Functional Analysis of a Conifer Protein that Interacts with the Global Transcriptional Regulator, Abscisic Acid Insensitive 3

by

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B.Sc., Simon Fraser University, 2009

Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Science

in the
Department of Biological Sciences
Faculty of Science

SIMON FRASER UNIVERSITY

Summer 2013

Approval

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Degree: **Master of Science**

Title of Thesis: ***Functional Analysis of a Conifer Protein that Interacts with the Global Transcriptional Regulator, Abscisic Acid Insensitive 3***

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Date Defended/Approved: May 16, 2013

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Abstract

ABI3 (*Ab*scisic acid *I*nsensitive 3) is an evolutionarily highly conserved transcription factor that is involved in seed development, stress signaling, and the induction and maintenance of seed dormancy. In previous work, the yellow-cedar (*Callitropsis nootkatensis*) ABI3 ortholog (*CnABI3*) was isolated and yeast two-hybrid approaches revealed CnAIP1 as a yellow-cedar protein that physically interacts with CnABI3. The CnAIP1 protein bears a high degree of similarity to eukaryotic heat shock protein associated factors, particularly those found in Arabidopsis (HIP1 and HIP2). *CnAIP1*::*GUS* reporter constructs expressed in Arabidopsis showed that the gene was temporally regulated during seed development, and its expression was enhanced in stressed seedlings. Seeds of *CnAIP1*-overexpressing Arabidopsis lines exhibited hypersensitivity to ABA and glucose as far as the inhibition of their germination was concerned, and their dormancy was enhanced. My results suggest that CnAIP1 is a positive regulator of seed development processes and works synergistically with CnABI3 to regulate dormancy.

Keywords: Abscisic Acid Insensitive 3; *Callitropsis nootkatensis*; Arabidopsis; CnAIP1; seed dormancy; abiotic stress

Acknowledgements

I would like to express my sincere gratitude to Dr. Allison Kermode, my senior supervisor, for her guidance, knowledge and insight. I've learned a lot about myself and my work in plant science and am thankful for the opportunity she gave me. I would like to thank Dr. Sherryl Bisgrove, my supervisor, for her helpful contributions towards my research work.

I am grateful to all the members of the Kermode Lab for contributing to a positive and enjoyable lab environment. I value all the feedback and experimental advice received. Special thanks to the "conifer group" – Kerstin Mueller, Ying Zeng and Tiehan Zhao – I am appreciative of all the troubleshooting, technical advice, feedback and result discussions that helped me reach this point.

My sincerest appreciation is extended to my family and close friends who always kept me motivated and believed in my success. A very special acknowledgement is extended to my mom and dad who have stood by me throughout this journey. Thank you for helping me reach my academic dreams and giving me the strength to persevere till the end.

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1. Introduction and Literature Review

1.1. Introduction to Seed Dormancy and Germination

The seed plays an important role in the plant life cycle in regards to maximizing survival of a given species. Seeds contain an embryo which gives rise to the new plant; they are structurally and physiologically prepared for dispersal following the completion of development. Within the confines of a seed are substantial food reserves to sustain germination and growth of a seedling until it is able to be an independent autotrophic organism. During the period of dispersal following seed development and maturation and before the establishment of a new seedling, dispersed seeds are often able to survive for extended periods of unfavourable environmental conditions without germinating (Bentsink and Koornneef, 2008). The mature quiescent seed will germinate if the surrounding environmental conditions such as light intensity, temperature, oxygen levels and water availability are appropriate for subsequent seedling growth. Germination is defined as the uptake of water by the quiescent seed and elongation of the radicle which results in its emergence from seed structures surrounding the embryo (Bewley and Black, 1994; Bewley 1997).

Regulation of seed dormancy and germination is vital to ensure the survival of the next generation. Primary dormant viable seeds do not germinate upon dispersal even under favourable conditions. The completion of germination relies on a balance between the growth potential of the elongating radicle and the restraining strength of the surrounding tissues (Koornneef *et al*., 2002). The conifer species that this work is focused on, yellow-cedar (*Callitropsis nootkatensis*), is one example of many plant species that produce dormant seeds. Dormancy is imposed on these seeds during their development and these seeds require specific environmental conditions such as prolonged periods of warm-dry conditions (after-ripening), moist chilling (cold stratification) and even smoke, in order to terminate dormancy (Adkins *et al*., 1986, Egerton-Warburton, 1998). Secondary seed dormancy can be induced in mature

nondormant dispersed seeds by prolonged exposure to unfavourable environmental conditions including low oxygen levels in soil, poor light intensity, reduced water availability and non-ideal temperatures (Bewley 1997). Seeds that enter secondary dormancy lose sensitivity to dormancy-breaking cues. As a result, some seeds may continually gain and lose dormancy if the surrounding conditions are not optimal for germination. This process is referred to as dormancy cycling.

