* mutant alleles, *tfl2-1* and *tfl2-2* were tested in response to maturation at 20°C and 15°C. These mutants were selected for this analysis given that they share a suite of phenotypes with *hos1* mutants, which include early flowering. *arp6* mutants display a number of constitutive warm phenotypes and these phenotypes are linked to the inability to remove H2A.Z deposition from the chromatin, which has an effect on the expression of the temperature transcriptome (Kumar and Wigge, 2010). The early flowering phenotype of *tfl2* is attributed to an increase in *FT* expression (Kotake et al., 2003). Increased *FT* expression is also seen in *hos1* seedlings (Lee et al., 2001, Lazaro et al., 2012).

Low dormancy levels were induced in *tfl2-1* and *tfl2-2* when matured at 20°C (Figure 5.12). When this temperature was reduced to 15°C, the *tfl2* mutants had a very similar striking low dormancy phenotype to *hos1* seeds, with seeds germinating significantly higher than wild-type. This result suggests that TFL2 is required for the induction of high dormancy levels. These results suggest that the low dormancy phenotype of *hos1* seeds is shared by *tfl2* mutant seeds.

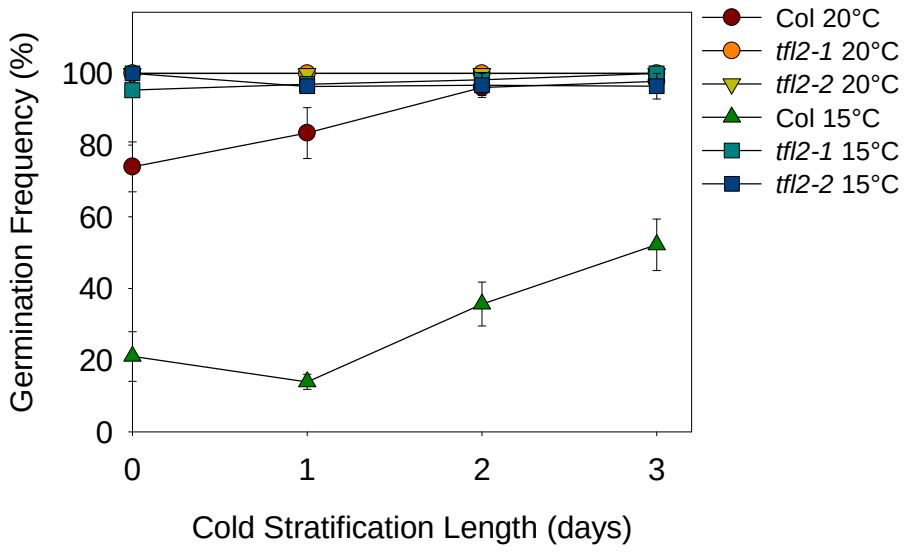

Figure 5.12: Germination of *tfl2* seeds.
Freshly harvested seeds matured at 20°C or 15°C were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

arp6-1 mutants also displayed low dormancy levels when matured at a warm temperature (Figure 5.13). The germination of *arp6-1* was slightly higher than wild-type. When the maturation temperature was reduced to 15°C dormancy was induced in *arp6-1* seeds, although germination of these seeds was higher than wild-type seeds. However, when *arp6-1* seeds were cold stratified, the dormancy was quickly reduced,

as seeds germinated to 100% following three days of cold stratification and this was significantly higher than wild-type. Therefore, this result suggests that *arp6-1* mutants also display reduced dormancy, but this phenotype is not as strong as the reduced dormancy phenotypes displayed by *hos1* and *tfl2* mutants.

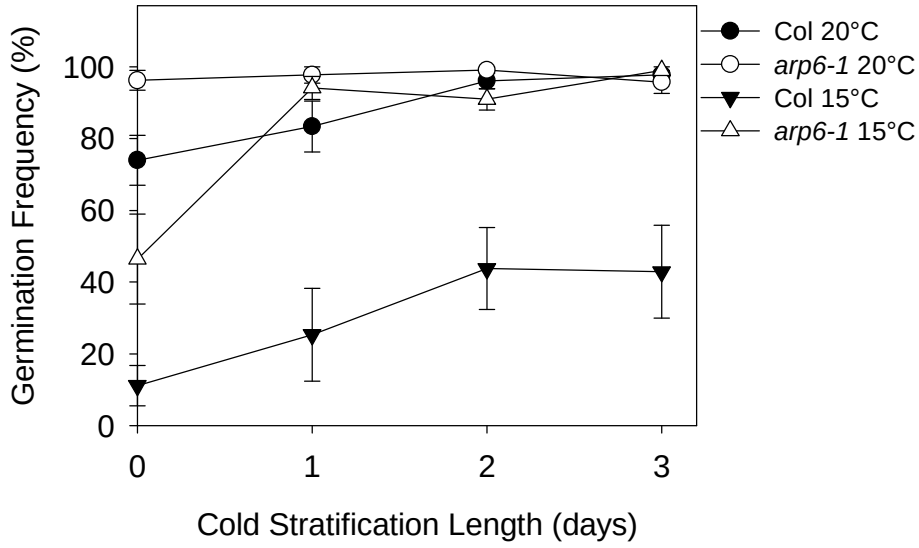

Figure 5.13: Germination of *arp6-1* seeds.
Freshly harvested seeds matured at 20°C or 15°C were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

5.2.9 Nuclear morphology as a regulator of dormancy

5.2.9.1 Characterisation of dormancy phenotypes of *linc1-2* seeds

A recent study has revealed a potential role for HOS1 as a component of the NPC (Tamura et al., 2010). LITTLE NUCLEI (LINC) are involved in regulating nuclear size and morphology and *linc1* and *linc2* nuclei are smaller than wild-type (Dittmer et al., 2007). Expression of *LINC1, LINC2* and *LINC3* is greater in dry seeds matured at 20°C

in comparison to those matured at 10°C (Kendall et al., 2011). A recent study has shown that dormancy levels are not correlated with nuclei size (van Zanten et al., 2011b). However, the effect of maturation temperature was not analysed in this study and so it is possible that temperature may have an effect on nuclear morphology.

Therefore, the ability to induce dormancy in *linc1-2* loss-of-function seeds was tested to determine whether dormancy induction in response to low temperature was compromised in *linc1-2* mutant seeds. Germination of *linc1-2* seeds was found to be high in response to maturation at 20°C, and this was higher than wild-type (Figure 5.14A). In this particular assay, germination of wild-type increased to approximately 50% when seeds were matured at 15°C and cold stratified for three days, whereas the germination of *linc1-2* seeds remained significantly lower (Figure 5.14B). The germination of wild-type seeds reached approximately 90% following seven days of cold stratification and secondary dormancy was induced in response to fourteen days of cold stratification or more (Figure 5.14B). In contrast, the germination of *linc1-2* seeds was promoted to a maximum of 20% in response to fourteen days of cold stratification and the germination decreased in response to further lengths of cold stratification (Figure 5.14B). Together these results suggest that LINC1 may act as a negative regulator of dormancy in response to low temperature.

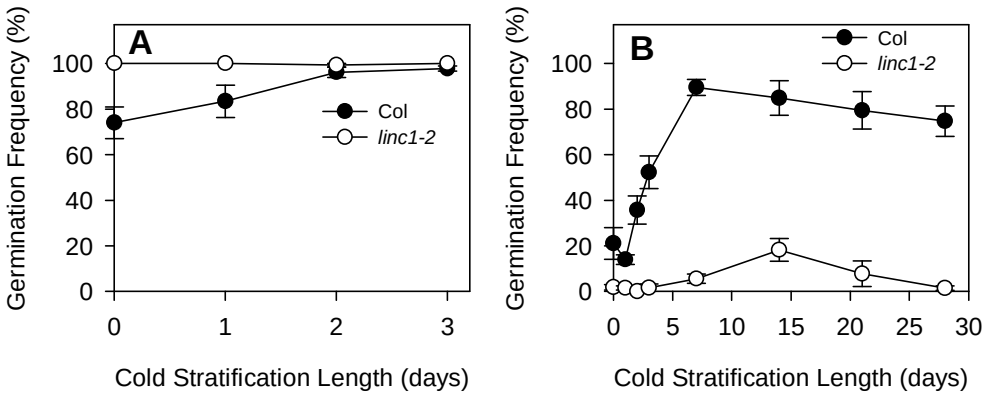

Figure 5.14: Germination of *linc1-2* seeds.
Freshly harvested seeds matured at 20°C (A) or 15°C (B) were cold stratified for up to three days (A) or twenty eight days (B). Germination was scored as radical protrusion following seven days at 22°C in 12 hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

5.2.9.2 Characterisation of nuclear volume in embryo and endosperm cells

5.2.9.2.1 Measurements in wild-type seeds

Following on from the observation that *linc1-2* mutant seeds show increased dormancy in comparison to wild-type when matured at low temperature (Figure 5.14B), the prediction that alterations to nuclear size could be part of the mechanism by which temperature regulates dormancy was investigated. Very little is known about how nuclear size and shape is regulated. Studies on fission yeast reveal that nuclear size is independent of DNA content but is highly proportional to cell size (Neumann and Nurse, 2007). DAPI was used to stain nuclei in wild-type embryo and endosperm cells from seeds matured at 20°C and 15°C and *hos1-3, hos1-4* and *hos1-5* embryo and endosperm cells from seeds matured at 15°C. Nuclear volume was calculated using 1D images and it was assumed that nuclei were spherical and so, the calculations are estimates.

The results showed that nuclei volume was unchanged by maturation temperature in embryo cells (Figure 5.15A, 5.16). In endosperm cells, nuclei from seeds matured at low temperature were significantly larger than nuclei from seeds matured at warm temperature (Figure 5.15B). Additionally, a comparison was made between nuclei circumference and cell circumference to determine if a positive correlation is present, as suggested by Neumann and Nurse (2007). The results show that there is no positive correlation between embryo nuclei circumference and embryo cell circumference in this experiment (R^2=0.160) (Figure 5.17). Therefore, the results suggest that nuclei volume is not regulated by maturation temperature in embryo cells. However, in endosperm cells temperature did have an effect on nuclei size.

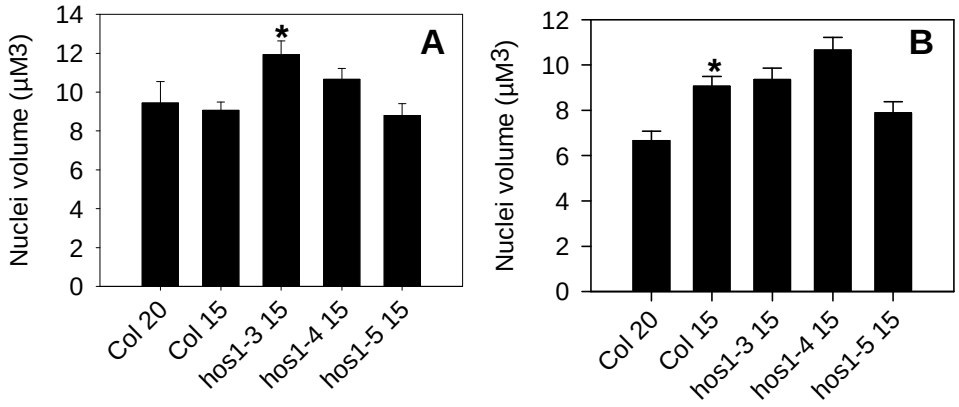

Figure 5.15: Measurements of nuclear volume.
Nuclear volume was calculated in DAPI stained wild-type and *hos1* mutant embryo root tip cells (A) and endosperm cells (B). Data points are the average of approximately two hundred nuclei from five biological replicates and error bars represent standard error. * Indicates significant difference to wild-type when P≤0.05 by students t-test.

5.2.9.2.2 Measurements in *hos1* seeds

Since a potential role for HOS1 as a component of the NPC has been proposed (Tamura et al., 2010), it was possible that the *hos1* mutation was compromising the NPC and so it was hypothesised that nuclear volume could be altered in *hos1* seeds. Although the results showed no changes to nuclei volume by temperature in wild-type embryo cells, there were alterations in endosperm cells (Figure 5.15A, B). The data presented in Figure 5.5 show that the low dormancy phenotype of *hos1* seeds is temperature independent and, therefore, it may not be surprising that the mechanism by which HOS1 acts through doesn't show changes in response to temperature. When nuclei volume was measured in *hos1* seeds that were matured at 15°C, the findings showed *hos1-3* and *hos1-4* embryo cells contained larger nuclei, but the difference was only significant for *hos1-3* (Figure 5.15A, 5.16). The nuclei of *hos1-5* cells were not different to wild-type. The situation with endosperm cells was different to embryo cells, with *hos1-4* nuclei being larger than wild-type and *hos1-5* nuclei being smaller, but these differences were not significant (Figure 5.15B). There was no difference in size of *hos1-3* nuclei in comparison to wild-type. Together, these results show that there are alterations in nuclear volume in the *hos1* mutants, but these aren't consistent for all three alleles. For this reason, the mechanism through which HOS1 affects seed dormancy is unlikely to involve the regulation of nuclei volume.

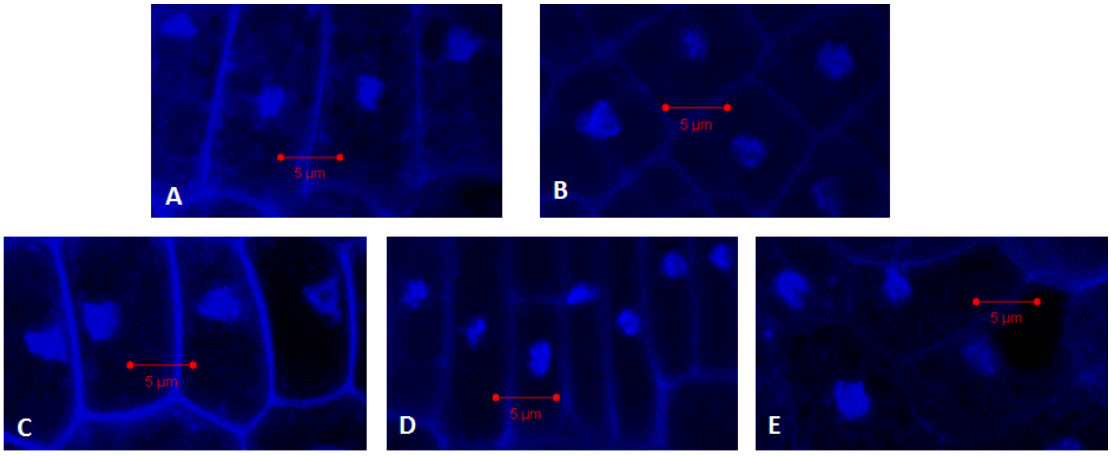

Figure 5.16: DAPI staining of embryo nuclei in root top cells.
Embryo root tip cells from Col matured at 20°C (A), Col matured at 15°C (B), *hos1-3* (C), *hos1-4* (D) and *hos1-5* (E) matured at 15°C. Scale bar represents 5µm.

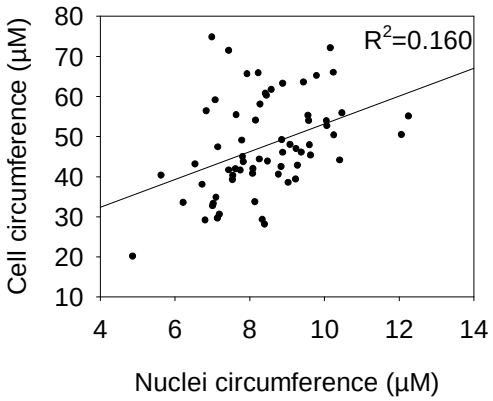

Figure 5.17: Comparison of nuclei size and cell size.
Nuclei and cell circumferences measured in DAPI stained Col embryo root tips.

5.2.10 Characterisation of dormancy phenotypes of NPC mutants

The proposal of HOS1 as a component of the NPC prompted the investigation into whether other NPC mutants display a low dormancy phenotype similar to *hos1* seeds. A role for other components of the NPC in cold signalling has been described (Gong et

al., 2005, Dong et al., 2006b), and this is important given the role of the CBFs in regulating dormancy levels. The *nup160-1* mutant, which displays defective mRNA transport, has increased freezing sensitivity (Dong et al., 2006b). Mutation to the DEAD box RNA helicase, *LOW EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 4* (*LOS4*), also leads to a reduction in mRNA transport and decreased freezing tolerance (Gong et al., 2005). Together the role for NPCs in freezing tolerance suggests that RNA export may have a critical role in cold stress responses and, therefore, could also be involved in regulating dormancy in response to temperature.

Seed of the *los4-1* mutant, which is in the C24 background, did not exhibit reduced dormancy when matured at 20°C in comparison to wild-type (Figure 5.18). There was no requirement to test the dormancy phenotype of *los4-1* seeds matured at low temperature since dormancy can be induced in the C24 background at warm temperatures. Next the dormancy phenotype of *nup160-2* was also tested. The dormancy levels of wild-type and *nup160-2* seeds were low when seeds were matured at 20°C (Figure 5.19). When the maturation temperatures were reduced to 17°C and 15°C, the *nup160-2* seeds displayed slightly higher levels of dormancy than wild-type, but the difference was not significant (Figure 5.19). Consequently, it can be concluded that the reduced dormancy phenotype of *hos1* is not characteristic of other NPC mutants.

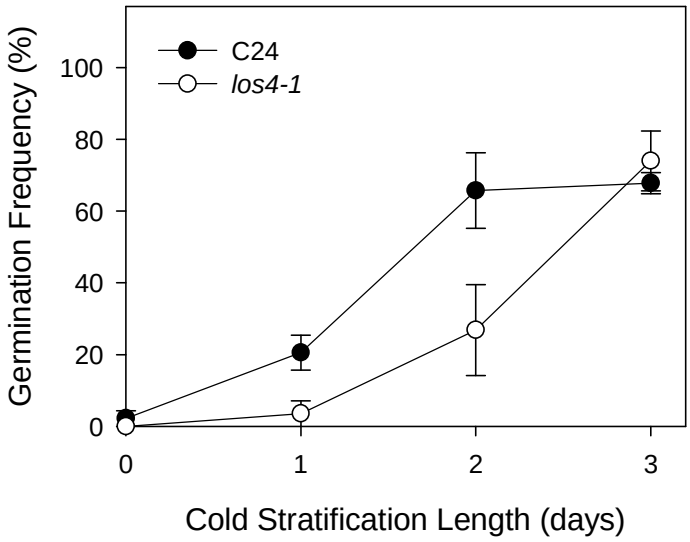

Figure 5.18: Germination of *los4-1* seeds.
Freshly harvested seeds matured at 20°C were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

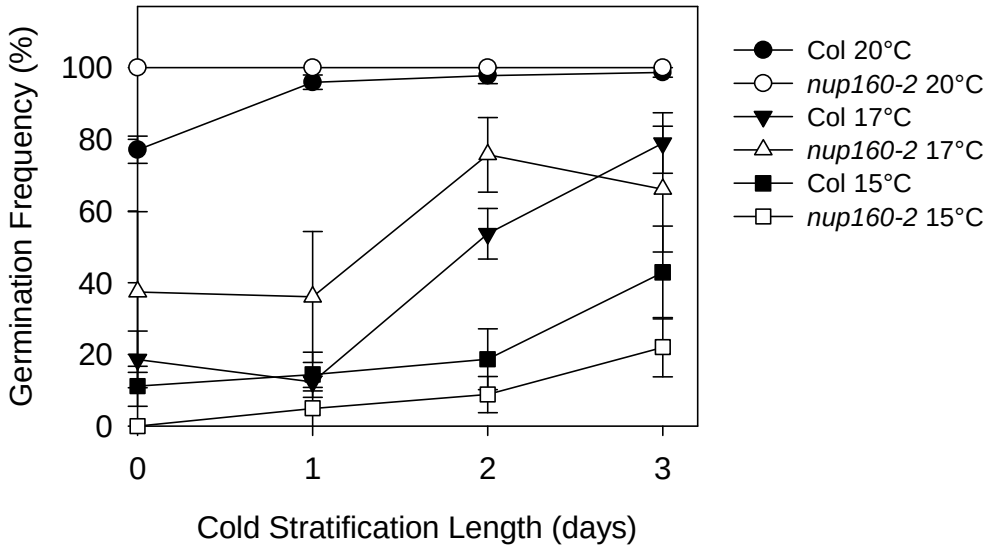

Figure 5.19: Germination of *nup160-2* seeds.
Freshly harvested seeds matured at 20°C, 17°C and 15°C were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

5.2.11 Transcriptome analysis of *hos1* seeds

To gain an understanding of the genes that are involved in regulating dormancy by HOS1, a transcriptome analysis was carried out using RNA-Sequencing (RNA-Seq). RNA-Seq is a technique which deep sequences cDNA fragments to create short reads that can then be aligned against a reference sequence (Wang et al., 2009b). The data obtained from RNA-Seq can give an indication of genes whose expression is differentially regulated between different experimental conditions or genotypes.