While the process of seed germination can be considered an all-or-nothing occurrence, induction and depth of dormancy can vary greatly from deeply dormant to nondormant depending on the surrounding conditions to which the seed is exposed. Seed dormancy is an adaptive trait because it allows the distribution of germination in time and space. In doing so, seed dormancy decreases competition for resources between and among species, allows the survival of species following environmental disturbances and ensures germination in the appropriate season to maximize survival. Seed dormancy is determined by genetic factors and influenced by environmental factors and thereby provides plant species the opportunity to adapt to diverse habitats (Graeber *et al*., 2012).

The two major types of dormancy are coat-enhanced dormancy and embryo dormancy. In coat-enhanced dormancy, it is the surrounding seed structures (endosperm, testa, megagametophyte) which prevent the embryo from germinating (Kermode 2005). When yellow-cedar embryos are removed from mature dormant seeds and placed in water they are able to germinate demonstrating that the enclosing seed structures have an inhibitory effect (Schmitz *et al*., 2002). The surrounding seed structures could prevent germination by mechanically blocking radicle emergence, inhibiting water uptake and gas exchange and/or producing germination inhibitors such as abscisic acid (ABA) (Kermode 2005). Coat-enhanced dormancy is the primary form of dormancy found in conifer species such as yellow-cedar (Ren and Kermode, 1999, 2000). In embryo dormancy, the embryo is unable to germinate when excised from its surrounding seed tissues and placed on water. Therefore it is the embryo that is dormant and dormancy is not influenced by surrounding seed tissues. Embryo dormancy may be due to the presence of germination inhibitors such as ABA. In some species, seeds may have both types of dormancy simultaneously or successively (Bewley and Black 1994).

Bewley (1997) questions whether dormancy is the result of an insufficiency of a cellular event required for germination or whether dormancy must be negated before germination can commence. To complicate matters, only a few cells surrounding the embryonic root axis may be involved in terminating dormancy and initiating germination (Bewley 1997). Additionally once dormancy is terminated, there is probably a signal transduction pathway that networks with other signal pathways to coordinate cellular responses. These types of signal transduction pathways can be elucidated by several studies that have performed "omic" analyses on Arabidopsis seed transcriptomes and proteomes (Holdsworth *et al*., 2008).

The regulation of seed dormancy and germination is critical for the agriculture and forestry industries. Dormancy can be considered an undesirable trait for the agriculture industry when there is a requirement for seeds to germinate uniformly and vigorously during expected time intervals. Seed populations generally do not germinate uniformly and the minimal stimulus required to reach threshold and promote germination varies among individual seeds. Although extensive breeding programs have attempted to maximize germination levels and uniformity, a balance between seed dormancy and germination is required. For example, reduced harvest dormancy of cereal crops can cause pre-harvest sprouting in the grains thereby reducing the value and quality of the grain product (Bewley and Black, 1994). Mutants of these cereals which are not dormant also have reduced seed longevity, i.e., a reduction in seed viability during long-term storage (Clerkx *et al*. 2003). In the forestry industry, nursery breeding programs test various lengthy treatments to break dormancy of deeply dormant seeds of some conifer species. From an industrial, agronomic and economic perspective, knowledge of the mechanisms that underlie seed dormancy and the requirements for transition from dormancy to germination can be useful to produce economically useful and valuable crops and breeding programs.

1.2. Role of abscisic acid (ABA) in Seed Development, Dormancy Inception and Maintenance

There are several lines of evidence to support a role for abscisic acid (ABA) in seed dormancy induction during seed development. ABA is known to be involved in

many key events during seed development and is responsible for maintaining seeds in a developmental state while still attached to the parent plant until sufficient reserves have accumulated for successful germination (Kermode 1995, 2005; Holdsworth *et al*. 1999; Finkelstein *et al*., 2002). In addition, seeds are kept in this developmental state until sufficient reserves have accumulated for successful germination (Kermode 2005). The biosynthesis of ABA and the accumulation of these storage protein reserves ensure that seeds continue to remain in the maturation stage of development thereby preventing precocious germination (Kermode 1990, 1995). ABA is a sesquiterpene phytohormone whose biosynthesis and catabolism create changes in hormone signaling pathways that subsequently affect gene expression. Fluctuations in ABA level thereby are able to control transitions from dormancy to germination and from germination to growth (Nambara and Marion-Poll, 2005). However levels of this hormone alone are not the key regulatory control.

Embryo ABA levels as well as the minimum threshold of ABA required to initiate or maintain seed developmental processes change during development. During early seed development when histodifferentiation, cell division and basic pattern formation occurs, ABA levels in the seed are quite low. Typically, ABA content peaks at mid-development during the expansion stage when protein and lipid storage reserves are being synthesized and dormancy is induced. There is then a drastic drop in ABA levels during the later stage of development associated with maturation drying and metabolic inactivity (Bewley and Black, 1994; Kermode, 1995; Meinke 1995; Bewley 1997). This hormone appears to regulate the deposition of storage reserves in seeds, induction and maintenance of seed dormancy, acquisition of desiccation tolerance and prevention of precocious germination (Kermode, 2005). The changes in ABA level during seed development positively correlate with the expression of the gene encoding the ABA biosynthesis enzyme zeaxanthin epoxidase (Audran *et al*., 1998; Seo and Koshiba, 2002).

Crosses between *aba* mutants and wild-type plants show that the mother plant and seed coat maintains ABA synthesis during seed development and maturation, but dormancy induction is dependent on ABA synthesized in the embryo and/or endosperm (Karssen *et al*., 1983). It is not known if maternal ABA penetrates the embryonic axis, but it does have effects on other aspects of seed development such as increasing seed

yield and rate of embryo growth as observed in *N. plumbaginifolia* and carrot (Frey *et al*., 2004, Homrichhausen *et al*., 2003).

There are also changes in embryo sensitivity to ABA during seed development and changes to the minimum threshold of ABA required to initiate or maintain seed developmental processes (Xu and Bewley, 1991; Kermode 2005; Jiang *et al*., 1996). Additionally, environmental conditions to which a seed is exposed to during development can have an effect on ABA levels and sensitivity. Changes in temperature, light intensity, gas exchange and/or water availability can alter ABA levels and consequently alter seed dormancy during development (Kermode, 2005).

Detailed transgenic and mutant studies have been performed to determine the role and contribution of ABA in seed developmental processes. Mutants from many species including *Arabidopsis* and maize were created that are either deficient in ABA due to defects in the ABA biosynthesis pathway or are insensitive to this hormone (i.e. response mutants). The former mutations result in decreased ABA levels and reduced seed dormancy which disrupts normal seed maturation and can even result in vivipary or precocious germination. Viviparous mutant seeds transition directly from development to germination while still attached to the parent plant without undergoing maturation drying. ABA-deficient mutants of *Arabidopsis* (*aba1*), tobacco (*aba2*) and maize (*viviparious*(*vp*)*5*) have reduced seed dormancy (Karssen *et al*.,1983; Tan *et al*., 1997; Frey *et al*., 1999; White *et al*., 2000). In the *aba2* mutant, ABA deficiency is due to mutation in the *ABA2* gene which encodes zeaxanthin epoxidase, a key enzyme required in the ABA biosynthetic pathway (Marin *et al*., 1996). Some mutants with reduced dormancy have weakened seed coat structures and reduced testa pigmentation. These mutants have reduced proanthocyanidins, polymers which become cross-linked during maturation to give rigidity to the seed coat, thereby reducing permeability to water, gases and hormones and creating a barrier to subsequent germination (Kermode 2005; Debeaujon *et al*., 2001). ABA-deficient and ABA-insensitive *Arabidopsis* mutants have a thinner mucilage layer surrounding the seed coat which then permits water and oxygen uptake (Karssen *et al*., 1983). Dormancy is reduced in ABA-deficient mutant seeds; chemical inhibition of ABA biosynthesis or the sequestering of ABA by antibodies in the seed has similar effects on dormancy (Lin *et al*., 2007; Nambara and Marion-Poll 2003). The ABA-deficient mutants are able to produce some

ABA due to the presence of redundant genes and similar pathways compensating for mutated genes. The maize mutant *vp14* has 30% ABA content when compared to wild-type seeds. The ABA content of these mutant seeds allows for normal development until the final stages of development; however precocious germination is observed at the later stages (Tan *et al*., 1997). The *Arabidopsis* insensitive mutants, *abi1* to *abi5* and *abi8* are able to germinate at higher ABA concentrations which are inhibitory to germination of wild-type seeds. Severe ABA-insensitive mutants of *Arabidopsis* (eg. *abscisic acid insensitive* (*abi*)*3-6* and the double mutant *abi/abi*) produce seeds which are desiccation intolerant. The less severe mutant *abi3-7* produces seeds with lower levels of particular storage proteins and reduced dormancy (Kermode 2005). These particular mutants are able to germinate at high concentrations of exogenous ABA while wild-type seed germination is inhibited at 1-2 μM ABA (Nambara *et al*., 1994). Similar to the deficient mutants, ABA insensitive mutants are able to compensate for defective genes depending on the severity of the mutation. Characterization of a subset of the ABA-insensitive mutants has revealed that some of the defective genes encode transcription factors of ABA signaling pathways (Kermode 2005).

ABA plays a role in maintaining dormancy of deeply dormant yellow-cedar seeds. These seeds are dormant at maturity and experience a 2-fold decrease in ABA content in the embryo following a dormancy-breaking treatment (Schmitz *et al*., 2000). In addition the embryos are 10-fold less sensitive to (+)-ABA. Both the decline in ABA levels and a decreased sensitivity to ABA are required to terminate dormancy.