RNA was extracted from Col green cotyledon seeds that were matured at 15°C and 20°C and *hos1-3* seeds that were matured at 15°C which were harvested five to seven hours after dawn. RNA from these seed samples was run on a bioanalyser system to assess the quality of the RNA. As can be seen in Figure 5.20, distinct bands are

present which indicate that the RNA integrity had been kept during the extraction procedure. This was further confirmed by studying the fluorescence of the samples (Figure 5.20). The nine samples that were tested showed good integrity. Had the samples had lower integrity, the RNA would have been more fragmented. The RNA was subsequently converted to cDNA and the samples were run on an Illumina HiSeq 2000 by Konrad Paszkiewicz at the University of Exeter.

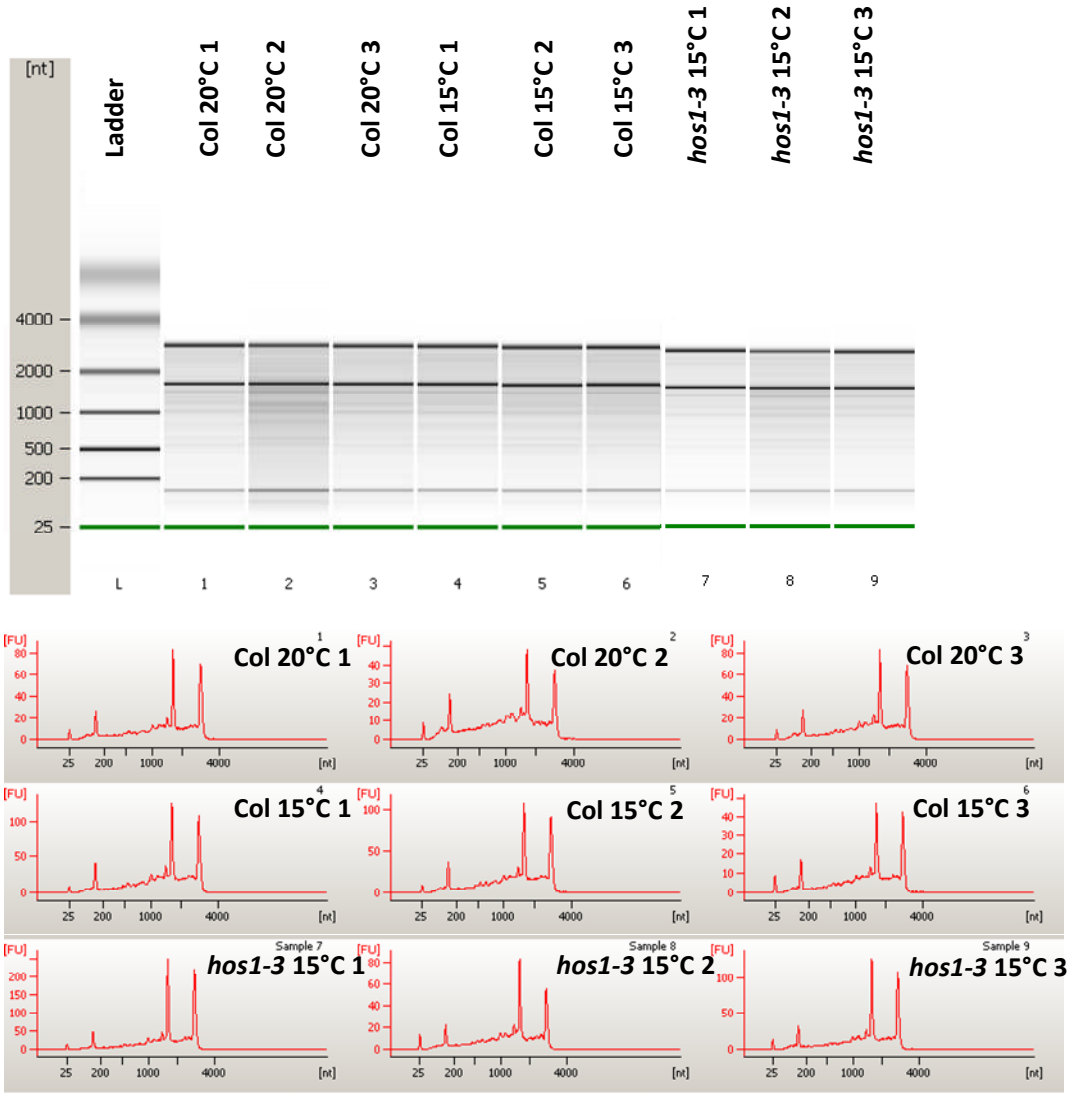

Figure 5.20: Bioanysler data for samples for RNA-Seq.
Quality control of RNA extracted from Arabidopsis plants to check for RNA integrity using an Aligent 2100 bioanalyser.

Lists of genes that were significantly differentially expressed by two-fold or more between the different genotypes/temperatures were obtained. A temperature reduction from 20°C to 15°C during seed maturation resulted in alterations in the transcription of a large number of genes. One thousand, two hundred and ninety two genes were upregulated at 15°C in comparison to 20°C in wild-type seeds (Figure 5.21A), whereas only two hundred and eighty three genes were down regulated (Figure 5.21B). There were one thousand, five hundred and sixty three genes that were downregulated in *hos1-3* seeds (Figure 5.21C), whereas three hundred and forty genes were upregulated (Figure 5.21D). Over a third of the genes that were upregulated in *hos1-3* seeds were upregulated in wild-type seeds matured at 20°C (Figure 5.22), thus suggesting that the expression of a large number of genes expressed in the *hos1-3* mutant represents warm conditions.

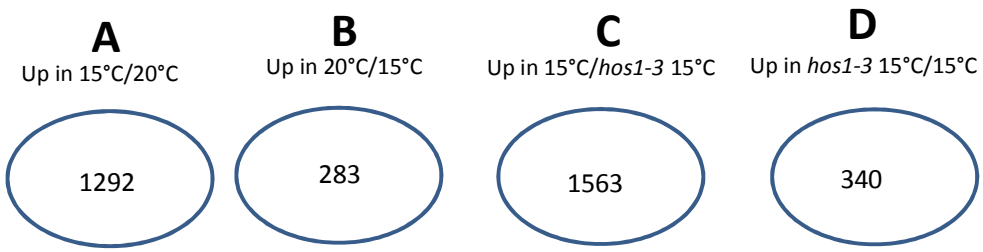

Figure 5.21: Comparison of differentially expressed genes by temperature and *hos1-3*.
Genes upregulated by low maturation temperature (A), downregulated by low maturation temperature (B), downregulated in *hos1-3* seeds (C) and upregulated in *hos1-3* seeds (D). Genes are significantly differentially expressed by two-fold or more.

To compare these data to the situation in L*er* dry seeds, a comparison was made to a data set in which seeds were matured at 20°C and 10°C (Kendall et al., 2011). An overlap of only sixty seven genes was found between the seeds matured at low temperature in the two experiments (Figure 5.23A). Only nineteen common genes were

upregulated in response to warm maturation temperature in the two experiments (Figure 5.23B). Therefore, there appeared to be a relatively small overlap in differentially regulated genes between the two datasets.

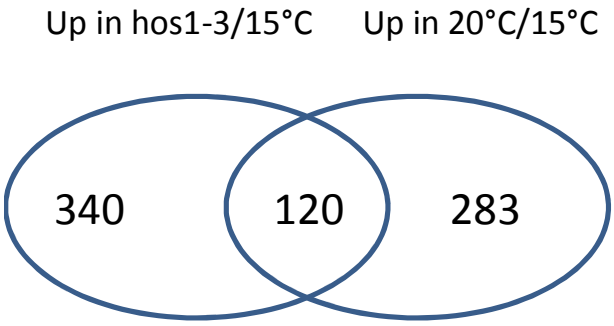

Figure 5.22: Comparison of genes upregulated in *hos1-3* and genes upregulated by warm maturation temperature.
Genes are significantly differentially expressed by two-fold or more.

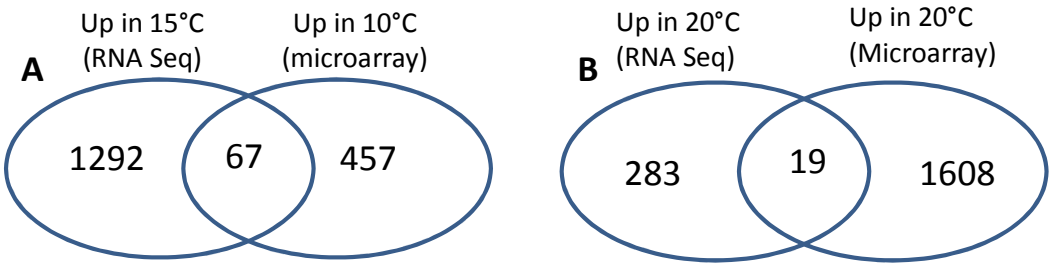

Figure 5.23: Comparison of transcript data from RNA-Seq and published microarray data.
Microarray data from Kendall et al., (2011) (genes are differentially expressed by three-fold or more). Overlapping genes upregulated in response to low temperature (A) and overlapping genes upregulated in response to warm temperature (B) determined by the two transcriptome studies.

The list of genes that were differentially expressed by temperature and differentially expressed in *hos1-3* seeds was scanned for genes known to be involved in regulating dormancy, germination and flowering time (Table 5.1, 5.2). Expression of *GA2ox6*,

which is involved in GA catabolism and *NCED6,* which is involved in ABA biosynthesis, were found to be upregulated in low temperature matured seeds in comparison to warm temperature matured seeds (Table 5.1). The expression of *GA2ox6* was also downregulated in *hos1-3* seeds (Table 5.2) and this is consistent with the reduced dormancy of these seeds (Figure 5.3). The expression of *GA2ox2*, which also catalyses GA deactivation, was unchanged in response to maturation temperature (Table 5.1).

Table 5.1: Differentially expressed genes between wild-type seeds matured at 20°C and 15°C.
Genes which are significantly up or down regulated by at least two-fold are shown at the top and bottom of the table, respectively. In between, selected genes with no significant change in expression or a fold change of less than two are listed. Values represent mean of the three replicates. The Q-value indicates the expected frequency of false positives present in a list of differentially expressed genes, and values of 1% or below are considered significant.

Locus	Gene Name	Average expression at 15°C	Average expression at 20°C	Fold Change 15°C/20°C	P-Value	Q-Value (%)
Upregulated						
AT5G57380	VIN3	6.15918	0.126774	48.58393677	0	0
AT3G55120	TT5	112.926	24.1423	4.677516227	1.15E-14	3.48E-12
AT1G02400	GA2OX6	1.91669	0.41299	4.641008257	0.000125	0.002096
AT5G15840	CO	3.62505	0.800287	4.529687475	1.60E-05	0.00039
AT1G01060	LHY	44.1467	11.1621	3.955053261	0	0
AT5G07990	TT7	321.291	93.4604	3.437723357	1.02E-06	3.91E-05
AT1G20440	COR47	49.775	17.2951	2.877982781	8.86E-09	6.57E-07
AT2G46830	CCA1	27.3566	9.85738	2.77524048	2.31E-11	3.41E-09
AT4G34000	ABF3	4.11895	1.49047	2.763524257	0.000165	0.002624
AT3G24220	NCED6	31.9766	11.8152	2.706395152	1.05E-07	5.62E-06
AT5G13930	TT4	475.424	197.222	2.41060328	0.001903	0.017334
AT1G18100	MFT	386.254	182.381	2.117841222	0.004771	0.033894
Unchanged						
AT1G65480	FT	13.6982	7.42611	1.844599663	0.009436	0.055204
AT4G09820	TT8	53.4041	30.0437	1.777547373	0.002057	0.018399
AT4G26080	ABI1	26.8916	15.9211	1.689054148	0.005237	0.036199
AT5G10140	FLC	72.5969	43.3498	1.6746767	0.00517	0.035848
AT2G39810	HOS1	6.76475	5.95877	1.135259458	0.503007	0.677755
AT4G36930	SPT	28.6195	27.9981	1.022194363	0.905043	0.947982
AT1G30040	GA2OX2	6.04581	5.9946	1.008542688	0.969272	0.983592
AT5G45830	DOG1	138.973	143.107	0.971112524	0.886918	0.938838
AT1G79460	GA2	21.6836	23.9832	0.904116215	0.582301	0.738936

AT5G35550	TT2	2.1431	2.39622	0.894366961	0.72998	0.843196
AT5G15960	KIN1	165.696	197.992	0.836882298	0.379439	0.574358
AT2G06050	OPR3	5.57344	10.0681	0.55357416	0.002503	0.021389
Downregulated						
AT5G51810	GA20OX2	14.2261	29.4936	0.482345322	0.000186	0.002888
AT1G78390	NCED9	15.9157	36.5788	0.435107221	2.33E-05	0.000529
AT1G03790	SOM	16.2984	47.5461	0.342791522	6.83E-07	2.77E-05
AT4G25470	CBF2	4.72285	13.8165	0.341826801	2.90E-05	0.000641
AT4G25490	CBF1	3.87814	11.6065	0.334135183	5.05E-05	0.001004
AT3G23250	MYB15	5.30086	17.8952	0.296216863	3.08E-08	1.94E-06
AT5G61270	PIF7	0.963196	3.77433	0.255196551	4.15E-05	0.000852
AT4G19170	NCED4	1.43605	6.2559	0.229551304	1.59E-08	1.08E-06

Table 5.2: Differentially expressed genes between wild-type and *hos1-3* seeds matured at 15°C.

Genes which are significantly up or down regulated by at least two-fold are shown at the top and bottom of the table, respectively. In between, selected genes with no significant change in expression or a fold change of less than two are listed. Values represent mean of the three replicates. The Q-value indicates the expected frequency of false positives present in a list of differentially expressed genes, and values of 1% or below are considered significant.

Locus	Gene Name	Average expression at 15°C	Average expression in *hos1-3*	Fold Change 15°C/*hos1-3*	P-Value	Q-Value
Upregulated						
AT1G65480	FT	13.6982	1.22789	11.1558853	4.82E-14	1.28E-11
AT5G15840	CO	3.62505	0.522175	6.94221286	1.54E-06	5.46E-05
AT2G39810	HOS1	6.76475	1.02855	6.576977298	2.44E-15	8.39E-13
AT5G57380	VIN3	6.15918	1.23311	4.9948342	2.16E-10	2.55E-08
AT5G13930	TT4	475.424	128.142	3.71013407	1.15E-06	4.30E-05
AT5G15960	KIN1	165.696	44.939	3.687131445	8.31E-10	8.30E-08
AT5G35550	TT2	2.1431	0.634688	3.376619693	0.002752	0.022914
AT4G34000	ABF3	4.11895	1.30202	3.163507473	4.69E-05	0.000942
AT2G06050	OPR3	5.57344	1.83081	3.044248174	8.27E-05	0.0015
AT5G07990	TT7	321.291	119.298	2.693180104	9.85E-05	0.001728
AT5G10140	FLC	72.5969	27.1568	2.673249426	2.88E-07	1.32E-05
AT1G18100	MFT	386.254	146.748	2.632090386	0.00017	0.002688
AT1G02400	GA2OX6	1.91669	0.734638	2.609026487	0.007824	0.048203
AT4G09820	TT8	53.4041	21.4555	2.48906341	1.99E-06	6.78E-05
AT3G55120	TT5	112.926	48.3722	2.334522722	1.22E-05	0.000312
AT1G20440	COR47	49.775	23.3119	2.135175597	6.09E-05	0.001171
Unchanged						
AT1G30040	GA2OX2	6.04581	3.30401	1.829840103	0.013448	0.070743
AT1G79460	GA2	21.6836	12.3737	1.752394191	0.002745	0.022866

AT4G36930	SPT	28.6195	16.595	1.724585719	0.003968	0.029789
AT2G46830	CCA1	27.3566	17.4527	1.567470936	0.001983	0.017883
AT5G51810	GA20OX2	14.2261	9.42033	1.510148795	0.044977	0.158312
AT4G26080	ABI1	26.8916	19.4275	1.384202805	0.087424	0.239639
AT1G01060	LHY	44.1467	33.0168	1.337098083	0.016813	0.082271
AT3G24220	NCED6	31.9766	25.2364	1.267082468	0.210507	0.406245
AT4G25490	CBF1	3.87814	4.16576	0.930956176	0.810091	0.892651
AT5G45830	DOG1	138.973	164.82	0.843180439	0.421062	0.611218
AT1G78390	NCED9	15.9157	21.129	0.753263287	0.144809	0.324844
AT3G23250	MYB15	5.30086	8.37845	0.632677882	0.06461	0.198573
Downregulated						
AT4G25470	CBF2	4.72285	9.59845	0.492042986	0.007377	0.046254
AT5G61270	PIF7	0.963196	3.64907	0.26395657	6.85E-05	0.001296
AT4G19170	NCED4	1.43605	7.65366	0.187629187	1.75E-10	2.11E-08
AT1G03790	SOM	16.2984	170.968	0.09533012	0	0

In contrast, *CYP707A2,* which is involved in ABA catabolism, is downregulated in low temperature matured seeds (Table 5.1) and this is consistent with what is observed in L*er* dry seeds (Kendall et al., 2011). The alterations to these genes which are involved in hormone metabolism is consistent with the increased ABA and decreased GA levels that are present in seeds that are matured at low temperature (Figure 3.7). Interestingly, *DOG1* expression was not differentially expressed in response to low temperature or in *hos1-3* seeds (Table 5.1, 5.2).

The expression of a number of *TT* genes was found to be upregulated by low maturation temperature (Table 5.1) and this is consistent with what was observed in dry L*er* seeds (Kendall et al., 2011). *TT4*,*TT*2,*TT7,TT8*,*TT5* expression was downregulated in *hos1-3* seeds in comparison to wild-type and this is consistent with the fact that these seeds show reduced dormancy (Figure 5.3). This suggests that the expression of *TT* genes may be important regulators of dormancy by temperature and HOS1.

The expression of both *CBF1* and *CBF2* was found to be downregulated, whereas expression of *COR47* was upregulated in low temperature matured seeds in comparison to warm temperature matured seeds (Figure 5.1). Consistent with the role of HOS1 as a negative regulator of cold acclimation, the *hos1-3* seeds display elevated expression of *CBF2*, whereas expression of *COR47* and *KIN1* is downregulated in these seeds (Table 5.2). In contrast, expression of *CBF1* remains unchanged in *hos1-3* seeds (Table 5.2). These results suggest that prolonged low temperature does not promote expression of the *CBFs* in seeds and that the regulation of *CBFs* in seeds is different to seedlings.