Changes in ABA levels in the plant are controlled through a balance between ABA biosynthesis and catabolism. Imbibition of the mature dormant seed is generally accompanied by ABA biosynthesis. An overexpression of ABA biosynthesis enzymes would cause enhanced dormancy or impede germination. ABA biosynthesis takes place in chloroplasts and other plastids. A few of the genes involved in the regulation of the ABA biosynthesis pathway and the corresponding mutants have been characterized. Zeaxanthin, the first carotenoid precursor, is converted into all-*trans*-violaxanthin by zeaxanthin epoxidase (ZEP), whose gene expression is ubiquitous in seed tissues during the maturation phase of seed development but becomes restricted to the embryo and endosperm during desiccation (Audran *et al*. 2001). *ZEP* expression levels peak in tobacco before mid-seed development, coinciding with ABA levels which are high during

maturation (Audran *et al*. 1998). Controlling gene expression of members of the 9-cis-epoxycarotenoid dioxygenase (*NCED*) gene family is required to catalyze the important subsequent regulatory step in the ABA biosynthesis pathway (Seo and Koshiba, 2002). The *NCED5* and *NCED6* genes in Arabidopsis are active during seed development until late maturation into the drying phase (Tan *et al*., 2003). Lefebvre *et al*. (2007) demonstrated that *AtNCED6* and *AtNCED9* encode the most abundant NCEDs and the resultant ABA that is synthesized within the embryo and endosperm contributes to dormancy induction. Seed double mutants (*Atnced6*/*Atnced9*) have increased germination rates and reduced dormancy compared to wild-type seeds (Lefebvre *et al*., 2007). NCED activity is considered the rate-limiting step in ABA biosynthesis (Rodriquez-Gacio *et al*., 2009). The AtABA2 protein, belonging to the Short Chain Dehydrogenase/Reductase1 (SDR1) family in Arabidopsis converts xanthoxin to abscisic aldehyde, the second to last step in ABA biosynthesis. Mutations in this gene cause severe ABA-deficiency (Nambara and Marion-Poll, 2005). The final step in ABA biosynthesis involves aldehyde oxidases (AAOs) (Seo and Koshiba, 2002). Null *aao3* mutants have significant ABA deficient phenotypes indicating the importance of AAO enzymes in ABA biosynthesis.

The transition from dormancy to germination requires an increase in the ratio of ABA catabolism to ABA biosynthesis. ABA is degraded via oxidation and conjugation mechanisms (Zaharia *et al*., 2005). Constitutive expression of an 8'-hydroxylase gene (cytochrome P450 *CYP707A* subfamily) encoding a major enzyme of ABA catabolism in transgenic seeds results in lower ABA levels at maturity and a reduction in after-ripening (Feurtado and Kermode 2007). CYP707A converts ABA to 8'-hydroxy ABA which is then subsequently autoisomerized to phaseic acid (PA) (Saito *et al*., 2004). Enhanced dormancy occurs in *cyp707a* mutants that have increased ABA levels due to a block in ABA degradation. *CYP707A2* is the main 8'-hydroxylase gene expressed during seed imbibition, which coincides with a decrease in seed ABA levels and dormancy release (Feurtado and Kermode 2007). An increased capacity for ABA catabolism is associated with dormancy termination in conifer seeds such as western white pine and yellow-cedar (Schmitz *et al*., 2000, 2002; reviewed in Feurtado and Kermode, 2007). Dormancy-breaking treatments cause a reduction in ABA levels and an increase in germination

capability. ABA levels continue to decrease during germination due to increased catabolism and reduced biosynthesis of ABA (Feurtado and Kermode, 2007).

1.3. ABI3 is an Important Transcription Factor of ABA Signal Transduction

The control of the transition from dormancy to germination by ABA involves many downstream events, including changes in transcription levels and recruitment of transcription associated factors, effects on RNA processing and posttranslational modifications of proteins.

The ABI3 (*Ab*scisic acid *I*nsensitive 3) transcription factor is an essential component of ABA signal transduction pathways in seeds and in seedlings under normal and stress conditions. The maize ortholog of *ABI3* is *VP1* (*Viviparous1*). Orthologs have also been identified in other plants such as rice (*Oryza sativa*), bean (*Phaseolus vulgaris)*, wheat (*Triticum aestivum*) and poplar (*Populus trichocarpa*). The yellow-cedar ortholog for *ABI3*, *Callitropsis nootkatensis ABI3* (*CnABI3*) was also cloned (Lazarova *et al*., 2002). There is a functional conservation between different ABI3/VP1 orthologs as shown by the successful complementation of the severe *abi3-6* Arabidopsis mutant by the transgenic expression of the conifer *CnABI3* gene (Zeng and Kermode, 2004) or by the *VP1* gene (Suzuki *et al*., 2001). The encoded transcription factors regulate the expression of many seed development genes, including storage reserve genes and genes involved in desiccation tolerance such as Late Embryogenesis Abundant (LEA) proteins, in response to ABA (Kermode and Finch-Savage, 2002; Finkelstein *et al*., 2002; Kroj *et al.*, 2003). In addition to regulating seed specific responses, these transcription factors act as regulators for the timing of major developmental transitions in the lifecycle of a plant.

ABI3/VP1 and its orthologs belong to the B3 domain family of transcription factors (Kermode 2005). Members of the B3 domain family contain four conserved domains: A1 is the transcriptional activation domain and B1, B2 and B3 are basic domains. The B1 domain physically interacts with bZIP transcription factors such as ABI5 that are able to bind to the ABREs (ABA Responsive Elements) of gene promoters

(Marcotte *et al*., 1989; Nakamura *et al*., 2001). The B2 domain is important for gene expression because it enhances DNA binding at ABREs by DNA binding proteins (Hill *et al*., 1996; Carson *et al*., 1997) and facilitates interaction with bZIP transcription factors (Hill *et al*., 1996). The B2 domain is responsible for the ABA-dependent activation of ABA-regulated genes, such as the Em gene encoding one of the LEAs. In addition the B2 domain has a putative nuclear localization signal (NLS) (Giraudat *et al*., 1992). Bies-Etheve *et al* (1999) found that the B2 domain is involved in the regulation of a LEA gene, *AtEm6*, and genes encoding two albumins, At2S1 and At2S2. Deletion of the VP1 B2 domain causes a loss of transactivation of the *Em* promoter (Hill *et al*., 1996), although this may be due to the loss of the NLS. The B3 domain of ABI3/VP1 binds to RY/Sph elements in the promoters of many seed maturation genes (Suzuki *et al*., 1997), but as Suzuki *et al*. (1997) reported the isolated B3 domain has this binding activity while the full length protein does not have specific DNA binding activity. Using the *Brassica napus* napin gene promoter, it was found that the interaction between B2 and B3 domains is necessary for transactivation in which the B2 domain mediates activation through ABRE and the B3 domain interacts with the RY/G-box complex.

Members of the B3 domain family of transcription factors are known to have many functions in seeds which include: (1) regulation of genes whose expression is controlled by ABA (eg. storage reserve proteins and desiccation protectants) (McCarty *et al*., 1991; Giraudat *et al*., 1992; Parcy *et al*., 1997; Zeng *et al*., 2003; Zeng and Kermode, 2004; Kagaya *et al*., 2005); (2) repression of post-germinative genes (Nambara *et al*., 2000; Gazzarrini *et al*., 2004) and (3) induction of dormancy and maintenance of seeds in a dormant state at maturity (Jones *et al*., 1997; Fukuhara *et al*., 1999; McKibbin *et al*., 2002; Zeng *et al*., 2003). Severe *abi3* mutants, in which the ABI3 protein is deficient, do not become dormant, are desiccation intolerant, show extreme insensitivity to ABA and have a wrinkled appearance due to reduced accumulation of protein storage reserves (Nambara *et al*., 1994).

The regulatory processes that control seed development likely involve ABI3 interacting with other proteins and transcription factors as noted in the previous section. Identification of these interacting proteins provides information on the mechanism of ABA and ABI3 functions (Kurup *et al*., 2000). ABI3 likely interacts with different proteins and transcription factors to elicit various gene expression responses. The yeast two-

hybrid assay system has been used to detect some of these protein-protein interactions in Arabidopsis, maize and wild oat. Nakamura *et al*. (2001) discovered that the ABI3 protein in Arabidopsis interacts with ABI5 using the yeast two hybrid approach. The Arabidopsis bZIP proteins, AtbZIP10 and AtbZIP25, were also found to interact with ABI3 and to regulate seed storage protein gene expression (Lara *et al*., 2003). This same approach was used to identify four proteins that have specific interactions with the B2 and B3 domains of ABI3 (Kurup et al., 2000). This group's findings report that the ABI3 interacting proteins (AIPs) were expressed throughout seed and plant development as opposed to the embryo-specific expression of ABI3. This may suggest that the interacting proteins play a general transcriptional role throughout development while ABI3 expression during embryo maturation provides temporal specific transactivation. AtAIP1 was later identified as TOC1, a protein with important functions in the circadian clock (Seung *et al*., 2011). AtAIP2 is an E3-ligase that polyubiquitinates ABI3 and targets it for protein degradation by the proteasome thereby negatively regulating ABA signaling pathways (Zhang *et al*., 2005). Interaction with AtABI3 was also shown for the RNA polymerase II RPB5 (Kurup *et al*., 2000). The same research group reported in a related study three proteins (AfVIPs) that interacted with the wild oat (Avena *fatua*) VP1 (Jones *et al*., 2000). These proteins interact with the B2 and B3 domains of AfVP1 and probably modulate the activities of AfVP1, which include controlling seed development, dormancy and desiccation and germination processes. AfVIP1 has so far not been assigned a function or group, while AfVIP2 is a zinc-finger protein (Jones *et al*., 2000). Interestingly, AtAIP3 was identified by its homology to AfVIP3 (Kurup *et al*., 2000; Jones *et al*., 2000) and turned out to be part of the prefoldin family (Hill and Hemmingsen, 2001), a group of proteins implicated in protein folding of cytoskeletal elements and in the stress response (Rodriguez-Milla and Salinas, 2009). Schultz *et al*. (1998), discovered that GF14, a 14-3-3 protein, interacts with VP1 and EmBP1 at the Em promoter and may perform chaperone or scaffolding functions at the transcription complex.