Of notable interest was the downregulation of *FT* in *hos1-3* seeds (Table 5.2). In contrast, expression of *FT* appeared to be unchanged in response to maturation temperature (Table 5.1) *ft-1* mutants in the L*er* background show an increase in dormancy levels in comparison to wild-type in response to short day photoperiods and low temperature, both of which promote dormancy (Penfield lab, unpublished). The downregulation of *FT* in *hos1-3* green cotyledon stage seeds could be confirmed using Real-Time PCR (Figure 5.24A). The expression of *FT* was normalised to the expression of an average of *TUB9* and *AT3G06240* expression. These control genes were selected since their expression was unchanged in the RNA-Seq analysis. The analysis of *FT* expression in *hos1-3* seeds was extended to investigate if *FT* expression was also downregulated in *hos1-3* seeds at additional seed stages. This analysis was carried out using wild-type and *hos1-3* seeds matured at 15°C. A small decrease in *FT* expression is observed in walking stick stage seeds, whereas there is a large decrease in expression at the green cotyledon stage (Figure 5.25A). In contrast, expression of *FT* could not be detected in *hos1-3* and wild-type dry stage seeds.

Another interesting gene that was found to be differentially regulated in *hos1-3* seeds was *MFT*. MFT has been shown to have a role in regulating germination (Xi et al., 2010). *MFT* expression was found to be upregulated in wild-type seeds matured at low temperature and downregulated in *hos1-3* seeds (Table 5.1, 5.2). Real-Time PCR was used to confirm these results (Figure 5.24B). The analysis of *MFT* expression in *hos1-3* and wild-type seeds that were matured at 15°C was extended to include walking stick stage and dry seeds. There was a slight increase in expression of *MFT* in *hos1-3* walking stick stage seeds in comparison to wild-type, whereas expression was decreased in *hos1-3* green cotyledon stage seeds in comparison to wild-type (Figure 5.25B). There appeared to be no difference in *MFT* expression in dry seeds between the two genotypes.

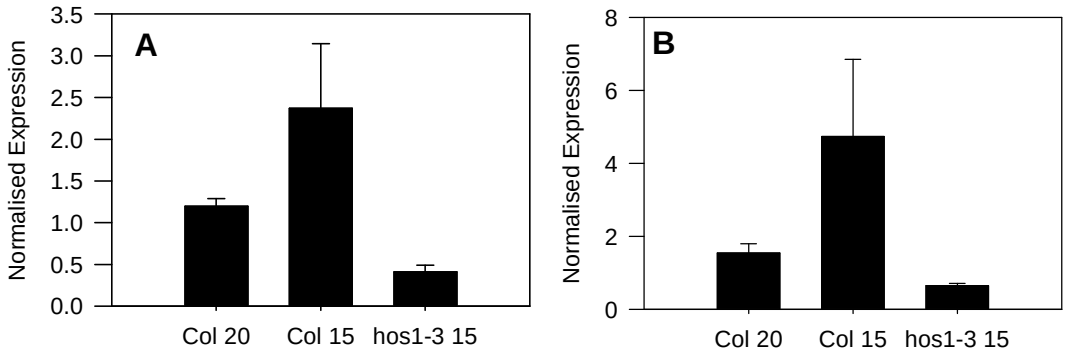

Figure 5.24: Confirmation of RNA-Seq results.
Expression of *FT* (A) and *MFT* (B) in green cotyledon stage wild-type seeds matured at 20°C and 15°C and *hos1-3* seeds matured at 15°C was measured using Real-Time PCR. Data points are the average of two biological replicates and expression is normalised to the average of two control genes *TUBULIN9* and *AT3G06240*. Error bars represent standard error.

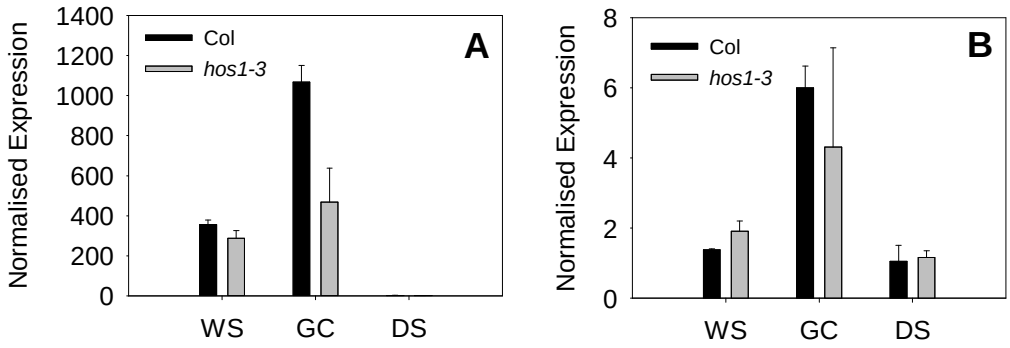

Figure 5.25: Expression of *FT* and *MFT* during seed development.
Expression of *FT* (A) and *MFT* (B) in wild-type seeds matured at 20°C and 15°C and *hos1-3* seeds matured at 15°C. Developmental stages are Walking Stick (WS), Green Cotyledon (GC) and Dry Seed (DS). Data points are the average of two biological replicates and expression is normalised to the average of two control genes *TUBULIN9* and *AT3G06240*. Error bars represent standard error.

To further the understanding of the role of ABA in regulating dormancy, the presence of the ABA RESPONSE ELEMENT (ABRE) motif element in the promoters of genes that were differentially regulated between the genotypes/conditions was analysed. This analysis revealed that a number of genes did contain the ABRE motif in their promoters (appendix 1, 2) suggesting the potential regulation of these genes by ABA.

5.2.12 Characterisation of dormancy phenotype of *ft-10* seeds

FT expression is downregulated in *hos1-3* seeds (Figure 5.24A, 5.25A) and so it was hypothesised that HOS1 could regulate dormancy levels through FT. To begin to understand the role played by FT in regulating dormancy the dormancy phenotype of *ft-10* mutant seeds (in the Col background) in response to maturation at 20°C, 17°C and 15°C was analysed (Figure 5.26). *ft-10* mutant seeds showed low levels of dormancy when matured at 20°C and this was similar to wild-type. In contrast, when the

maturation temperature was reduced to 17°C, dormancy was induced in the *ft-10* seeds and the levels were similar to wild-type (Figure 5.26). When the maturation temperature was reduced further to 15°C, the initial levels of dormancy induced in *ft-10* seeds were higher than wild-type. However, when cold stratification was applied, the dormancy levels of *ft-10* were similar to wild-type (Figure 5.26). These results suggest that HOS1 does not regulate dormancy levels solely through FT given the lack of apparent dormancy phenotype.

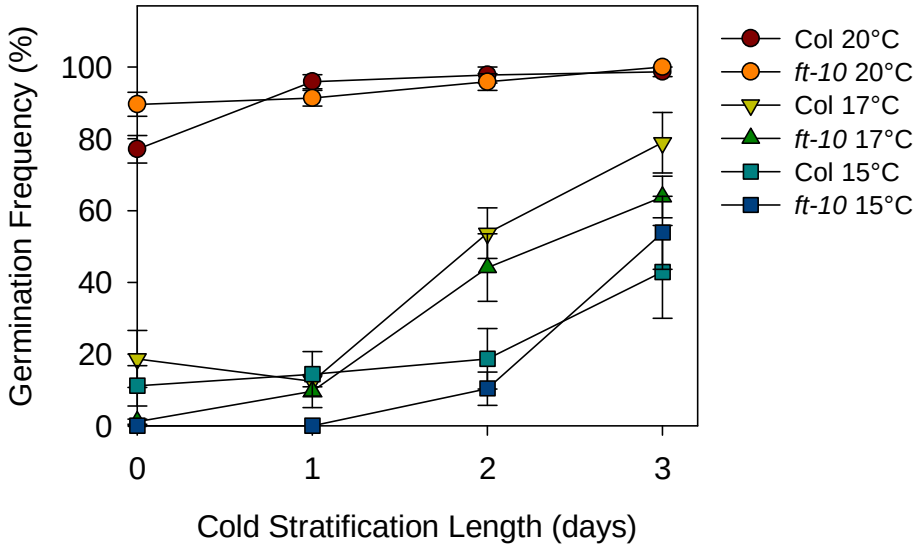

Figure 5.26: Germination of *ft-10* seeds.
Freshly harvested seeds matured at 20°C, 17°C or 15°C were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

5.2.13 Characterisation of dormancy phenotypes of *tt* seeds

The results from the transcriptome analysis showed that expression of *TT* genes is differentially regulated in *hos1-3* seeds in comparison to wild-type (Table 5.2) Additionally, a number of the *TT* genes is upregulated in seeds that were matured at low temperatures in comparison to warm temperatures (Table 5.1). To investigate the potential role of TT in regulating dormancy in response to temperature *tt4-1, tt5-1* and *tt6-1* loss-of-function seeds were matured at 20°C and 15°C and the dormancy levels were analysed.

When matured at 20°C, *tt4-1* and *tt5-1* showed very low dormancy levels and their germination was like wild-type (Figure 5.27A). Germination of *tt6-1* was slightly lower initially, but following two days of cold stratification it reached wild-type levels. When the seeds were matured at low temperature, a clear low dormancy phenotype was observed for *tt5-1*, which germinated to significantly higher levels than wild-type (Figure 5.27B). In contrast, only slightly lower dormancy levels were observed for *tt6-1* in comparison to wild-type however, germination was significantly increased when seeds were cold stratified for two days or more in comparison to wild-type. Dormancy levels in *tt4-1* were higher than *tt5-1* and *tt6-1,* although seeds did germinate slightly higher than wild-type at some time points. Taken together these results show that TT5*,* and to a lesser extent, TT6 have important roles in defining dormancy levels in response to low temperature. Since the expression of a number of *TT* genes is downregulated in *hos1-3* mutant seeds (Table 5.2) and loss-of-function of a number of *tt* mutants leads to reduced dormancy, the results suggest that HOS1 may function through TT to regulate dormancy.

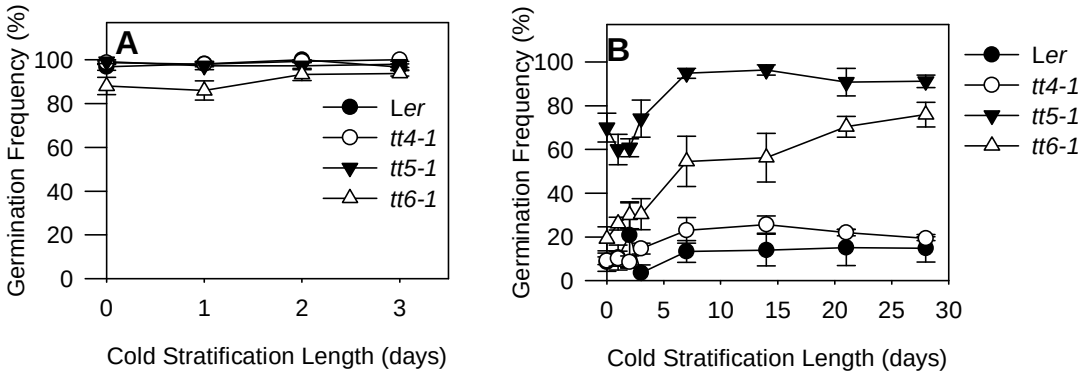

Figure 5.27: Germination of *tt* seeds.
Freshly harvested seeds matured at 20°C (A) or 15°C (B) were cold stratified for up to three days (A) or up to twenty eight days (B). Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

5.3 Discussion

5.3.1 HOS1 regulation of dormancy is not temperature specific

The results presented in this chapter have shown that *hos1* mutant seeds have a striking reduced dormancy phenotype (Figure 5.3, 5.4, 5.5). The inability of *hos1* mutants to induce dormancy was shown to be independent of the maturation temperature (Figure 5.5), thus suggesting that HOS1 has a role in regulating dormancy, but its effect is downstream of the input of temperature into the mechanism. Based on this observation, it is likely that *hos1* mutant seeds would be deficient in inducing high dormancy levels in other situations that promote high dormancy such as a short day photoperiod. When *HOS1* expression was measured in seeds matured at low and high temperatures, differences were only identified at the walking stick stage (Figure 5.6). This result suggested that *HOS1* expression is not generally temperature regulated, but also that *HOS1* expression does not correlate with dormancy levels. However, it cannot be ruled out that there may be differences in HOS1 protein levels between the two temperatures, and this would be important to confirm in the future.

These results show that a lack of functional *HOS1* expression in two ecotypes, Col and C24, leads to the inability of the seeds to enter the highly dormant state (Figure 5.3, 5.4, 5.5). It will be interesting to determine whether introgressing the *hos1* mutation into additional highly dormant ecotypes, such as Cvi, will result in the same reduced dormancy phenotype. Additionally, it would also be interesting to determine if variation in HOS1 expression or protein levels occurs between different ecotypes.

5.3.2 Additional flowering time mutants have dormancy phenotypes

The dormancy phenotypes of *tfl2* and *arp6* mutants were analysed because they display early flowering phenotypes which are similar to *hos1* mutants (Larsson et al., 1998, Deal et al., 2005, Lazaro et al., 2012). The analysis of dormancy phenotypes of the *tfl2* mutants revealed that these mutants were also unable to induce high dormancy levels in response to maturation at low temperature and this phenotype was similar to *hos1* mutants (Figure 5.11, 5.2). A dormancy phenotype was also displayed by *arp6*, although this was not as striking as that of *hos1* or *tfl2* (Figure 5.13). Given the suite of phenotypes shared between *hos1, tfl2* and *arp6* it is perhaps unsurprising that these genes could also be important for the regulation of dormancy. To address this further, crosses could be made between *hos1, arp6* and *tfl2.* Although it will be difficult to assess if these mutants have an additive effect on the dormancy phenotype of *hos1* given that this mutant germinates highly. It will also be important to determine if the effect of TFL2 and ARP6 in regulating dormancy is through a maternal pathway like HOS1.

5.3.3 Regulation of GA levels is important for the *hos1* dormancy phenotype

The results in this chapter showed that there was no difference in the sensitivity of *hos1* seeds to either ABA or PAC in comparison to wild-type (Figure 5.11). Other reduced dormancy mutants also display wild-type sensitivity to ABA and GA (Peeters et al., 2002). However, levels of both GA and ABA were found to be altered in *hos1* mutants (Figure 5.10). The levels of GA were significantly higher in *hos1* mutants and this correlates with the low dormancy phenotype (Figure 5.10A, 5.3). Surprisingly, levels of ABA were also increased in seeds of one of the *hos1* mutant alleles (Figure 5.10B) and

this result does not correspond with the reduced dormancy phenotype. However, it is possible that the increase in ABA levels is part of a feedback loop in response to the highly elevated levels of GA.

To investigate the mechanism by which HOS1 affects dormancy it will be necessary to determine the nature of these increases in GA levels and whether they are sufficient for the low dormancy phenotype. The effect of the *hos1* mutation on dormancy has been shown to be through a maternal pathway (Figure 5.8). Therefore, the site of GA synthesis could be in the maternal plant or in the maternal tissues of the seed. To identify where the increased GA is synthesised, GA levels could be measured in the different tissues. Another way in which this could be investigated would be to alter GA levels by adding exogenous PAC. Developing *hos1* siliques could be dipped with PAC, and this would prevent the synthesis of GA in the developing seed and siliques. If germination was reduced in these seeds, then it would suggest that the GA is being produced in the silique or seed coat and that GA is necessary for the *hos1* mutant dormancy phenotype.

To determine whether GA is sufficient for the reduced dormancy of *hos1* seeds the *hos1 ga1* double mutant should be created and the dormancy analysed. If increased GA levels are important for the *hos1* mutant phenotype then one would expect dormancy levels to be increased in this double mutant in comparison to *hos1* seeds.

5.3.4 Nuclear size is not a regulator of dormancy in response to temperature

The results presented in this chapter show that maturation temperature had no effect on nuclei volume in embryo cells, whereas there was a negative correlation between

temperature and nuclei volume in endosperm cells (Figure 5.15, 5.14). The results also showed that cells from a number of the *hos1* mutant alleles contained larger nuclei however, the alterations are not consistent between embryo and endosperm cells (Figure 5.15). Together these results suggest that dormancy levels do not consistently correlate with alterations to nuclei volume. This is somewhat surprising given that expression of *LINC* genes is higher in seeds matured at 20°C (Kendall et al., 2011), and so one might have expected the size of the nuclei of seeds matured at warm temperatures to be greater. Additionally, the results presented in Figure 5.14 show that *linc1-2* seeds do display an increased dormancy phenotype, suggesting a potential role for LINC1 in the regulation of dormancy. To investigate this further, alternative measures of nuclear morphology such as chromatin condensation could be examined between the two maturation temperatures.

A recent study also showed that changes in dormancy levels did not correlate with alterations to nuclei size (van Zanten et al., 2011b). Nuclear size was analysed in the highly dormant ecotype Cvi and *dog1* and *rdo2 mutants* and no differences were observed between these different genotypes. However, changes to nuclei size and chromatin condensation were shown to be important parts of the seed development programme associated with desiccation tolerance. A reduction in nuclear size during seed maturation was identified and this was dependent on ABI3. During germination, a further reduction in nuclear size was determined and this was shown to require LINC1 and LINC2 (van Zanten et al., 2011a).

The dormancy phenotypes of two NPC mutants; *los4-1* and *nup160-2* were tested and no differences were found in comparison to wild-type (Figure 6.17, 6.18). This result shows that the reduced dormancy phenotype of *hos1* seeds is not common to all NPC

mutants. However, there are a large number of additional NPC mutants that could be tested to confirm this. Therefore, together these results suggest that there is no role for the control of nuclear morphology or the NPC in regulating the temperature regulation of dormancy.

5.3.5 Transcriptome analysis reveals differential expression of *FT* in *hos1* seeds

A transcriptome analysis was carried out using RNA-Seq to determine genes that are important for the temperature regulation of dormancy. A small number of overlapping genes which were differentially expressed by temperature in seeds could be identified by comparing the RNA-Seq and a previously published microarray data set (Kendall et al., 2011). The fact that only a small number of genes was found to overlap between the two data sets was somewhat surprising (Figure 5.23). However, there were number of differences between the experimental designs of the two analyses. Firstly the seed stages that were sampled differed between the two experiments, with this analysis using green cotyledon seeds and the published data set used dry seeds. The green cotyledon stage was chosen as dormancy is induced during seed maturation, and by sampling the dry seed stage genes involved in this induction may have been missed. Additionally, there was a 5°C maturation temperature difference between the two low temperature treatments. A drop in temperature from 15°C to 10°C during seed maturation has large effects on the seed dormancy levels that are induced (Figure 3.2) and, therefore, it is likely that the expression of different genes would be important for this. Lastly, the ecotypes which were used differed. This analysis was carried out using Col seeds whereas the published data set was based on L*er* seeds.

The expression of *CYP707A2* has been shown to be downregulated by low temperature during seed maturation in both transcriptome studies and was confirmed independently by Real-Time PCR (Figure 3.16D). Additionally, *GA2ox6* was found to be differentially upregulated by low maturation temperature in both transcriptome analyses. However, this upregulation of *GA2ox6* was more difficult to detect in green cotyledon seeds by Real-Time PCR as expression levels appeared to be very low (Figure 3.16B).

A notable difference between the two data sets was the expression of *NCED4*. In the L*er* dry seed data set *NCED4* expression was upregulated in seeds matured at 10°C in comparison to 20°C, whereas in the Col green cotyledon dataset, *NCED4* expression is the opposite, with higher levels of expression at the warm maturation temperature (Table 5.1). Since *NCED4* functions in ABA biosynthesis and ABA levels are higher in low temperature matured seeds, the upregulation of *NCED4* by low maturation temperature is consistent. However, there is an upregulation of *NCED6* by low temperature in the Col green cotyledon seed data set and this could be the predominant *NCED* gene involved in ABA biosynthesis in this ecotype. The analysis of *NCED4* expression in green cotyledon stage seeds using Real-Time PCR showed that there was little difference in expression of *NCED4* in Col, whereas in WS there was a slight decrease in expression in response to low temperature (Figure 3.16C).