1.4. ABI3 Interacts with other Transcription Factors of ABA Signaling in Seed Development

The various functions of ABI3/VP1 transcription factors noted in the previous section are mediated by ABI3 interacting with other transcription factors and with

transcription associated proteins (Finkelstein *et al*., 2002). Together these transcription factors can activate or deactivate downstream genes whose protein products are involved or required for normal seed developmental processes. In addition to ABI3/VP1, other transcription factors required for ABA regulated gene expression include ABI4, ABI5, LEC1, LEC2 and FUS3 (Finkelstein *et al*., 2002). The ABI3, ABI4 and ABI5 transcription factors form transcription complexes which regulate gene expression (Finkelstein *et al*., 2002). ABI5, a basic leucine zipper transcription factor interacts with the B1 domain of ABI3 (Nakamura *et al*., 2001) and is expressed late in seed maturation. ABI5 is also able to bind to ABREs at an ACGT core motif thereby targeting ABI3 to promoters containing RY elements which are common in seed-specific gene promoters (Finkelstein *et al*., 2002). *ABI4* gene expression is detected much earlier in seed maturation and expression is very high in the embryo (Soderman *et al*.,2000). Mutants of *abi4* and *abi5* have reduced expression of various *LEA* genes such as *AtEm6* and *AtEm1* in *Arabidopsis*. Mutations at the *ABI4* and *ABI5* gene loci affect seed developmental processes and ABA sensitivity, although null mutations at the *ABI3* locus create more severe phenotypes (Finkelstein *et al*., 2002). In addition, Finkelstein and Lynch (2000) found that *ABI5* gene transcripts are downregulated in *abi1*, *abi2*, *abi3*, *abi4* and *aba1* mutants therefore suggesting that *ABI5* is regulated by these loci and ABA.

ABI4, ABI5, LEC1, LEC2 and FUS3 exhibit protein-protein interactions and/or protein-DNA interactions. LEC 1, LEC 2 and FUS3 are defined by mutations of the *Arabidopsis LEAFY COTYEDON* (*LEC*) genes. These genes act upstream of ABI3 and positively induce its expression and can regulate ABI3 protein levels (Parcy *et al*., 1997; Kagaya *et al*., 2005; Mendoza *et al*., 2005). LEC1, LEC2 and FUS3 are involved in gene expression during the early and final stages of seed development including the stages of dormancy induction and acquisition of dessication tolerance (Keith *et al*., 1994; Meinke *et al*., 1994; Holdsworth *et al*., 1999). The lec1 and fus3 mutants are not dormant and do not acquire desiccation tolerance. In addition, the embryos of these mutants have reduced synthesis of protein and lipid storage reserves and exhibit post-germinative growth even while maturing on the parent plant (Kermode 2005).

LEC2 and FUS3 both belong to the B3 domain family of transcription factors which includes ABI3 (Leubner-Metzger *et al*., 1998; Stone *et al*., 2001). FUS3 and LEC

1 regulate normal seed embryogenesis and cotyledon identity (West *et al*., 1994). In *lec* mutants, developmental rate is altered with the embryo showing some characteristics of seedlings indicating that precocious germination and postgerminative growth has occurred. LEC1 and LEC2 are both involved in promoting embryogenesis and the two seem to have similar and partially redundant functions during seed development (Stone *et al*., 2001). Interactions between ABI3, LEC1 and FUS3 are thought to regulate sensitivity to ABA, accumulation of anthocyanins and the expression of genes that belong to the 12S storage protein gene family during seed development (Parcy *et al*., 1997), although it has been found that *lec1* mutants are not sensitive to ABA. The LEC proteins bind directly to RY promoter sequences of seed-specific genes while ABI3 binds indirectly through interactions with basic leucine zipper transcription factors such as ABI5. In addition to the above mentioned transcription factors, the roles of ABA in seed development are achieved through the combined actions of protein modifying and processing enzymes, proteins involved in G-protein coupled signal transduction, and transport proteins (Kermode 2005).

1.5. ABI3 has Functions Outside of Seeds: Lifecycle Transitions and Abiotic Stress

ABI3/VP1 also has functions outside the seed, with functions in vegetative tissues, regulation of lifecycle transitions (Rohde *et al*., 2000) and in abiotic stress responses, such as those to osmotic, drought or glucose stress (Wang *et al*., 2004). Studies now show connections of ABI3 with the control of cellular differentiation, meristem quiescence and phase transitions (Rohde *et al*., 2000). It is possible that since ABI3 plays roles in different tissues at different times that ABI3 may function as a global regulator with broader functions that control developmental transitions throughout the lifecycle of plants. Experiments with dark-grown *abi3* mutant plants indicate that *ABI3* plays a role in plastid differentiation in vegetative tissues and suggests that *ABI3* is partly regulated by light (Rhode *et al*., 2000). Additionally, *ABI3* is expressed in the quiescent apex of Arabidopsis seedlings grown in the dark (Rohde *et al*., 1999). *ABI3* is observed to repress the outgrowth of axillary meristems (Robinson and Hill, 1999). *ABI3* has also been observed to play a role in regulating the transition from vegetative to reproductive growth. The *abi3-4* mutant flowers early suggesting that *ABI3* delays flowering (Kurup *et*

al., 2000). The ectopic expression of *ABI3* in vegetative tissues causes the these plants to accumulate seed specific mRNAs such as *At2S3* and *AtEm1* in response to ABA (Parcy and Giraudat, 1997). As previously mentioned, ABI3 functions in vegetative meristem differentiation and activity and has been suggested to play a major role in vegetative arrest, especially bud dormancy (Rohde *et al*., 2000).