Additionally, expression of *DOG1* was found to be unchanged in response to low maturation temperature in the RNA-Seq dataset (Table 5.1), whereas expression was upregulated in dry L*er* seeds (Kendall et al., 2011). Analysis of *DOG1* expression in green cotyledon stage seeds using Real-Time PCR showed that there was an upregulation of *DOG1* expression in Col seeds, whereas in WS there was a slight

decrease in expression in response to low temperature (Figure 3.16A). The transcriptome analysis using RNA-Seq used seeds which were matured at 15°C and 20°C, whereas the Real-Time PCR was carried out on seeds which were matured at 15°C and 22°C. Therefore, the smaller temperature range used for the transcriptome analysis could have had an effect on the differential expression of *DOG1*.

A number of *TT* genes were found to be upregulated in low temperature matured seeds in comparison to wild-type seeds matured at warm temperature and *hos1-3* seeds (Table 5.1, 5.2). This result suggests that seed coat morphology could be altered in these seeds. However, when seed coat morphology was analysed the results showed no difference between seeds matured at warm and low temperatures (Figure 3.5). Yet, the results from an experiment analysing the dormancy of *tt4-1, tt5-1* and *tt6-1* showed that TT5 is important for the induction of dormancy in response to low temperature since *tt5-1* mutants were unable to enter highly dormant states (Figure 5.27). However, it is possible that altered seed coat morphology could be part of the mechanism by which HOS1 regulates dormancy levels. To investigate this, sections of seed coat from *hos1* and wild-type should be analysed to identify if there are differences in seed coat thickness or structure.

FT was identified as being downregulated in *hos1-3* seeds and this reduction in expression was confirmed by Real-Time PCR (Figure 5.24, 5.25). This differential regulation of *FT* in *hos1-3* seeds is interesting given the involvement of FT as a positive regulator of flowering. A recent study investigated the role of HOS1 as an integrator of temperature and photoperiod signals in the regulation of flowering time (Lazaro et al., 2012). This study found that HOS1 interacts with CO to regulate *FT* expression (Lazaro et al., 2012).

The reduced expression of *FT* in *hos1-3* seeds is in contrast to what is observed in vegetative tissues, where *hos1* seedlings showed an upregulation of *FT* (Lazaro et al., 2012). The downregulation of *FT* in *hos1-3* seeds was somewhat surprising given the fact that the *ft-1* mutant shows increased seed dormancy in response to low maturation temperature and short-days (Penfield lab, unpublished). This would suggest that in the L*er* background FT is a negative regulator of dormancy and, therefore, a decrease in expression of *FT* in *hos1* would lead to an increase in dormancy levels. Of course, the opposite is true in *hos1* mutants, which display a reduced dormancy phenotype (Figure 5.3). However, when the dormancy phenotype of the *ft-10* mutant was analysed, the seeds showed wild-type levels of dormancy in response to the three maturation temperatures tested (Figure 5.26). This result suggests that HOS1 cannot be acting solely through FT to regulate dormancy levels. Dormancy levels should now be investigated in the *hos1 ft-10* double mutant in response to low maturation temperature to try and understand this relationship more thoroughly.

MFT expression was found to be upregulated by low maturation temperature in comparison to warm maturation temperature and downregulated in *hos1-3* seeds in comparison to wild-type (Table 5.1, 5.2). These results could be confirmed by Real-Time PCR (Figure 5.24B, 5.25B). This temperature regulation of *MFT* in green cotyledon seeds is consistent with the results of a study which analysed the effect of temperature on the dormancy levels of seeds in the soil bank (Footitt et al., 2011). This study showed that *MFT* expression is increased by low temperature in *Arabidopsis* seeds in the soil bank. Additionally, a study investigating the effect of low temperature during seed development of wheat also found that the wheat *MFT* homologue was upregulated in response to low temperature (Nakamura et al., 2011). In contrast, *MFT*

expression was not upregulated in *Arabidopsis* L*er* dry seeds which were matured at 10°C (Kendall et al., 2011).

Although MFT has been shown to have a positive effect on promoting germination in after-ripened seeds (Xi et al., 2010), freshly harvested *mft* loss-of-function mutants show reduced dormancy (Graham lab, unpublished). Thus, the dormancy phenotype of *mft* mutant seeds should be investigated in response to low maturation temperature. One may predict that *mft* mutant seeds may show a reduced dormancy phenotype in response to maturation at low temperature. Additionally, the dormancy levels of the *hos1 mft* double mutant should be analysed in response to low maturation temperature to identify if HOS1 affects dormancy levels through MFT.

5.3.6 Conclusions

Experiments in this chapter have demonstrated that *hos1* mutants display highly reduced dormancy phenotypes in situations when high dormancy levels are induced in wild-type. The data suggest that HOS1 defines a novel essential maternal pathway that controls seed dormancy that involves the regulation of GA levels in the mature seed, but not sensitivity to ABA or GA in the imbibed seed. A transcriptome analysis using RNA-Seq reveals that expression of *FT*, *MFT*, and *TT* is different and, therefore, could be involved in dormancy regulation with HOS1.

Chapter 6 Characterisation of *cold stratification insensitive* mutants

6.1 Introduction

Germination of *Arabidopsis* is promoted by exposure to a period of low temperature during imbibition and in the laboratory situation this is known as “cold stratification”. Cold stratification is used to ensure the germination of a population of seeds occurs in synchrony, which is important in both laboratory and horticultural situations. Uniform germination is important to ensure that the emergence of the plant and hence the different stages of a plants lifecycle (i.e. flowering) occur simultaneously within a population. Coordination between plants in a population is particularly important in crops such as cereals where the majority of the seeds must be at the same stage of maturity when harvested.

Low temperature can have both positive and negative effects on *Arabidopsis* development. While cold temperatures promote seed germination and winter annual flowering through vernalisation they can also repress plant growth and induce seed dormancy in the subsequent generation. Although low temperature during imbibition promotes germination, seedling establishment at low temperature can result in chilling injury and seed maturation at low temperatures represses germination. The understanding of how the low temperature signal is able to promote germination is limited however, the regulation of GA metabolism through changes in *GA3ox1* expression is important (Yamauchi et al., 2004).

Here, a forward genetic screen was used to isolate *Arabidopsis* mutants which do not respond to the germination promoting effects of low temperature. Forward genetic

screens can be carried out by either chemical treatment or physical damage which leads to alterations in the DNA. Commonly EMS is used as a chemical mutagen. EMS is a base modifying agent which works by adding an ethyl group to the hydrogen-bonding oxygen in guanine, which leads to a G/C to A/T transition. Approximately 5% of the mutations created by EMS will result in the creation of a stop codon. In addition, approximately 65% and 30% of mutations will be missense or silent changes respectively (McCallum et al., 2000). Therefore, it is likely that the majority of mutations in a screen created by EMS will be loss-of-function mutations rather than gain of function mutations.

By isolating mutants incapable of germinating in response to low temperature it was hoped that more could be learnt about the mechanisms that regulate the process of cold stratification. A characterisation of five of these mutants is presented in this chapter along with some preliminary genetic analyses.

6.2 Results

6.2.1 Description of mutant screen

A forward genetic screen was used to isolate mutants that were unable to germinate in response to cold stratification during imbibition (Figure 6.1). Briefly, EMS mutagenised freshly harvested Col seed was exposed to cold stratification (three days at 4°C, dark) and then transferred to a twelve hour light, twelve hour dark light regime at 22°C. After one week, germination was analysed and any seeds which did not germinate were taken forward to the next part of the screen where they were dried and then after-ripened for one month. Seeds that germinated following after-ripening were taken through to the next generation and the resulting progeny was re-tested. Through this selection process fourteen mutants were isolated, named *cold stratification insensitive1-14* (*cosi1-14*), which showed a strong insensitivity to the cold stratification treatment but were able to germinate following after-ripening. The mutant screen and isolation of fourteen *cosi* mutants was carried out by Steve Penfield, prior to the start of this project.

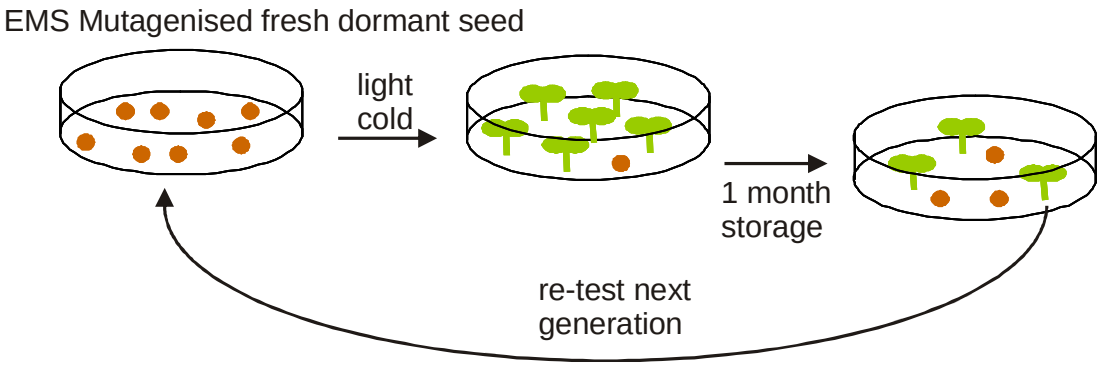

Figure 6.1: Schematic of forward genetic screen.
Freshly harvested EMS mutagenised Col seeds were cold stratified for three days and moved to twelve hour light, twelve hour dark light regime at 22°C. After one week, germination was analysed and seeds which did not germinate were taken forward to the next part of the screen where they were dried and after-ripened for one month. Seeds that germinated following after-ripening were taken through to the next generation and the resulting progeny was re-tested.

6.2.2 Characterisation of *cosi* mutant phenotype in response to cold stratification

To test the robustness of the phenotype of each *cosi* mutant, the response of the fourteen selected mutants to cold stratification was analysed further. Freshly harvested seed (harvested from plants grown in glasshouse conditions) was exposed to up to three days of cold stratification and then transferred to light/dark conditions at 22°C. Germination of the fourteen different *cosi* mutants was highly variable and the mutants could be split into three broad classes; those that showed a low, intermediate and high insensitivity to the cold stratification (Figure 6.2). *cosi11* and *cosi12* germinated to wild-type levels or above and so, showed no insensitivity to cold stratification. *cosi6, cosi8, cosi10* and *cosi13* also showed low insensitivity to cold stratification. *cosi5, cosi7, cosi9* and *cosi14* all showed germination which was highly insensitive to cold stratification. These six mutants which showed a low or medium insensitivity to the cold stratification

were removed from further characterisation. Eight mutants that showed high insensitivity to cold stratification remained and the five mutants with the strongest phenotypes (*cosi1-5*) were selected for further analysis based on their strong insensitivity to cold stratification.

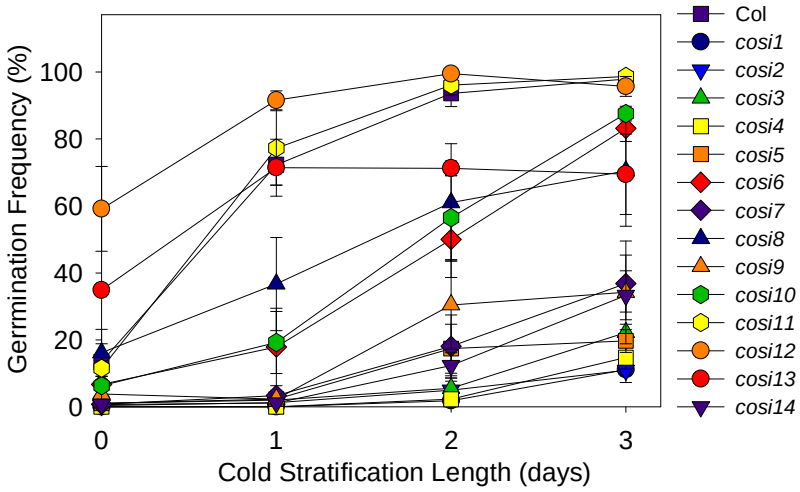

Figure 6.2: Germination of mutants isolated from forward genetic screen. Freshly harvested seeds generated under glasshouse conditions were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

The initial characterisation of the fourteen *cosi* mutant cold stratification phenotypes that have been described was carried out using seed generated from plants that were grown under glasshouse conditions (Figure 6.2). However, after testing multiple generations of seeds, which were generated in both glasshouse and growth room conditions, it was observed that variability in environmental conditions was affecting the phenotypes observed (Figure 6.3A, B). The data presented in Figure 6.2, 6.3A and 6.3B represent seed from independent increasing generations. Problems with insect infestation meant that not enough seed was produced by *cosi3* to analyse the

germination and hence germination data for only four *cosi* mutants is presented in Figure 6.3A. In some cases the cold stratification insensitive phenotype of the mutant disappeared over generations, whereas in other cases the phenotypes were extremely dependent on the environmental conditions in which the mother plant was grown and the seeds matured. In December the temperature range experienced by plants in the glasshouse was 13.8°C to 24.8°C, whereas in July the range was 17.7°C to 31.6°C. Similarly large differences in the total amount of light were observed between winter and summer. The growth of *cosi1* plants generated under glasshouse conditions was found to be poor and seed production was vastly reduced in comparison to wild-type. The reduction in the number of seeds produced by *cosi1* meant that germination experiments were difficult to carry out as the seed number was limiting. Therefore, to eliminate the effect of differences in environmental conditions, such as temperature and light, on the mutant phenotype all plants for further experiments were grown in growth cabinets where conditions were tightly regulated. It was also hoped that the growth and seed production of *cosi1* could be improved by growing the plant in more controlled conditions.

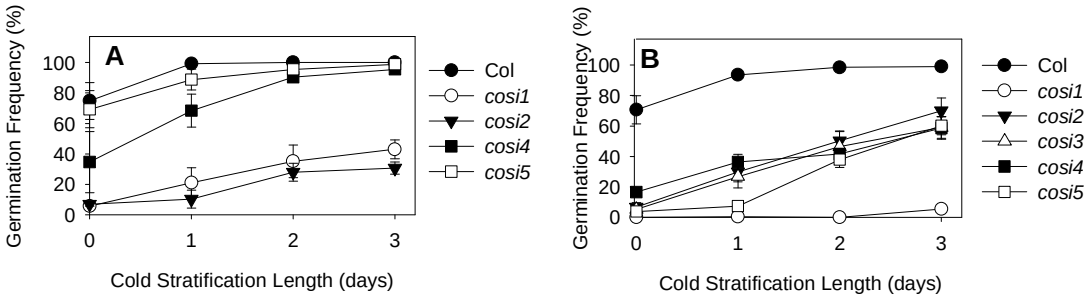

Figure 6.3: Germination of five selected *cosi* mutants.
Freshly harvested seeds generated under glasshouse conditions (A) or in a growth room (B) were cold stratified for up to three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error. Problems with insect infestation resulted in low seed production for *cosi3* meaning that the dormancy assay (A) could not be carried out for this mutant.

6.2.3 Response of *cosi* mutants to extended cold stratification

The results have shown that the five selected *cosi* mutants show reduced germination in response to a relatively short exposure to low temperature (Figure 6.2, 6.3). It is possible that the *cosi* mutants are able to respond to low temperature but they require a longer period of exposure for germination to be promoted. This could be due to the seeds having a higher chilling requirement and so a greater threshold of exposure to cold must be met for dormancy to be overcome. Secondly, there could be a lag in the response to cold stratification. Seeds with high dormancy levels which are induced in response to low temperature during seed maturation show an increase in germination when exposure to 4°C is extended from three days to fourteen days (Penfield and Springthorpe, 2012) and further extension of cold stratification can often induce seeds into secondary dormancy (Finch-Savage et al., 2007, Penfield and Springthorpe, 2012).

To investigate if extended cold stratification can promote higher levels of germination in the *cosi* mutants an experiment was carried out where cold stratification was extended to twenty eight days (Figure 6.4). Seeds for this experiment were generated in a growth cabinet. Three days of cold stratification promoted high germination levels in wild-type seeds (Figure 6.4). The germination of wild-type seeds could not be increased with further exposure to low temperature as the germination had already reached maximum levels in response to three days of cold stratification. Long exposure to cold (i.e. twenty one/twenty eight days) did not lead to a reduction in germination in wild-type and hence entry into secondary dormancy was not promoted (Figure 6.4). In this experiment *cosi5* responded like wild-type to the cold stratification, germinating to approximately 90% following three days cold stratification, suggesting that the phenotype that this mutant had been selected for was highly variable (Figure 6.4). Since the seeds for this experiment were generated in a growth cabinet, it is unlikely that environmental effects were the cause of the increased germination. Germination of *cosi5* was increased to almost 100% when seeds were exposed to further cold (Figure 6.4). The increase in cold stratification was able to promote high germination levels in *cosi2* seeds, although low germination levels were still observed in response to short cold stratification times for this mutant (Figure 6.4). Extended cold stratification did promote increased germination for *cosi3* and *cosi4* seeds, but germination remained significantly lower than wild-type (Figure 6.4). After seven days of cold stratification *cosi4* seeds exhibited reduced germination indicating seeds had entered secondary dormancy. After twenty eight days of cold stratification germination levels of *cosi3* had reached approximately 60%, a marked increase from the 20% observed following three days of cold stratification. This suggests that *cosi3 does* require an increased amount of cold in comparison to wild-type. In contrast to the other *cosi* mutants, *cosi1* seeds showed no

significant increase in germination in response to the increased cold stratification treatment.

Together these results suggest that by extending cold stratification the germination of *cosi2* and *cosi5* can be increased to wild-type levels, although levels of germination of *cosi5* were high in comparison to previous experiments (Figure 6.2, 6.3A, 6.3B). On the other hand, the cold stratification extension failed to increase the germination of *cosi1, cosi3* and *cosi4* to wild-type levels.

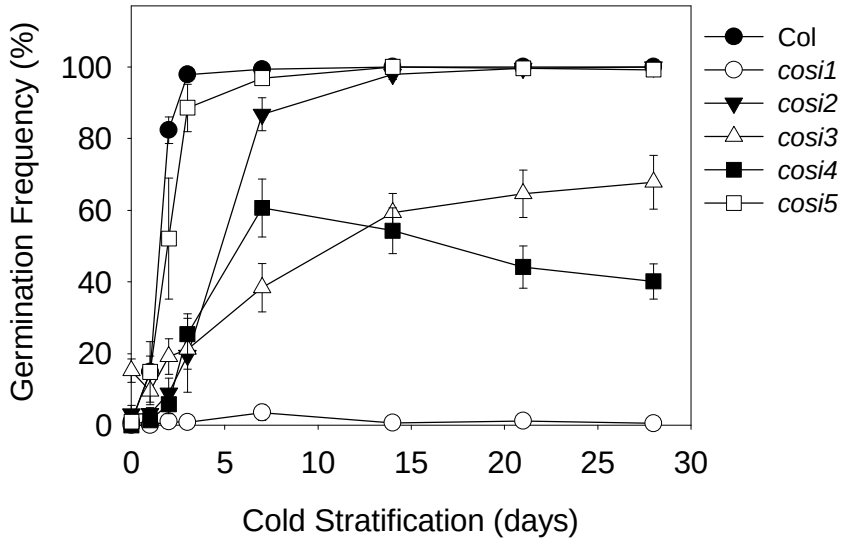

Figure 6.4: Germination of *cosi* mutants in response to extended cold stratification.
Freshly harvested seeds generated in a growth cabinet were cold stratified for up to twenty eight days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

6.2.4 Involvement of *cosi* mutants with hormones

6.2.4.1 Germination response of *cosi* mutants to ABA and PAC

The phytohormones, ABA and GA, are known to play an important role in regulating germination and, therefore, an experiment was carried out to determine whether sensitivity to ABA or GA is altered in the *cosi* mutants. To examine this hypothesis, the sensitivity of *cosi* mutants to ABA and PAC, a GA biosynthesis inhibitor was analysed. Altered sensitivity to ABA and PAC is a common phenotype for a number of seed germination mutants such as *dog1-2*, (Bentsink et al., 2006). The results from the ABA and PAC sensitivity experiment would also contribute to the understanding of how the pathway in which germination is promoted in response to low temperature interacts with ABA and GA signalling pathways.

cosi mutant seeds which were generated under glasshouse conditions were after-ripened for approximately three months and germinated in the presence of varying concentrations of ABA, PAC or a control (methanol). Seeds were after-ripened to ensure high levels of germination were obtained in response to control conditions so the effect of ABA and PAC on reducing germination could be observed. The five *cosi* mutants showed a varied response to both ABA and PAC, although the majority of mutants appeared to show hypersensitivity in comparison to wild-type (Figure 6.5). Hypersensitivity to both ABA and PAC is consistent with the reduced germination phenotype which is observed, i.e. the seeds are more sensitive to the negative effects of ABA and PAC. Specifically, *cosi2, cosi4* and *cosi5* were significantly sensitive to intermediate concentrations of ABA, whereas *cosi3* showed a similar response to wild-type (Figure 6.5A). *cosi1* showed high sensitivity to low concentrations of ABA, but a similar response to wild-type in response to high levels of ABA. Unfortunately, the

sensitivity of *cosi1* to ABA and PAC was only tested using one individual seed batch, rather than the usual five as there were problems with seed production.