Abiotic stresses induce ABA biosynthesis that initiates signaling pathways that ultimately upregulate many genes, especially stress response genes, osmoprotectants and/or antioxidants depending on the stress imposed (Lee and Luan, 2012). It is likely that plants have strategies to tolerate stresses and manage in various abiotic stress conditions. Identification of ABA receptors and downstream components of ABA signal transduction would aid in understanding the key players in abiotic stress response. The focus here will be on glucose stress. The defective genes associated with mutants isolated for their altered glucose sensitivity (*gin* mutants) have turned out to be allelic to genes controlling ABA biosynthesis or ABA signaling (Rolland *et al*., 2002). ABI3 is one of the ABA-signaling components that seem to be involved in both seed development/dormancy and in the glucose response: The *abi3* mutant is both non-dormant and insensitive to glucose.

Low levels of glucose have been found to delay seed germination in Arabidopsis, with the delay becoming more severe as concentrations increase (Price 2003; Dekkers 2004). At high glucose concentrations, *aba2* and *aba3* are resistant to the germination delay, indicating a role for ABA biosynthesis in the reaction to high glucose stress. The *abi2*, *abi4,* and *abi5* mutants do not have a glucose phenotype during seed germination (Dekkers 2004). However, the *abi3* mutant is less sensitive to glucose than wild-type (Zeng & Kermode 2004; Yuan & Wysocka-Diller, 2006; Dekkers *et al*., 2008), and overexpression of *CnABI3* in the severe Arabidopsis *abi3-6* mutant background leads to hypersensitivity to glucose (Zeng & Kermode 2004). Glucose-induced delay of seed germination thus seems to be mediated by ABI3.

Seed germination delay is not specific to glucose, but is also triggered by the metabolism-resistant sugar analog 3-OMG (Dekkers 2004). This suggests that no further metabolism is needed to initiate a sugar response and makes it unlikely that enzyme hexokinase (HXK) plays a role.

However, HXK1 and ABI4 signaling is involved in seedling developmental arrest by glucose with the respective mutants showing an insensitivity phenotype. Seedlings will begin to show phenotypes at concentrations of about 5% glucose. Two opposing effects of glucose on seedling growth have been described: developmental arrest with bleaching or anthocyanin production, and growth promotion.

Glucose-inhibited seedlings display reduced rates of storage lipid biosynthesis (To 2002). This effect cannot be induced by exposure of the seedlings to 3-O-methylglucose and thus seems to be linked to glucose metabolism via HXK1. As *abi4* does not show the inhibition, the signaling most likely involves ABI4. Plants overexpressing *HXK1* or *ABI4* in an ABA-deficient background (*aba2*) show the glucose insensitive seedling development phenotype of the background lines (Zhou 1998; Dekkers 2008), demonstrating that *de novo* ABA synthesis is required for the seedling arrest. In addition to ABI4, other factors of the ABA signaling pathways seem to be involved in glucose signaling during seedling arrest, with ABI5 and ABI3 being the most likely candidates. ABI5 was shown to play a role in glucose signaling in seedlings: ABI5 is induced by glucose and *abi5* has a glucose insensitive seedling phenotype (Arenas-Huertero, 2000; Dekkers 2008), as does *abi3* (Dekkers 2008; Zeng & Kermode 2004). Seedlings whose growth is arrested by glucose show induction of a number of *LEA* genes (Dekkers 2008), all of which are under the control of ABI3. The *abi3* mutant was found to be impaired in this induction.

1.6. ABA Hormonal Cross-Talk in the Regulation of Dormancy and Germination

The role of ABA in dormancy maintenance and seed development is partly achieved through interactions with other hormonal signaling pathways. There is hormonal cross-talk between ABA and hormones such as gibberellins (GAs), ethylene, cytokinins, auxin and brassinosteriods. GAs act antagonistically to ABA in dormancy breakage and germination (Karssen and Lacka, 1986; Karssen 1995). They are involved in the promotion and maintenance of germination by increasing the growth potential of the embryo and mediating the weakening of tissues enclosing the embryo (Bewley 1997). The localization of GA biosynthesis in the radicle of *Arabidopsis* seeds is