In response to PAC, *cosi1* was significantly hypersensitive, although it should be noted that the germination of this mutant was low (only approximately 40%) in response to the control treatment (Figure 6.5B). Consequently, the extreme hypersensitivity observed in *cosi1* may be a reflection of the general high dormancy. *cosi4* also showed significant hypersensitivity to PAC at the mid range concentrations, although increases in concentration from 0.1µM to 1µM ABA appeared to have little effect on the germination of this mutant. *cosi5* showed significant hypersensitivity to PAC at concentrations of 1µM and 5µM PAC. *cosi2* and *cosi3* also showed significant hypersensitivity to PAC at concentrations of 0.5µM to 5 µM in comparison to wild-type. Germination of all five *cosi* mutants and wild-type in response to 20µM PAC was approximately 0%.

Therefore, there is a high amount of variability in the responses of the different *cosi* mutants to ABA and PAC, but most mutants do show a hypersensitive phenotype, which is consistent with the reduced germination phenotype of the *cosi* mutants.

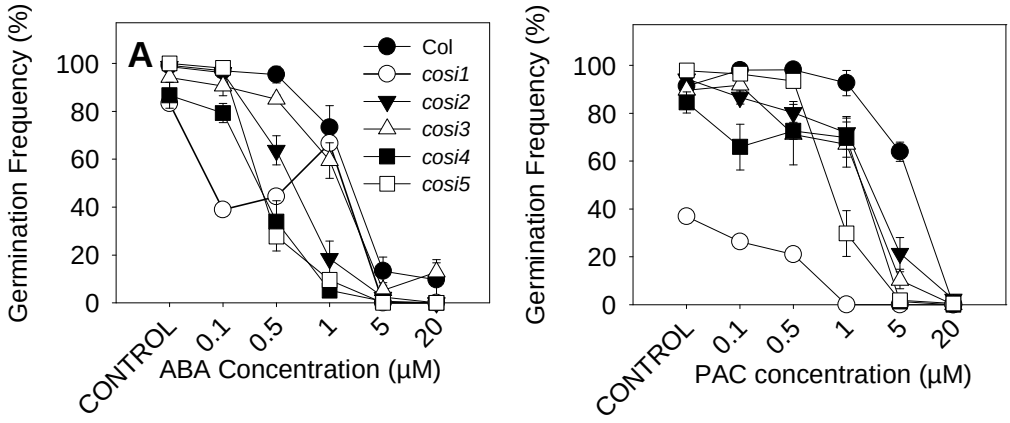

Figure 6.5: ABA and PAC sensitivity of *cosi* mutants.
Seeds generated in glasshouse conditions were after-ripened for approximately three months. Cold stratified seeds were germinated in the presence of increasing concentrations of ABA (A) and PAC (B) and germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error. An exception to this is *cosi1* and data points represent one single seed batch.

6.2.4.2 Germination response of *cosi* mutants to GA and NOR

Results presented in chapter three showed that the germination of highly dormant wild-type seeds can be increased with the addition of endogenous GA or NOR, an inhibitor of ABA biosynthesis (Figure 3.9). Therefore, an experiment was carried out to determine whether the addition of exogenous GA or NOR would increase the germination of unstratified and cold stratified *cosi* mutant seeds which were generated in a growth cabinet.

Germination of unstratified wild-type seeds was significantly increased with the addition of GA to approximately 85%, whereas the addition of NOR only increased germination to approximately 25% (Figure 6.6). For these seeds the combined addition of GA and NOR significantly increased the germination further to approximately 90% (Figure 6.6). Germination of wild-type cold stratified seeds in response to the control reached the

same level as unstratified seeds treated with GA, thus suggesting the exogenous application of GA can replace the action of cold in promoting germination.

In unstratified seeds, the application of GA led to an increase in germination of *cosi2-5* and this increase was significant for *cosi4* and *cosi5.* In contrast, the application of GA to unstratified *cosi1* seeds had little effect on promoting germination. In cold stratified seeds, the application of GA significantly increased the germination of *cosi1,* and *cosi4,* whilst it had no significant effect on the germination of *cosi2, cosi3* and *cosi5.* The reason for the inability of GA to promote germination in these seeds may be due to the fact that cold stratification did increase the germination of these seeds. However, the effect of the application of GA on germination of *cosi* seeds did not lead to wild-type levels of germination.

In contrast to the effects of the application of GA, the germination of unstratified seeds was not significantly increased by the application of NOR. An exception to this was the response of *cosi3* seeds to NOR, which led to a significant increase in germination that was greater than the response to applied GA. In cold stratified seeds, the application of NOR only led to a significant increase in germination for *cosi1* seeds. Again, NOR had a greater effect on promoting germination in *cosi3* cold stratified seeds in comparison to GA, but neither of these treatments were significantly different to the control treatment.

The application of both GA and NOR led to a significant increase in germination of *cosi* mutant unstratified seeds that was greater than the effect of GA or NOR alone. An exception to this was the germination of *cosi3* unstratified seeds, where NOR alone did have a greater effect on germination. The synergistic effect of GA and NOR is most striking on *cosi1* unstratified seeds, which show low germination in response to either

GA or NOR alone, but wild-type levels of germination in response to both GA and NOR. Germination of cold stratified *cosi* mutant seeds is promoted to wild-type levels in response to both GA and NOR. An exception to this is germination of *cosi2* stratified seeds, as the application of GA and NOR did not significantly increase the germination further than the control treatment.

These results reveal that the *cosi* mutants cannot be deemed GA insensitive since germination responds to GA in all cases. The results show that generally GA has a greater effect on promoting germination than NOR. However, promotion of wild-type levels of germination for *cosi* mutants generally requires the synergistic effect of both GA and NOR. Consequently, the results suggest that GA may have a more important role in promoting germination in response to low temperature, although there is also a role for ABA.

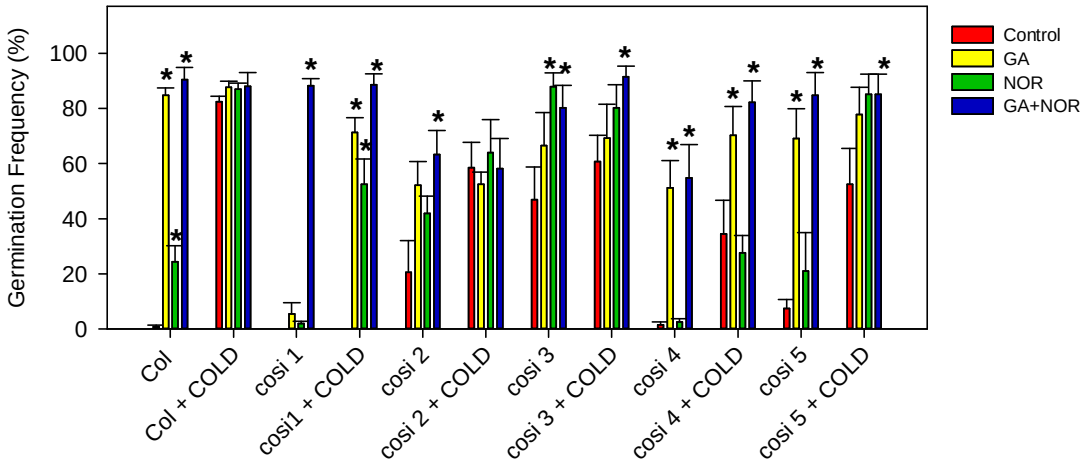

Figure 6.6: Germination of *cosi* mutants in response to GA and NOR.
Seeds generated in a growth cabinet were cold stratified for zero or three days (COLD) and germinated in the presence of GA (100µM), NOR (50µM), GA + NOR or control (100µM methanol). Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error. * Indicates significant difference to the control treatment when P≤0.05 by students t-test.

6.2.4.3 Response of *cosi3* growth to exogenous GA application

cosi3 mutants show reduced germination in response to cold stratification (Figure 6.2, 6.3A, 6.4B, 6.5). Germination of this mutant is increased in response to extended lengths of cold stratification (Figure 6.4). In addition to these phenotypes *cosi3* mutant plants also display a dwarfed phenotype and are dark green in colour, which is similar to the phenotypes displayed by the GA-deficient mutant *ga1-3* (Koornneef and Veen, 1980). Exogenous application of GA reverses the dwarf phenotype in *ga1-3* to that of wild-type (Koornneef and Veen, 1980). Therefore, to determine whether the dwarfed appearance of *cosi3* plants could also be reversed by the exogenous application of GA, *cosi3* seeds were germinated on medium containing GA and seedling growth was

analysed (Figure 6.7). Additionally, the *cosi3* plants were directly sprayed with GA (Figure 6.8). Application of GA to both *cosi3* seeds and plants may have had a slight promotional effect on the size of seedlings and plants, but the dwarfed phenotype was not completely rescued to wild-type as would have been expected if *cosi3* were a GA-deficient mutant like *ga1-3*. Wild-type seedlings and plants showed a similar response whereby treated plants were slightly bigger than control plants (Figure 6.8). This suggests that the GA treatments were effective and that the small response of *cosi3* to the GA confirms the result that *cosi3* is not insensitive to GA which is shown in Figure 6.6.

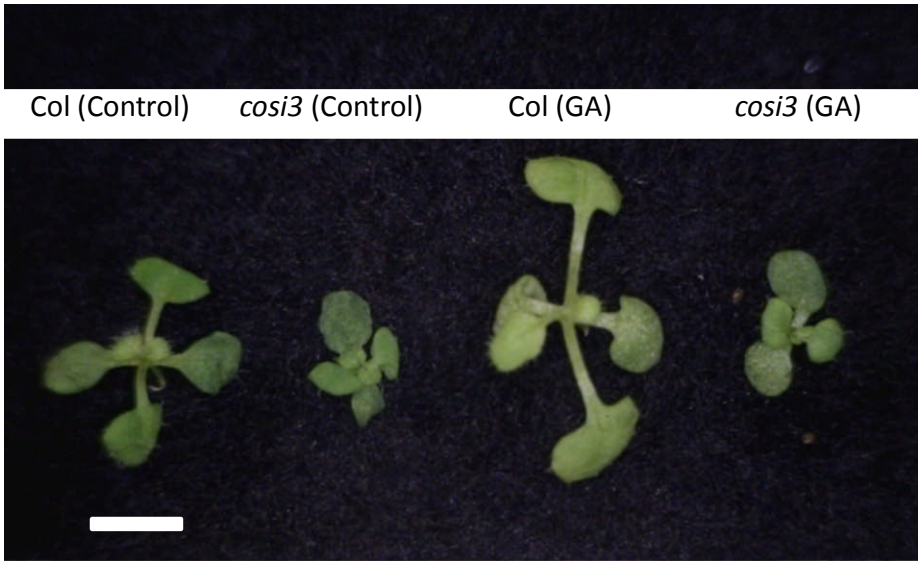

Figure 6.7: Seedling growth of *cosi3* in response to exogenous GA.
Wild-type and *cosi3* seedlings were germinated on water-agar with 100µM Methanol (control) or 100µM GA. Seeds were given three days cold stratification and then transferred to 22°C in twelve hour white light/dark cycles. Fourteen day old seedlings were photographed. Scale bar indicates 2.5mm.

Figure 6.8: Exogenous application of GA to *cosi3* plants.
Wild-type and *cosi3* plants were grown in long day conditions with a temperature regime of 20°C/18°C. Control plants were treated with 2% Methanol and +GA plants were treated with 100µM GA in 2% Methanol. Plants were sprayed on a weekly basis from seventeen days old. Five week old plants were photographed.

6.2.5 Analysis of additional *cosi* mutant germination phenotypes

6.2.5.1 Response of *cosi* mutants to different imbibition temperatures

The aim of this screen was to identify mutants that were unable to respond to low temperatures, specifically during imbibition. However, it is possible that *cosi* mutants may show a more general inability to respond to a range of temperatures and not just to low temperature. To test this hypothesis freshly harvested seeds, which were

generated in a growth cabinet, were imbibed at four temperatures; 12°C, 17°C, 22°C and 27°C and germination after seven days was analysed (Figure 6.9). In wild-type seeds, low imbibition temperatures promote high levels of germination whereas at higher imbibition temperatures the process of thermoinhibition prevents germination. Seeds of winter annual ecotypes of *Arabidopsis* are shed during the spring, but their germination is prevented by high temperature during summer, meaning these seeds germinate in the autumn.

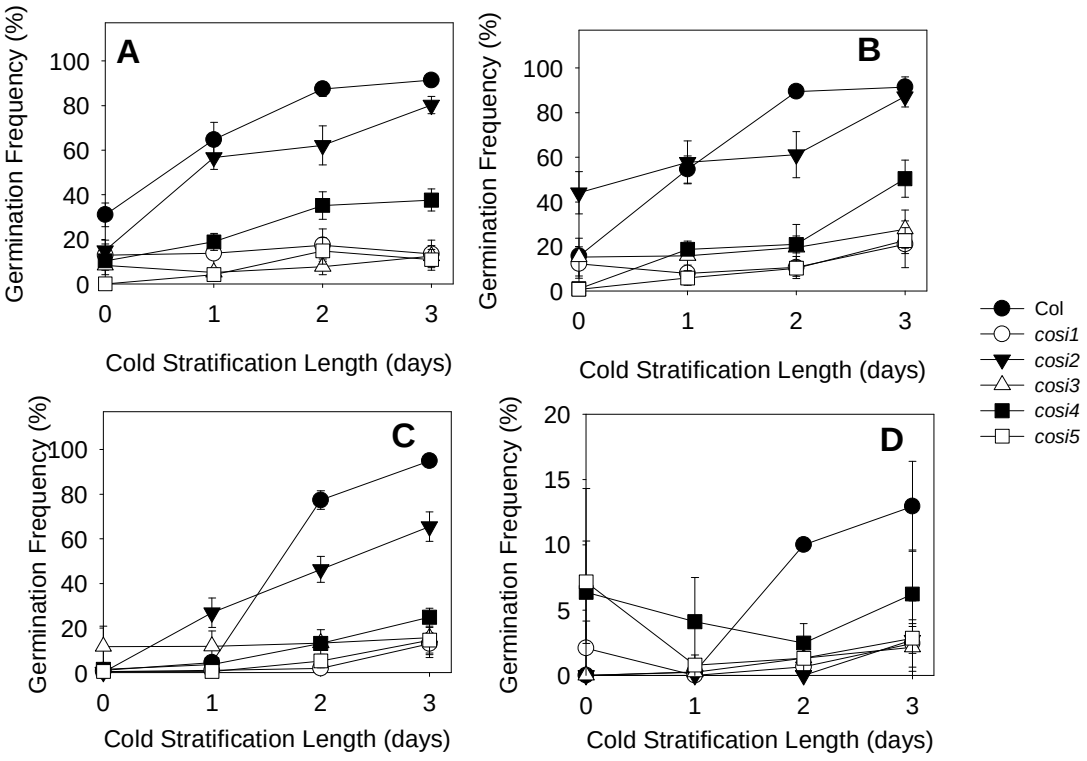

Figure 6.9: Germination of *cosi* mutants in response to different imbibition temperatures.
Seeds generated in a growth cabinet were imbibed at 12°C (A), 17°C (B), 22°C (C) and 27°C (D). Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

When wild-type seeds were exposed to increasing days of cold stratification before imbibition at 12°C, 17°C or 22°C, the negative effect of increasing imbibition temperature was reduced. Following three days cold stratification, the germination of seeds imbibed at 12°C and 22°C was no longer significantly different (Figure 6.9A, C). In contrast, although cold stratification promoted germination when seeds were imbibed at 27°C, germination was still significantly suppressed by high temperature following three days of cold stratification in comparison to seeds imbibed at 22°C (Figure 6.9D). These results suggest that increases in temperature in the ambient range do not influence the germination potential of seeds. Seeds are, however, still respondent to the negative effect of imbibition at high temperatures.

The five different *cosi* mutants showed a large amount of variation in their responses to different imbibition temperatures. Most noticeably is the response of *cosi3* to different imbibition temperatures. The results for this mutant are shown alone with wild-type for an easier comparison (Figure 6.10). Germination of *cosi3* seeds do not show any significant differences in response to an increase in imbibition temperature from 12°C to 27°C (Figure 6.10B). *cosi1* and *cosi5* also show a similar phenotype to *cosi3*, but cold stratification does promote some germination when seeds are imbibed at 22°C and germination is suppressed significantly when seeds are imbibed at 27°C. Germination of *cosi4* is significantly lower than wild-type at the four imbibition temperatures and some promotion of germination by cold stratification is observed. In contrast to the other *cosi* mutants, *cosi2* shows a similar phenotype to wild-type when imbibed at 12°C and 17°C (Figure 6.9A, B). Even when *cosi2* was imbibed at 22°C, it still germinated to approximately 60% following three days of cold stratification. In addition, cold stratification did not promote any significant increase in germination of *cosi2* at all four imbibition temperatures tested (Figure 6.9). Therefore, the results highlight *cosi3* as

being particularly interesting since it displays an imbibition temperature insensitivity phenotype. However, this does suggest that the temperature sensing defect of this mutant may not be specific to low temperature.

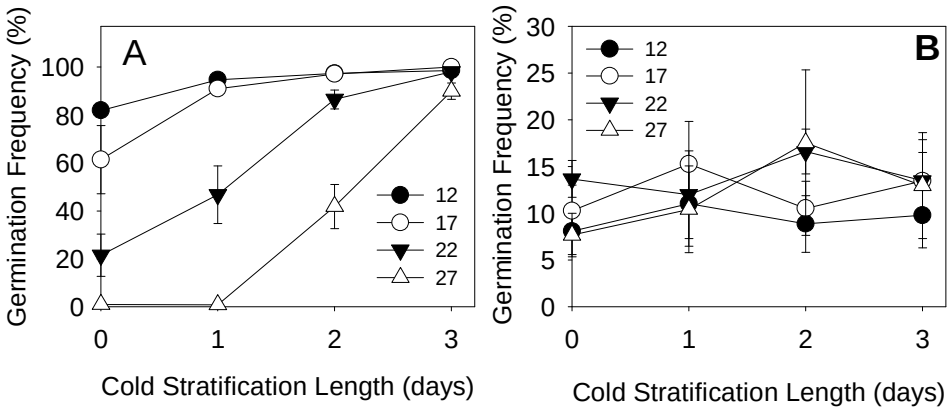

Figure 6.10: Germination of *cosi3* in response to different imbibition temperatures.
Wild-type (A) and *cosi3* (B) seeds generated in a growth cabinet were imbibed at 12°C, 17°C, 22°C and 27°C. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error. The data in this Figure is taken from Figure 6.9.

6.2.5.2 Response of cosi mutants to after-ripening

The five *cosi* mutants were selected on the basis that although they do not germinate in response to cold stratification, a period of after-ripening was able to reduce dormancy levels such that seeds were able to germinate. These selection criteria meant that the selected mutants show a specific germination defect in the response to cold stratification. Results presented in chapter three show that seeds which have high levels of dormancy are in some cases unresponsive to a short period of cold stratification (i.e. three days) and also show little response to relatively short periods of after-ripening (approximately two months) (Figure 3.2, 3.3). Since the selected mutants should germinate in response to after-ripening, it can be ruled out that the mutants are

simply ‘more dormant’ and so don’t respond to the cold stratification. Therefore, it was important to quantify the response to after-ripening and so germination of *cosi* mutant seeds, which were generated in a growth cabinet, in response to increasing lengths of after-ripening was analysed.

The response of *cosi3* seeds to after-ripening was significantly quicker than that of wild-type seeds (Figure 6.11). The fact that this mutant responds well to after-ripening suggests that the observed germination defect is a specific response to cold stratification. *cosi1* seeds showed no significant response to after-ripening and, consequently, this suggests that although after-ripening had promoted germination in the initial screen, the mutation may cause a more generalised germination defect that is not specific to the cold stratification response. Similarly, after-ripening had little effect on the germination of *cosi4* seeds, whose germination reached a maximum of approximately 20% following fourteen weeks of after-ripening, which was significantly lower than wild-type. Again this indicates that the defect in this mutant is likely to be part of a broader mechanism regulating germination as a whole and not specific to the temperature response during imbibition. Initially, *cosi2* showed a quicker response to after-ripening in comparison to wild-type, although after six weeks of after-ripening, wild-type germinated to higher levels. Interestingly, *cosi5* did respond to after-ripening, but germination began to decrease following nine weeks of after-ripening. This could be due to a loss of viability within the tested seed batches. Or conversely this result could suggest that dormancy cycling is occurring in these seeds. Therefore, responses of the *cosi* mutants to after-ripening shows variation and the results from this experiment highlight mutants whose phenotypes are not specific to a defect in responding to cold stratification (i.e. *cosi1* and *cosi 4*). It is surprising that these mutants are unable to germinate in response to after-ripening since this was one of the criteria of the original

screen however, the seeds for this experiment and the original screen were generated under different environmental conditions.

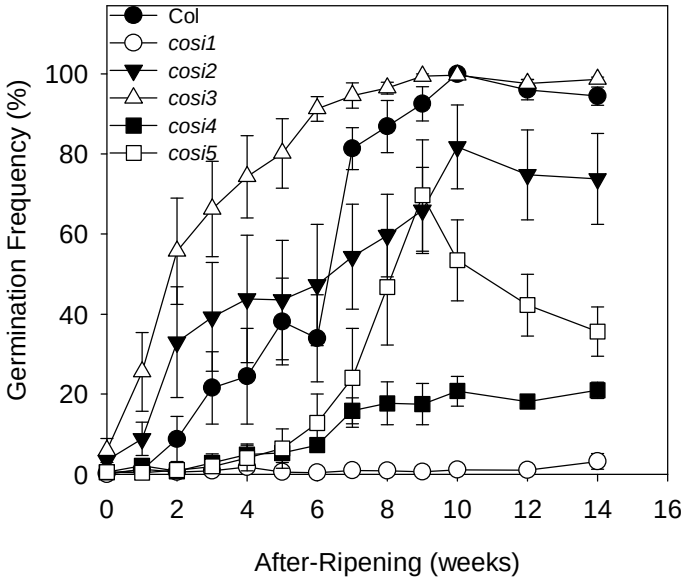

Figure 6.11: Germination of *cosi* mutants in response to after-ripening.
Seeds generated in a growth cabinet were after-ripened for up to fourteen weeks. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles. Data points are the average of five individual seed batches and error bars represent standard error.

6.2.6 Characterisation of *cosi* growth phenotypes

6.2.6.1 Analysis of *cosi* mutant hypocotyl elongation

Light is a key regulator of germination, and is known to regulate a number of other processes in the plant including the inhibition of hypocotyl elongation (de-etiolation), stimulation of cotyledon expansion and regulation of flowering time. *cosi1* displayed a number of phenotypes that are typical of light signalling mutants, including early flowering, long hypocotyl and long petioles and, therefore, to quantitate whether *cosi1* along with the other *cosi* mutants displayed any abnormal responses to light, hypocotyl

elongation in response to white, red, far red and blue light was investigated in comparison to light signalling mutants (Figure 6.12). *phyB* mutants are unable to repress hypocotyl elongation in response to red light, *phyA* mutants to far red light and *cry1* mutants to blue light (Koornneef et al., 1980, Whitelam et al., 1993). For these reasons, these mutants have been included in the seedling growth assays.

Both *cosi3* and *cosi4* have significantly shorter hypocotyls in response to far red light (Figure 6.12D) but respond like wild-type to white and blue light (Figure 6.12A, C). *cosi3* also has a significantly shorter hypocotyl in response to red light (Figure 6.12B). *cosi2* and *cosi5* have significantly longer hypocotyls in response to white, red and blue light, but respond like wild-type to far-red light (Figure 6.12 A,B, C, D). The response of *cosi2* to the different light regimes is very similar to what is observed for *phyB*; however, the hypocotyls of *cosi2* under white and red light were significantly shorter than *phyB* (Figure 6.12A, B). This suggests that *cosi2* may have some involvement in a light signalling pathway perhaps specifically through red light.

The long hypocotyl phenotype of *cosi1* that had been observed when growing these plants was confirmed, and this phenotype was apparent under each light regime tested. In comparison to *phyA* and *phyB* in the red and far red light treatments respectively, *cosi1* had significantly increased hypocotyl elongation (Figure 6.12B, D). However, in response to blue light, the hypocotyl elongation of *cosi1* did not exceed that of *cry1* (Figure 6.12C), thus suggesting that altered responses of multiple phytochromes but not cryptochromes are likely to be the basis of the hypocotyl elongation phenotypes that are observed in *cosi1.* Therefore, again a variety of phenotypic responses is displayed by the *cosi* mutants with regards to the de-etiolation process. *cosi1* shows a

long hypocotyl phenotype in response to all light treatments and this suggests that the gene that is mutated in *cosi1* may be a key component of a light signalling pathway.

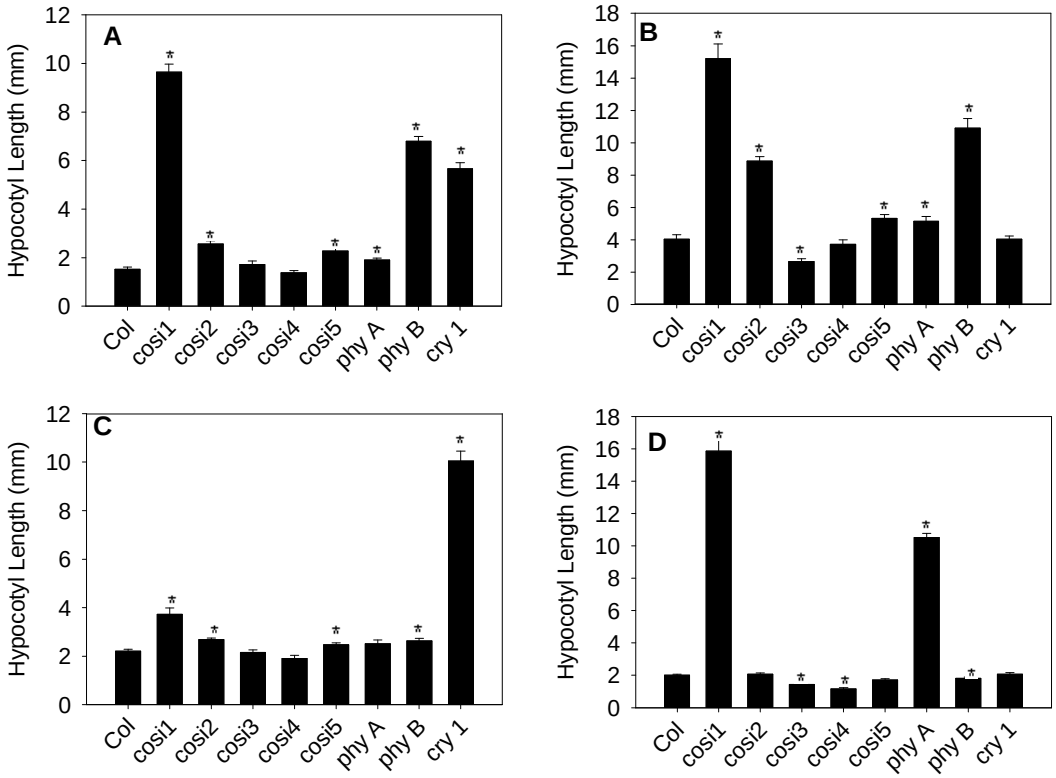

Figure 6.12: Hypocotyl elongation of *cosi* mutants.
Seeds were cold stratified for three days, transferred to a growth cabinet at 22°C with constant white light for two days and then the light was switched to the appropriate light treatment for a further seven days. Light treatments were white light (A), red light (B), blue light (C) and far-red light (D). Data points are the average of approximately twenty seedlings and error bars represent standard error. * Indicates significant difference to wild-type when P≤0.05 by students t-test.

6.2.6.2 Analysis of *cosi* mutant growth rate

Growth rate has been shown to be tightly coupled to temperature and a mutation in *SPT*, which is a repressor of seed germination, shows an increased growth rate phenotype at low temperature but no difference at warmer temperatures (Sidaway-Lee

et al., 2010). Additionally, *phyAphyBphyDphyE* plants show slow rosette leaf production in comparison to wild-type at 16°C (Halliday et al., 2003). Therefore, an experiment was carried out to understand if any of the *cosi* mutations were involved in growth regulation in response to temperature. To investigate this, plants were grown at 15°C and total rosette leaf area was used as a measure of growth rate. *cosi1* and *cosi5* plants showed significantly increased growth rate phenotypes after seven weeks of growth in comparison to wild-type (Figure 6.13). After seven weeks of growth, total rosette area of *cosi5* was 50% larger than wild-type. This increase in growth rate is not dissimilar to that of *spt-2* (Sidaway-Lee et al., 2010). Conversely, *cosi3* and *cosi4* showed a significantly reduced growth rate after seven weeks of growth in comparison to wild-type. It was not surprising that *cosi3* displayed such a phenotype, since this plant is dwarfed (Figure 6.8). In contrast, the growth rate of *cosi2* was largely similar to wild-type, although a small decrease in growth rate was observed at seven weeks. Together these results suggest that growth rate is altered in a number of the *cosi* mutants.

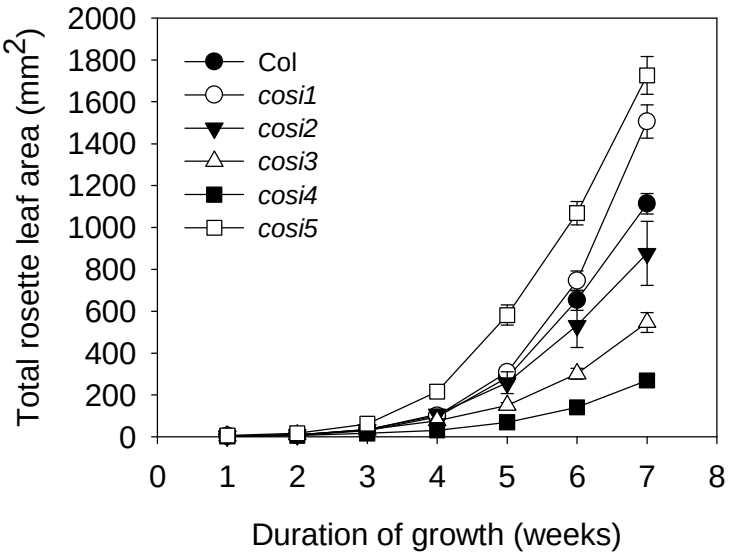

Figure 6.13: Growth rate of *cosi* mutants.
Growth rate was based on total rosette leaf area of approximately ten plants grown at 15°C under a long-day light regime. Photographs of plants were taken on a weekly basis from one week to seven weeks. Data points are the average of ten plants and error bars represent standard error.

6.2.6.3 Analysis of *cosi* mutant flowering time

Since a number of mutants which have roles in regulating germination have been shown to have altered flowering time phenotypes (Penfield and Hall, 2009), an experiment was carried out to determine whether any of the *cosi* mutants was involved in regulating flowering time. The flowering time of plants grown at 15°C was measured by analysing the number of rosette and cauline leaves produced at first anthesis (Figure 6.14). The reduced growth rate of *cosi4* (Figure 6.13) was coupled with significantly later flowering in comparison to wild-type (Figure 6.14). This mutant flowered twenty five days later than wild-type and had approximately ten more rosette leaves that wild-type. On the other hand, *cosi3* displayed a wild-type flowering

phenotype, with the total days to flowering and number of rosette leaves being extremely similar to wild-type. *cosi2* flowered with a similar number of rosette leaves, but had a significantly greater number of cauline leaves and took eight more days to flower than wild-type. The increase in days to flowering of *cosi2* could reflect the slightly lower growth rate towards the end of the experiment (Figure 6.13). Although *cosi5* flowered with a larger number of leaves than wild-type, the days to flowering was shorter and this is likely to be due to its increased growth rate phenotype that is observed (Figure 6.13). *cosi1* was also early flowering (in terms of days and rosette leaf number) (Figure 6.14). These results suggest that the germination phenotype of some of the *cosi* mutants can be coupled with altered flowering time. In a number of cases, altered flowering time can be correlated with an altered growth rate. It is also possible that these two phenotypes are unrelated.

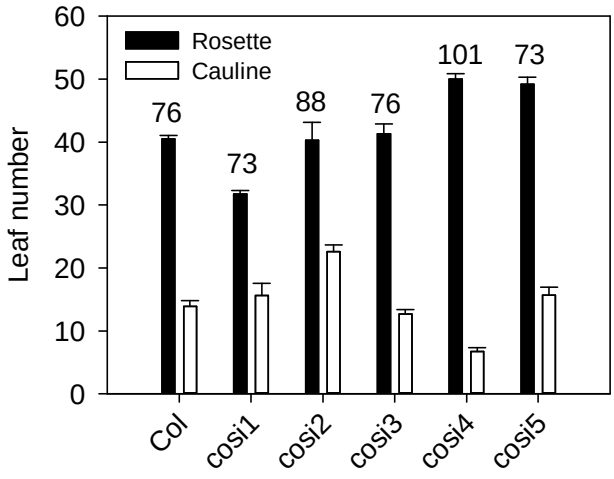

Figure 6.14: Flowering time of *cosi* mutants.
Plants were grown at 15°C under a long-day light regime. Flowering time was measured by number of rosette leaves, cauline leaves, and days post germination (above bars) until first anthesis. Data points are the average of ten plants and error bars represent standard error.

6.2.7 Genetic analyses of *cosi* mutants

6.2.7.1 Identification of *cosi1* as *hy2*

cosi1 displays a significantly longer hypocotyl in response to all light treatments in comparison to wild-type suggesting the mutant is incapable of inhibiting hypocotyl elongation (Figure 6.12). The long hypocotyl phenotype of *cosi1* in response to the various light treatments is much more striking than that of any single phytochrome mutants (Figure 6.12), suggesting that *cosi1* is not allelic to a single phytochrome mutant. Additionally, the ability to flower early in low temperature conditions suggests that multiple phytochromes must be absent or altered in this mutant (Figure 6.14). The long hypocotyl phenotype of *cosi1* can be likened to the *hy1, hy2* and *hy6* mutants (Koornneef et al., 1980, Chory et al., 1989), which have reduced levels of all phytochromes and exhibit an inability to inhibit hypocotyl elongation. A diallel cross was performed between *cosi1* and *hy1-1* and *hy2-1.* Hypocotyl elongation in response to red light was measured in the resulting F_1 progeny and these phenotypes confirmed that *cosi1* is allelic to *hy2 (*Figure 6.15). Therefore, it is likely that the insensitivity to cold stratification that is observed in *cosi1* may not reflect a specific defect in the response to the low temperature, but more so that dormancy levels cannot be overcome by the positive effects of light, since no active phytochromes are present.

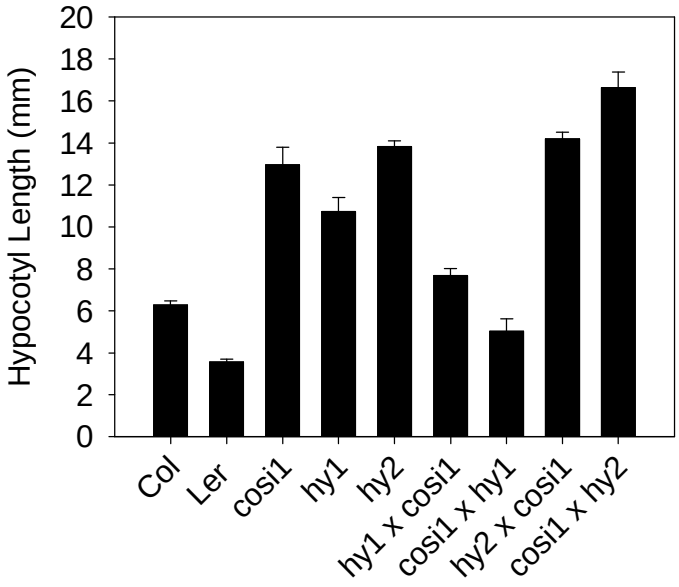

Figure 6.15: Alleism test of *cosi1* with *hy1-1* and *hy2-1*.
Hypocotyl elongation was measured in response to red light. All crosses were at the F_1 stage. Seeds were cold stratified for three days before being moved to constant light at 22°C for two days. Light was then changed to constant red light for seven days. Data points represent the average of seeds from five independent crosses and error bars represent standard error.

6.2.7.2 Reciprocal backcrossing of *cosi* mutants

Backcrossing of the *cosi* mutants to Col was carried out to determine the Mendelian segregation of the five *cosi* mutants that had been selected. Freshly harvested independent F_2 seed batches were cold stratified for up to three days and germination was analysed after seven days. A number of mutants had morphological phenotypes (such as the long hypocotyl of *cosi1*) that could be identified in the population and segregated with the cold stratification phenotype. However, for other mutants, such as *cosi3*, morphological phenotypes (dwarfing) did not segregate with the cold stratification insensitive phenotype. Although homozygous mutants could be identified for *cosi1* (Figure 6.16A), this mutant has already been identified as an allele of *hy2* and so no

further analysis was required. Identifying homozygotes in the backcrossed populations was particularly difficult for *cosi2* and *cosi5* (Figure 6.16B, E). *cosi2* itself didn't show a particularly strong cold stratification insensitive phenotype in this experiment and none of the backcrossed seed batches germinated to a lower level than wild-type (Figure 6.16B). Similarly, all *cosi5*xCol seed batches showed germination that was higher than wild-type (Figure 6.16E). Germination data for *cosi3* and *cosi4* looked as though the mutations were segregating in a dominant and recessive fashion respectively (Figure 6.16C, D).

Since *cosi3* appeared to have the most interesting and robust phenotype throughout the analyses presented in this chapter, the dominant nature of the mutation needed to be confirmed and so, the experiment was repeated. However, when this was repeated, *cosi3* germinated to approximately 60%, which was considerably higher than had previously been observed (Figure 6.17A). Consequently, it was difficult to determine homozygous mutants in this population. To try and avoid problems associated with identifying homozygotes, the F_3 seed was matured at either 12°C or 17°C before being swapped to 20°C approximately two weeks before harvest to try and accentuate the mutant phenotype. However, there was variation in the germination response of the *cosi3* backcrossed population and so again this made the identification of homozygotes within the population problematic (Figure 6.17B, C). When matured at 17°C, only two seed batches germinated to less than 90% (Figure 6.17B). Whereas when matured at 12°C, a large number of seed batches showed germination that was similar to *cosi3* (Figure 6.17C). The germination responses of seed batches that were matured at 12°C could be split into three groups, but did not reflect a sensible segregation pattern. It is extremely important to understand the way in which a mutation is segregating in order

to map it correctly. Since robust data could not be obtained for any of the *cosi* mutants map based cloning could not be carried out.

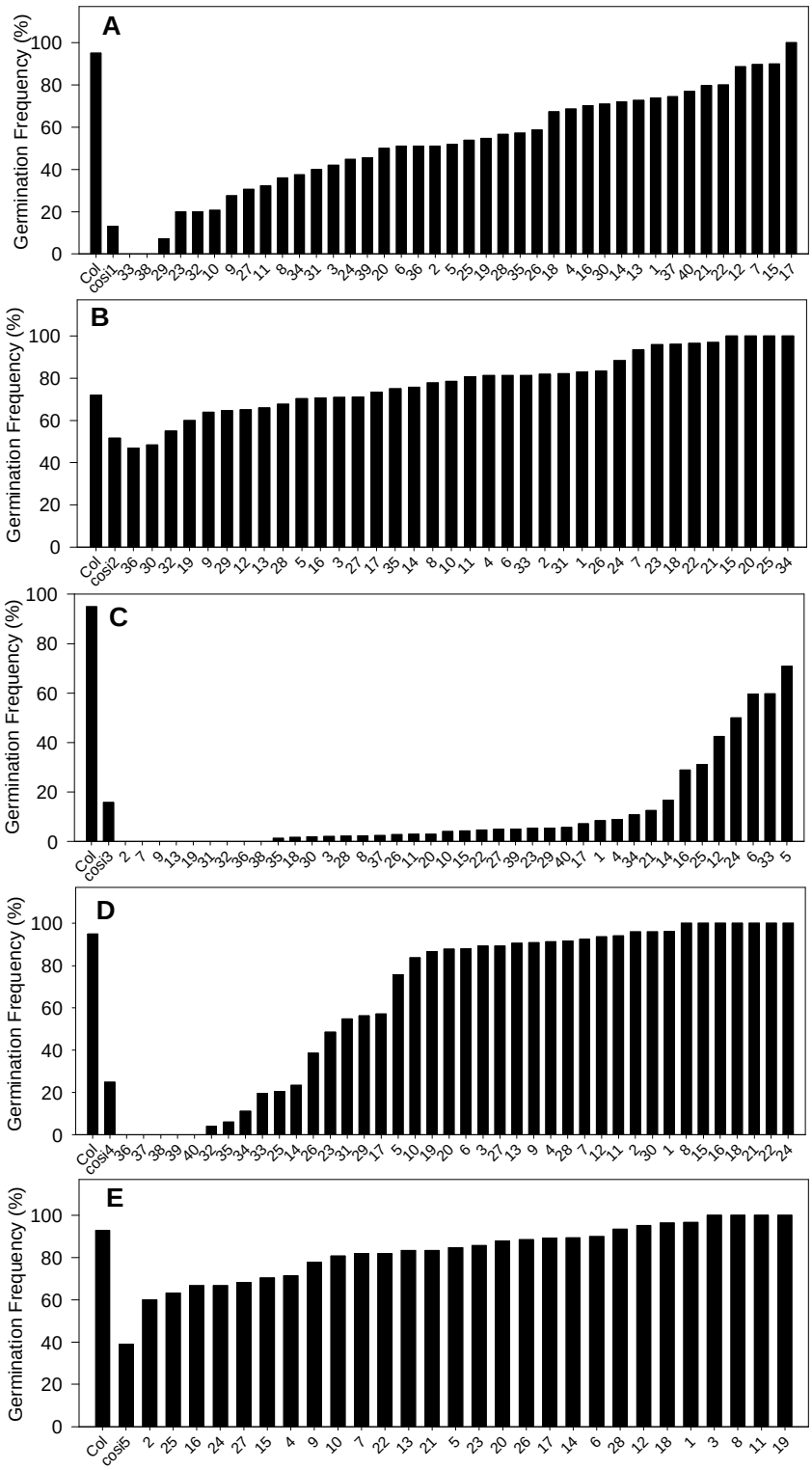
A
Germination Frequency (%)
0
20
40
60
80
100
Col
cosi1
33 38 29 23 32 10 9 27 11 8 34 31 3 24 39 20 6 36 2 5 25 19 28 35 26 18 4 16 30 14 13 1 37 40 21 22 12 7 15 17
B
Germination Frequency (%)
0
20
40
60
80
100
Col
cosi2
36 30 32 19 9 29 12 13 28 5 16 3 27 17 35 14 8 10 11 4 6 33 2 31 1 26 24 7 23 18 22 21 15 20 25 34
C
Germination Frequency (%)
0
20
40
60
80
100
Col
cosi3
2 7 9 13 19 31 32 36 38 35 18 30 3 28 8 37 26 11 20 10 15 22 27 39 23 29 40 17 1 4 34 21 14 16 25 12 24 6 33 5
D
Germination Frequency (%)
0
20
40
60
80
100
Col
cosi4
36 37 38 39 40 32 35 34 33 25 14 26 23 31 29 17 5 10 19 20 6 3 27 13 9 4 28 7 12 11 2 30 1 8 15 16 18 21 22 24
E
Germination Frequency (%)
0
20
40
60
80
100
Col
cosi5
2 25 16 24 27 15 4 9 10 7 22 13 21 5 23 20 26 17 14 6 28 12 18 1 3 8 11 19

Figure 6.16: Germination of backcrossed *cosi* mutants to wild-type.
Germination of approximately forty cosi1xCol F_2 (A), cosi2xCol F_2 (B), cosi3xCol F_2 (C), cosi4xCol F_2 (D) and cosi5xCol F_2 (E) freshly harvested seed batches. Seeds were cold stratified for three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles.

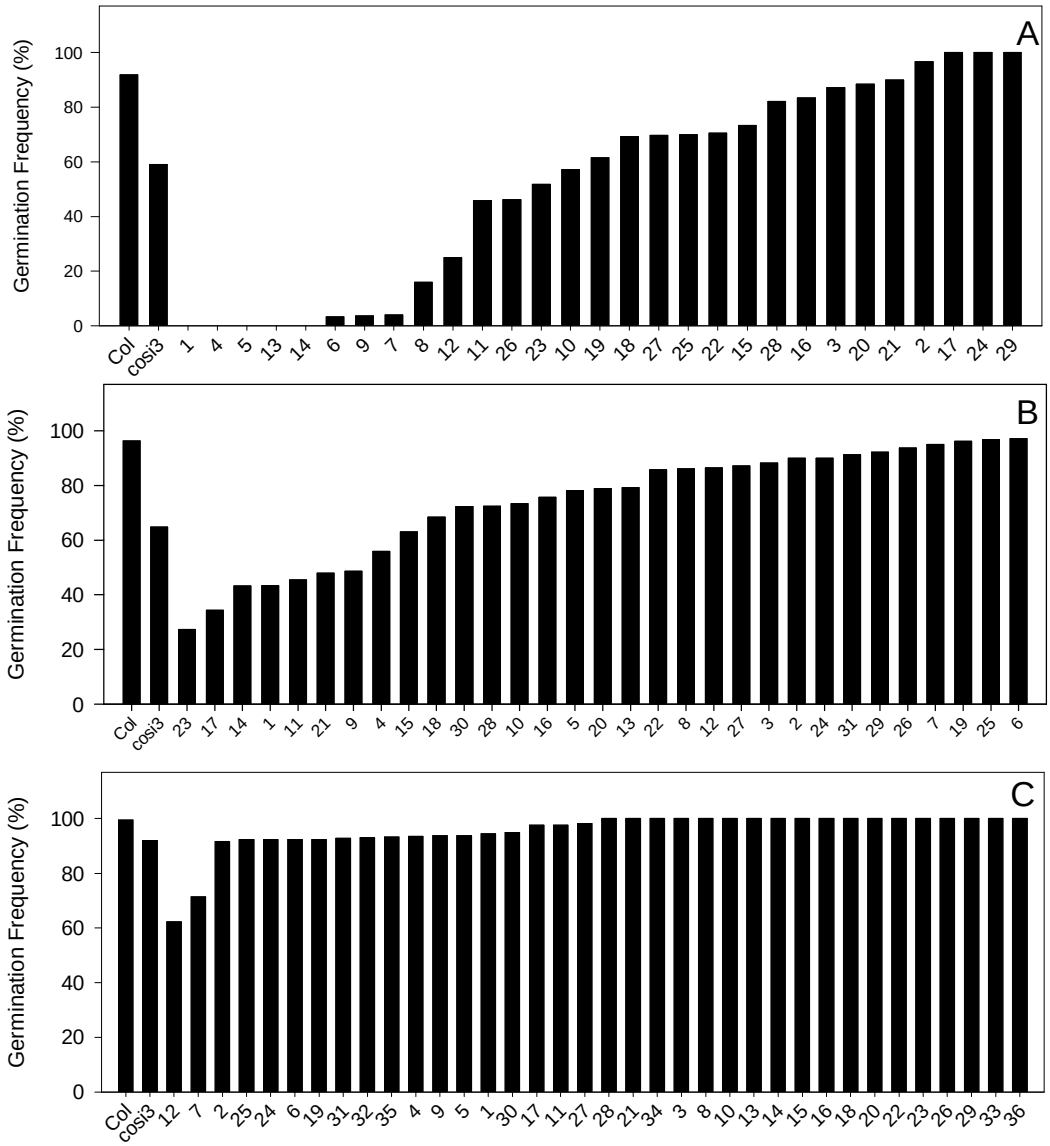

Figure 6.17: Germination of *cosi3*xCol F_3 seed batches.
Seeds were generated at the following temperatures: 20°C/16°C (A), 12°C (B) and 17°C (C). Freshly harvested seeds from approximately forty plants were cold stratified for three days. Germination was scored as radical protrusion following seven days at 22°C in twelve hour white light/dark cycles.

6.3 Discussion

6.3.1 Growth conditions affect *cosi* mutant phenotypes

A thorough characterisation of five *Arabidopsis* mutants, which were selected from a group of fourteen mutants that showed low germination in response to cold stratification, has been presented in this chapter. Initially, seeds were harvested from plants grown under glasshouse conditions; however, after testing multiple generations, phenotypes of these mutants were found to be highly variable between different experiments (Figure 6.2, 6.3). One reason for this could be due to the fluctuating environmental conditions that are experienced by plants grown in glasshouse conditions. Both light and temperature levels differ greatly between different times of year in the glasshouses.

When plants were grown in a growth room the mutant phenotypes could be reproduced to an extent, although some *cosi* mutants still germinated to approximately 70% (Figure 6.3B). Although conditions in the growth room are much more controlled than in the glasshouse, there are still instances where environmental fluctuations may occur. Therefore, it was decided that all future plants would be grown in a growth cabinet where conditions could be carefully controlled. Although it was now more certain that the environmental conditions remained the same for each experiment carried out, large variability in the phenotypes observed between experiments was still observed. For example, *cosi5* seeds germinated to approximately 90% following three days of cold stratification in the extended cold stratification experiment (Figure 6.4), whereas *cosi5* germinated to only approximately 10% in the imbibition temperature experiment (Figure 6.9). This is a difference of 80% between the two experiments. It is also possible that

the *cosi* mutants are not homozygous mutants and that the variation in the cold stratification insensitive phenotypes which are observed between experiments could be due to the mutants being heterozygotes.

6.3.2 *cosi3* shows ambient temperature insensitivity during imbibition

An interesting question that this chapter aimed to address was whether *cosi* mutants were insensitive to low temperature specifically during imbibition or whether they were insensitive to a range of different temperatures as a part of a broader temperature sensing defect. To investigate this, *cosi* mutant seeds were imbibed at 12°C, 17°C, 22°C and 27°C (Figure 6.9). Strikingly, *cosi3* displayed a lack of a germination response to these different imbibition temperatures, (Figure 6.9, 6.10) which suggests that this mutant may have defects in detecting differences in temperature correctly. It would have been interesting to determine whether the insensitivity to temperature of *cosi3* seeds is only during the imbibition period or occurs throughout plant development. This could have been analysed by looking at growth rate or flowering time in response to differing temperatures, however the time constraints of this work did not enable these experiments to be performed.

Germination of *cosi3* seeds did increase in response to extended cold stratification (Figure 6.4), which can be used to break strong primary dormancy (Penfield and Springthorpe, 2012). This suggests the low temperature signal may be sensed in *cosi3*, but the amount of cold required to cross the threshold at which germination is promoted is increased in this mutant. It is possible that germination of *cosi3* seeds could be increased further by extending cold stratification length past twenty eight days. On the other hand however, *cosi3* seeds may have been simply responding to the extended

'time' of imbibition in the dark and not in actual fact to temperature. Therefore, *cosi3* may be able to sense severe temperatures (i.e. 4°C) but is not able to detect temperatures in the range of 12°C to 27°C.

6.3.3 *cosi* mutants show altered hormone signalling

Since ABA and GA are known to be important regulators of germination, sensitivity of the *cosi* mutants to ABA and PAC were tested. Although there was variation in the response of the different *cosi* mutants to ABA and PAC, there were a large number of mutants that displayed hypersensitivity to ABA. The dwarfed appearance and dark green colouration of *cosi3* was typical of phenotypes displayed by GA biosynthesis mutants (Koornneef and Veen, 1980). Therefore, an experiment was carried out to investigate whether the dwarfed phenotype could be rescued by adding exogenous GA and showed that the growth could not be rescued to wild-type (Figure 6.8). Germination of *cosi3* seeds in the presence of GA was increased, and so the mutant was not insensitive to GA (Figure 6.6). Mutants that display a dwarfed phenotype are not limited to being involved in the GA pathway. BR mutants also display a dwarfed phenotype, and this can be reversed by the application of BR (Jang et al., 2000). BR is a positive regulator of germination and germination of the BR biosynthetic mutant *deetiolated2-1* (*det2-1*) and the BR insensitive mutant *brassinosteroid insensitive1-1* (*bri1-1*) is hypersensitive to ABA (Steber and McCourt, 2001).Therefore, *cosi3* could play a role in BR signalling, and germination responses of *cosi3* to exogenous BR could be tested in the future.

Lower germination levels of *cosi* mutants following cold stratification could reflect reduced expression of *GA3ox*, which are involved in the promotion of GA biosynthesis and is upregulated in response to cold stratification (Yamauchi et al., 2004). This

increase in *GA3ox1* expression contributes to an increase in bioactive GA levels. The addition of exogenous GA is potentially able to overcome the reduced germination of the *cosi* mutants and hence high germination levels can be promoted (Figure 6.6). Since exogenous GA generally has a greater effect on germination than NOR (Figure 6.6), this may suggest that the increase in GA biosynthesis during cold stratification may be more important than the decrease in levels of ABA i.e. higher GA levels promote higher germination than lower ABA levels do.

6.3.4 cosi1 is allelic to *hy2*

The phenotype that *cosi1* was selected because it appeared to become stronger with increasing generations. Germination of *cosi1* did not increase in response to after ripening (Figure 6.11), although germination was promoted to high levels in the presence of exogenous GA and NOR (Figure 6.6). This confirms that the seeds are viable, but the seeds do not respond to other germination promoting effects (e.g. prolonged cold stratification). The results have shown that *cosi1* was early flowering (Figure 6.14) and this is similar to *hy2* plants, which contain reduced levels of all phytochromes and exhibit an inability to inhibit hypocotyl elongation (Koornneef et al., 1980, Chory et al., 1989). *hy2* mutants flower with a reduced number of rosette leaves in comparison to wild-type in both short and long days (Koornneef et al., 1995). Low temperature (16°C) abolishes the early flowering phenotype of the *phyAphyBphyD* triple mutant. In contrast, the *phyAphyBphyDphyE* quadruple mutant does flower early at 16°C and thus suggests a role for phyE in temperature-dependent regulation of flowering (Halliday et al., 2003). The prominent role of PhyE at low temperature is also important for germination, whereby PhyE is required for promotion of germination specifically at low temperature (Heschel et al., 2007). The early flowering phenotype of

cosi1 at low temperature suggests that *cosi1* must lack several phytochromes (including PHYE), as when only the other phytochromes are absent (i.e. wild-type levels of PHYE), plants display a wild-type flowering time (Halliday et al., 2003). By making reciprocal crosses to *hy2-1, cosi1* could be identified as a novel allele of *hy2*. Germination of *hy2-1* seeds is reduced in response to five days of cold stratification (Donohue et al., 2008), although germination of *hy2-1* seeds is considerably higher than the germination of *cosi1* which has been shown in these experiments. These two mutants are in different ecotypes (*hy2-1* is in the L*er* background) and so this could explain the differences in germination responses observed.

6.3.5 Identification of homozygotes of backcrossed mutants is not robust

Unfortunately the segregation data that were produced from backcrossing the *cosi* mutants to Col were not robust and we were unable to identify the types of mutations (i.e. recessive or dominant) present in the mutants. *cosi3* had some particularly interesting phenotypes, but when backcrossed, it was difficult to identify homozygous mutants from the resulting population (Figure 6.17). It was, therefore, unfeasible to continue with further characterisation of the *cosi* mutant phenotypes as map based cloning would not have been able to be used to identify the location of the mutations. The importance of the *COSI* genes in the role of regulating temperature-dependent germination had to be questioned since there was a large amount of phenotypic variation and high dependence on certain environmental conditions observed throughout the experiments. Some growth conditions (i.e. long days at 20°C) induce such low levels of dormancy that germination can be as high as 100% without any cold stratification for wild-type seeds. Therefore, in these growth conditions the pathway involved in promoting germination in response to low temperature is not required to

promote germination since dormancy levels are low. So, it is important that the plant growth conditions in which seeds are generated induce enough dormancy so that cold stratification is required to promote germination. The plant growth conditions (in the growth cabinet) were long days with a day time temperature of 20°C and a night time temperature of 16°C. The lower night time temperature of 16°C appeared to promote the induction of dormancy levels, as germination of wild-type seeds generated under this temperature regime had higher dormancy levels than those generated at only 20°C (Figure 6.3B, 6.4). These dormancy levels induced by a lower night time temperature required cold stratification for germination promotion (Figure 6.4).

6.3.6 Conclusions

The data presented in this chapter have provided a thorough characterisation of five mutants that were isolated from a forward genetic screen that identified mutants whose germination was not increased in response to cold stratification. The results presented in this chapter highlight the importance of regulating the environmental conditions for seed production. One of the mutants, *cosi1* was found to be allelic to *hy2*. The genetic location of the remaining mutants could not be identified due to the inability to identify homozygotes when crossed to Col.

Chapter 7 General Discussion

The aims of this study were to increase the understanding of the role of temperature in regulating two important developmental processes: seed dormancy and germination in *Arabidopsis*. Although the importance of temperature as a key regulator of dormancy and germination has been known for many years, the mechanisms through which temperature controls these processes is not well understood. Thus, this study aimed to try and determine components of these mechanisms, primarily by identifying genes which have important roles in the regulation of dormancy and germination through the analysis of mutant dormancy phenotypes and a forward genetic screen.

A forward genetic screen was carried out to isolate mutants whose germination was not promoted by cold stratification and therefore to identify genes which have a role in this process. Understanding the way in which germination is regulated is of imperative importance as future temperature change has the potential to alter the germination of agricultural and horticultural plants. This screen revealed a number of interesting phenotypes for the five selected *cosi* mutants, especially for *cosi3*. However, the segregation of these mutants (based on the germination phenotype), when backcrossed to Col could not be robustly identified, and so mapping was not performed. In addition to the different germination phenotypes investigated, a number of the *cosi* mutants had flowering time and growth phenotypes which could be investigated further. It is possible that these phenotypes may be easier to detect in the backcrossed populations, and therefore mapping could be carried out on these mutants.

The experiments carried out to characterise the *cosi* mutants highlighted the importance of environmental effects on dormancy phenotypes and directed studies to

understanding how temperature regulates dormancy during seed maturation. Specifically, the roles of components of the cold acclimation pathway and the hormones ABA and GA in regulating dormancy in response to temperature was investigated. A summary of the results from this work which indicates pathways through which temperature regulates dormancy is shown in Figure 7.1.

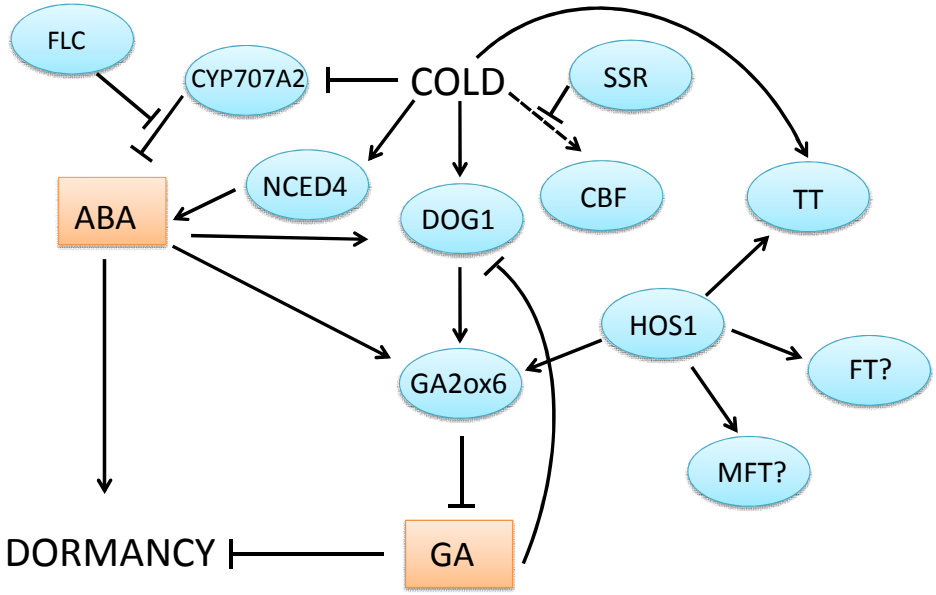

Figure 7.1: Proposed pathways mediating the effect of seed maturation temperature on dormancy.
Cold induction of *CBF* transcription is inhibited by an unknown seed-specific repressor of *CBF* expression (SSR), although CBFs are still required for normal dormancy levels. Cold induces high levels of dormancy through more than one mechanism, which includes the elevation of *DOG1* expression and the action of DOG1 in the promotion of *GA2ox6* expression. *GA2ox6* expression is also promoted by ABA during seed maturation (Seo *et al*., 2006). GA represses expression of *DOG1*, whilst ABA promotes its expression. Cold promotes expression of *NCED4* and represses expression of *CYP707A2*, which may require regulation by FLC (Chiang *et al*., 2009). ABA promotes dormancy whilst GA represses it. Cold and HOS1 promote expression of *TT* genes. Although HOS1 is not involved in the temperature regulation of dormancy, it may feed into the pathway downstream of the cold signal, where it induces expression of *GA2ox6*. The role of HOS1 in regulating dormancy may involve MFT and FT.

The results presented in this study show that a reduction in temperature during seed maturation leads to an increase in dormancy levels (Figure 3.2). Increases in dormancy levels delay germination, thus shifting the time frame of development due to differences in environmental conditions. In agricultural situations it is important that correct

dormancy levels are induced, to ensure germination occurs at the right time (i.e. avoid pre-harvest sprouting but attain uniform germination in the field). In the future it will be important to analyse the effect of higher temperatures (such as 25°C/27°C) on dormancy levels, as temperatures during seed maturation are beginning to increase in response to climate change. Given that thermoinhibition represses germination at high temperatures during imbibition, one may predict that high maturation temperatures may induce higher dormancy levels.

Increases in dormancy levels in response to temperature are coupled with an increase in the levels of ABA and a reduction in GA levels in dry seeds (Figure 3.7), suggesting that the regulation of ABA and GA metabolism is important for the mechanism that controls the temperature regulation of dormancy. Altered levels of ABA and GA by temperature are coupled with the promotion of *DOG1, GA2ox6* and *NCED4* expression and repression of *CYP707A2* expression by low maturation temperature in dry seeds (Figure 3.16). *cyp707a2-1* mutant seeds showed increased dormancy when matured at 17°C and 15°C (Figure 3.13), whilst *dog1-2* seeds display decreased dormancy levels when matured at low temperature (Figure 3.14). Roles for DOG1 and CYP707A2 in regulating dormancy have been shown in the past (Kushiro et al., 2004, Bentsink et al., 2006, Nakabayashi et al., 2012). A recent study investigating the role of DOG1 in dormancy regulation also showed that expression of *DOG1* was upregulated by low temperatures during seed maturation and that DOG1 protein levels were elevated in response to the low temperature (Nakabayashi et al., 2012).

NCED4 expression was found to be upregulated in *DOG1* NIL seeds, one of a number of *DOG* NILs which were derived from crosses between L*er* and accessions from different world regions (Bentsink et al., 2010). However, the dormancy phenotypes of

nced4 mutants are yet to be tested. Knock down and overexpression of *GA2ox6* leads to a reduction and increase in the dormancy levels which are induced respectively (Wang et al., 2004). *ga2ox6-1* mutant seeds show decreased sensitivity to low concentrations of PAC and the increased dormancy of seeds overexpressing *GA2ox6* cannot be rescued by the application of exogenous GA (Wang et al., 2004), which suggests that the reduction in GA levels is not the sole contributor to the dormancy phenotype of these seeds. The dormancy phenotypes of loss-of-function *ga2ox6* and *nced4* mutants in response to low temperature during maturation have not been characterised in this study. One may predict that these mutants would display reduced dormancy phenotypes in response to maturation at low temperature. This would need to be tested to fully understand the role played by NCED4 and GA2ox6.

CBF-deficient seeds show a decrease in the dormancy levels that are induced when seeds are matured at 15°C (Figure 4.2). Loss of functional *CBF* expression primarily results in a decrease in *DOG1* and *GA2ox6* expression (Figure 4.3). Given the reduced expression of *GA2ox6* in *dog1-2* mutant seeds (Figure 3.18B), one may hypothesise that the CBFs principally regulate *DOG1* expression alone and the reduced *GA2ox6* expression in *CBF*-deficient seeds is a consequence of reduced *DOG1* expression. However, GA levels were found to be either unaltered or higher in *CBF*-deficient seeds in comparison to wild-type (Figure 4.4B) and *dog1-2* seeds display reduced levels of GA in comparison to wild-type (Figure 3.19). This suggests that the regulation of GA levels by the CBFs cannot solely be through regulation of *GA2ox6* expression by DOG1. In seedlings, overexpression of *CBF1* leads to the upregulation of *GA2ox3* and to a lesser extent *GA2ox6* (Achard et al., 2008). Results from this study show that *GA2ox3* is also upregulated in *CBF*-overexpressing seeds, but there is not a corresponding downregulation in *CBF*-deficient seeds (Figure 4.8F, 4.3F). In fact for

cbf2 and *CBF3* RNAi seeds, expression of *GA2ox3* is upregulated in comparison to wild-type (Figure 4.3F).

Although maturation at 15°C reveals a dormancy phenotype for the *CBF*-deficient seeds, the *CBFs* do not regulate dormancy through the temperature regulation of their expression (Figure 4.10). Consistent with this is the fact that the results from the transcriptome analysis suggest that only *COR47* expression is upregulated by low maturation temperature in wild-type seeds (Table 5.1). Conversely the expression of both *CBF1* and *CBF2* is downregulated in low temperature matured wild-type seeds (Table 5.1). Together, these results suggest that the expression of *CBFs* is not promoted by either short or long exposure to low temperature in seeds. Therefore, the regulation of *CBF* expression by temperature in seeds is different to what is observed in seedlings, where exposure to low temperature promotes expression of *CBFs* (Gilmour et al., 1998). The promotion of *CBF* expression by low temperature is essential for cold acclimation, since *CBF1* and *CBF3* RNAi plants display increased sensitivity to freezing temperatures (Novillo et al., 2007). However, *cbf2* mutant plants display increased freezing tolerance and this is due to increased expression of *CBF1* and *CBF3* (Novillo et al., 2004, Novillo et al., 2007). Consequently, these results suggest that the temperature regulation of *CBF* expression is not involved in the regulation of dormancy levels by low temperature.

In vegetative tissues, HOS1 is a negative regulator of the cold acclimation pathway as it targets ICE1, which promotes *CBF3* expression, for ubiquitination (Ishitani et al., 1998, Lee et al., 2001, Chinnusamy et al., 2003, Dong et al., 2006a, Lazaro et al., 2012). *hos1-1* seedlings display increased expression of *RD29A, CBF2* and *CBF3* (Lee et al., 2001) however, in seeds only expression of *CBF2* was upregulated in comparison to

wild-type in *hos1-3* seeds (Table 5.2). *CBF1* was also found to be downregulated in *hos1* seeds, but the differential expression was not significant (Table 5.2). As mentioned, there is evidence to suggest that CBF2 acts as a negative regulator of *CBF1* and *CBF3* expression (Novillo et al., 2007) and, therefore, this increase in *CBF2* expression could be involved in a negative feedback loop with *CBF1* and *CBF3.* High levels of *CBF* expression would not correspond with the low dormancy phenotype of *hos1* seeds and so this may suggest that the regulation of *CBF* expression by HOS1 is quite different in seeds to seedlings.

Of course it is possible that another negative regulator of *CBF* expression, a transcription factor called MYB15 (Agarwal et al., 2006), could be responsible for lack of temperature regulated *CBF* expression in seeds. Expression of *MYB15* peaks during the later stages of seed development (Figure 4.15). If loss-of-function *myb15* seeds contained elevated levels of *CBF* expression, one may hypothesise that the dormancy levels of these seeds would be elevated. Loss-of-function *ice1-2* seeds matured at 10°C appear to be less dormant than *CBF*-deficient seeds matured at 10°C (Figure 4.14, 4.2), thus suggesting that ICE1 may be a more important regulator of dormancy. In contrast to its role in promoting *CBF3* expression in vegetative tissues, it is possible that ICE1 may have additional targets in seeds. Therefore, it is possible that the regulation of *CBFs* in seeds may not involve components of the cold acclimation pathway and this is possible given that temperature transcriptomes of seeds and seedlings are very different (Kendall et al., 2011).

Since low temperature during imbibition promotes germination, and the overexpression of *CBFs* leads to an increase in dormancy levels (Figure 4.10), one hypothesises that inhibition of *CBF* expression by low temperature during imbibition could be part of the

mechanism by which low temperature promotes germination. *CBF*-overexpression in vegetative tissue represses growth through a decrease in bioactive GA levels due to increased expression of *GA2ox3* and to a lesser extent *GA2ox6* (Achard et al., 2008). The promotion of germination of dormant seeds by low temperature during imbibition would not be predicted to occur in the presence of strong cold regulated *CBF* expression, as this requires an increase in GA levels (Yamauchi et al., 2004). Thus, the repression of *CBF* expression by low temperature in seeds is likely to represent an essential component of the pathway by which low temperature promotes germination. The fact that *CBF1* and *CBF2* were found to be downregulated by low maturation temperature (Table 5.1) suggests that the inhibition of *CBF* expression by low temperature may be initialised during seed development.

Seeds are able to cycle in and out of dormancy until the environmental conditions are favourable for seedling establishment. Although expression of *CBF1* was found to be very low in imbibed seeds (Figure 4.10A), it would be interesting to determine if expression of *CBFs* is induced by environmental conditions which would promote the transition to secondary dormancy. For example, is *CBF* expression promoted by prolonged exposure to low temperature during imbibition which can promote entry into secondary dormancy (Finch-Savage et al., 2007, Penfield and Springthorpe, 2012)? The mechanism that regulates the promotion of secondary dormancy by low temperature during imbibition must involve a component which is able to act upon crossing the threshold of 'cold days' required for the induction of secondary dormancy. This mechanism could share components with the vernalisation pathway by which an accumulation of cold days promotes flowering due to the silencing of *FLC*, which involves upregulation of *VIN3* expression (Sung and Amasino, 2004).

The regulation of dormancy by HOS1 has been determined as being through a maternal pathway (Figure 5.8). However, the nature of the regulation of dormancy by the CBFs has not been determined, given the fact that there was variation in both *CBF*-deficient and wild-type dormancy phenotypes when the seeds were matured at low temperature, although the data which are presented in this study are representative of a large number of experiments. However, it is possible that the CBFs could also act maternally to control seed dormancy. Since *CBF* expression is not temperature regulated in seeds, it is possible that *CBF* expression could be temperature regulated in vegetative tissue, which could be transmitted to the developing seed through downstream signalling.

Other genes have been identified that are involved in the maternal control of seed dormancy and germination. Two zinc finger transcription factors DOF ACTIVATING GERMINATION (DAG1) and DAG2 act as negative and positive regulators of germination respectively (Papi et al., 2000, Gualberti et al., 2002, Papi et al., 2002). Although seed coats of *dag1* seeds are structurally unaltered, they are more permeable and mucilage is released quicker following imbibition, which suggests that the seed coat could be weaker than wild-type (Papi et al., 2002) and this would be consistent with the fact that these seeds show increased germination. The seed coat acts as an important regulator of dormancy, by acting as a mechanical constraint for radicle protrusion and by regulating the permeability for water and oxygen uptake. *DAG1* and *DAG2* are not expressed in developing seeds but exclusively in the vasculature of vegetative tissues (Papi et al., 2000, Gualberti et al., 2002, Papi et al., 2002), which suggests that the DAG1 protein may re-locate from the vasculature to the seed to regulate dormancy during seed development.

There is evidence for signals which are involved in regulating flowering time moving from their site of synthesis to their site of action. The floral promoter FT is an example of a mobile signal which acts over a long range (Jaeger and Wigge, 2007). The FT protein is synthesised in the vasculature of the leaves, but moves to its site of action, the shoot apical meristem (Jaeger and Wigge, 2007). The TFL1 protein is another example of a mobile signal which is synthesised in the inner cells of mature shoot meristems and moves to the outer cells to repress flowering (Conti and Bradley, 2007).

Additional evidence for the maternal control of seed dormancy comes from the study of mutants of the *TT* group of genes which have reduced flavonoid pigmentation and show reduced dormancy levels (Debeaujon et al., 2000). The results in this study also implicate a role for TT in regulating dormancy in response to low temperature. A number of *TT* genes are upregulated in response to low maturation temperature and are downregulated in *hos1-3* seeds in comparison to wild-type (Table 5.1 and 5.2). Additionally, *tt5-1* and *tt6-1* show a reduced ability to induce high dormancy levels in response to maturation at low temperature (Figure 5.27). However, when seed coat morphology was analysed in wild-type seeds matured at 20°C and 10°C, no differences were observed (Figure 3.5). However, a more thorough analysis of the seed coat should be carried out to determine if differences in temperature or the *hos1* mutation lead to alterations in permeability and colour.

A number of flowering time genes has been shown to have dormancy phenotypes. In this study, the dormancy phenotypes of *arp6-1* and *tfl2* mutants have been characterised. Dormancy induction by low temperature is abolished in *tfl2* mutants, whilst some dormancy is still induced in *arp6-1* mutants (Figure 5.12, 5.13). Additionally, the *lhycca1* double mutant and the *early bolting in short days* (*ebs*) mutant

which are also early flowering display reduced dormancy phenotypes (Gómez-Mena et al., 2001, Penfield and Hall, 2009). Therefore, these results suggest that in a number of examples early flowering is coupled with reduced dormancy. Genes which are involved in regulating flowering time could be part of a common mechanism to regulate dormancy, although the analysis of dormancy phenotypes of double mutants would need to be carried out to determine this. On the other hand, the link between early flowering and reduced dormancy may be a consequence of altered expression of target genes which are involved in regulating dormancy, which would suggest that the flowering time genes do not have a direct role in regulating dormancy.

This work has highlighted a number of genes which are likely to have important roles in regulating dormancy. In the future, improving the understanding of the role of HOS1 in the maternal control of dormancy will be important. In particular, determining whether GA is required for the low dormancy phenotype of *hos1* mutants will be key to this. Additionally, further characterisation of the role of TT in regulating dormancy in response to temperature will aid the understanding of the function of the seed coat. Understanding how CBFs are regulated by low temperature during seed development and imbibition, and how this contributes to the regulation of dormancy and germination is another area of work that should be continued. This should also be followed up with determining the role of CBFs in crops such as maize and soybean whose germination is sensitive to chilling.

The work carried out in this thesis has focussed on understanding how dormancy is regulated by temperature at the transcriptional level. In the future it will also be important to extend this to understanding post-transcriptional and post-translation mechanisms that may have a role in regulating this process.

Chapter 7: General Discussion

The depth of dormancy and timing of germination has the potential to effect the time at which various developmental processes occur and the environmental conditions which are experienced during these processes. The data in this thesis has shown that temperature is a key regulator of dormancy, and the dormancy levels which are induced control when germination occurs. Future temperature changes have the potential to alter the environment in which seeds are matured on the plant, thus altering dormancy levels and leading to implications for seed quality, viability and germination timing. Therefore to ensure that quality seeds which germinate in a uniform manner can be consistently produced it will be important for the implications of temperature to be well understood. Additionally, it is possible that some of the important dormancy regulating genes which have been identified in this study could be used as targets for breeding programmes.

To summarise, the results in this thesis have shown that temperature is an important regulator of the levels of dormancy that are induced during seed maturation. The regulation of dormancy by temperature involves regulation of ABA and GA metabolism through expression of *GA2ox6, NCED4* and *CYP707A2*. This work has also revealed roles for the CBFs and HOS1 in regulating dormancy through a temperature independent mechanism. HOS1 acts through a maternal pathway to regulate dormancy and this involves alterations in GA levels. Finally, a forward genetic screen was carried out to investigate how cold stratification promotes germination, and although none of the characterised mutants could be mapped, the screen did identify some interesting phenotypes.

Abbreviations

ABA	Abscisic Acid
BR	Brassinosteroid
CFP	Cyan Fluorescent Protein
cDNA	Complementary DNA
DAPI	4’,6-diamidino2-phenylindole
dNTP	Dinucleotide triphosphate
DTT	Dithiothreitol
EGTA	Ethylene Glycerol Tetraacetic Acid
EMS	Ethyl Methyl Sulphonate
FAME	Fatty Acid Methyl Esther
GA	Gibberellin
GFP	Green Fluorescent Protein
HCl	Hydrochloric Acid
JA	Jasmonic Acid
KCl	Potassium Chloride
KOH	Potassium Hydroxide
LC-MS	Liquid Chromatography – Mass Spectrometry
LiCl	Lithium Chloride

Abbreviations

M	Molar per Litre
Mm	Millimole
mm	Millimetre
mm^2	Millimetre squared
µM	Micromole
µm	Micrometre
µl	Microliter
MES	4-Morpholineethanesulfonic acid
MS	Half strength Murashige and Skoog medium
mRNA	Messenger RNA
NASC	National Arabidopsis Stock Centre
NOR	Norflurazon
NPC	Nuclear Pore Complex
OPDA	12-oxo-phytodienoic acid
PAC	Paclobutrazol
PCR	Polymerase Chain Reaction
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
T-DNA	Transfer DNA
tRNA	Transfer RNA
UPLC-MS	Ultraperformance Liquid Chromatography-Mass Spectrometry

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