consistent with its function of overcoming the mechanical restraint of the seed covering layers by GA-induced cell wall hydrolases (Yamaguchi *et al*. 2001). When GA deficiency is induced by inhibition of biosynthesis in cultured immature maize embryos, the expression of many ABA induced genes including those encoding storage reserve proteins and LEAs are upregulated (White and Riven, 2000). If a GA deficiency is created in ABA synthesis mutants, the developing seeds do not germinate precociously and exhibit desiccation tolerance (White *et al*., 2000). The experiments with ABA-deficient and ABA-insensitive mutants in combination with GA biosynthesis inhibitors suggest that GA promotes vivipary. It has been proposed that accumulation of GA allows germination and the expression of post-germination genes in the absence of significant ABA levels. A reduction of GA levels re-establishes an ABA/GA ratio required for suppressing germination. It is the ABA/GA ratio that controls the regulation of vivipary as opposed to the actual hormone levels in the seed.

Ethylene is involved in the promotion of germination and counteracts the effects of ABA during seed development. The biosynthesis of ethylene alone is not sufficient for seed dormancy release. The main roles of ethylene could include promoting radicle cell expansion, increasing seed respiration or increasing the seed water potential (Kucera *et al*., 2005). The *ethylene insensitive2* (*ein2*) mutants of *Arabidopsis* are hypersensitive to ABA and are characterized by increased seed dormancy. The *ein2* mutant has been found to be a suppressor mutant of the ABA-insensitive mutant *abi1-1* (Beaudoin *et al*., 2000). The increased dormancy of the *ein2-45* mutant is counteracted by severe ABA-insensitive mutants, such as *abi3-4* (Beaudoin *et al*. 2000). The *ein2 abi3-4* double mutant has a nondormant phenotype equivalent to the *abi3-4* single mutant suggesting that ethylene suppresses seed dormancy by inhibiting ABA activity and influences seed sensitivity to ABA. Ethlyene reduces the inhibitory effects of ABA on seed germination processes by interfering with ABA signaling.

Brassinosteroids (BR) are involved in promoting seed germination by influencing cell elongation and cell division. BRs are not considered primary regulators of seed dormancy and germination, although Arabidopsis BR mutants reveal an increased sensitivity to ABA when compared to wild-type seeds (Steber and McCourt, 2001). The BR hormomes are thought to act synergistically with GA through distinct pathways to promote germination and counteract the actions of ABA.

Cytokinins are involved in embryogenesis, in embryonic pattern formation and in the promotion of cell division in the embryo. Auxins also play a role in embryogenesis by providing positional information for proper cellular pattern formation. In addition to its role in embryogenesis, auxin is modulated by FUS3 in its regulation of embryo growth and maintenance of embryonic trait expression. FUS3 is responsible for modulating the ABA/GA ratio, thereby maintaining important developmental processes such as reserve deposition, acquisition of desiccation tolerance and induction of primary dormancy (Raz *et al*. 2001; Gazzarrini *et al*. 2004).

1.7. Characteristics of CnAIP1 and Domains of ABI3 that Interact with CnAIP1

As previously noted, the transcriptional regulator ABI3 plays a major role in seed maturation processes. Recently, the yeast two-hybrid approach was used to identify three proteins that interact with the yellow-cedar ortholog of ABI3 (CnABI3) (Zeng 2005) and one of these proteins, CnAIP2 has been functionally characterized (Zeng *et al*., 2013).

The focus of my thesis work is on CnAIP1. Because my thesis work has relied heavily on some previous work conducted by Y. Zeng in the Kermode lab (Zeng, 2005 and unpublished), this is a discussion of this background information. To be clear, none of this data has been generated by me; however, it is well worth reviewing. Also, I have made sequence comparisons between the *CnAIP1* gene and other putative eukaryotic orthologs. It is Chapters 2 and 3 that report on my Masters research.

The full length of the CnAIP1 cDNA sequence is 1227 bp which encodes a 408-amino acid protein with a molecular mass of of 44.1 kDa and a theoretical pI of 5.17. The online program PSORT was used to predict the localization of CnAIP1; this indicated that CnAIP1 is very likely to be located in the nucleus (88%). CnAIP1 also contains a putative nuclear localization signal. This was expected considering that CnAIP1 interacts with CnABI3, a nuclear transcription factor.

CnABI3 and its ABI3/VP1 orthologs possess four highly conserved domains, which include an N-terminal acidic A1 domain and three basic domains, B1-B3. In order

to determine which domains of CnABI3 interact with CnAIP1, six deletion constructs of CnABI3 in the pSOS bait vector were created in different combinations (Figure 1-1). As shown, seven different combinations of deletion constructs of CnABI3 were tested for interaction with CnAIP1. Each of the different deletion constructs in pSOS were cotransformed with CnAIP1 in yeast pMyr twice to ensure the validity of the results. The results indicate that CnABI3 and CnAIP1 are able to interact only when the B1 and B2 domains of CnABI3 are present. The deletion of A1 and/or B3 did not affect the protein-protein interaction between CnABI3 and CnAIP1 as yeast growth was still observed (Zeng, 2005).

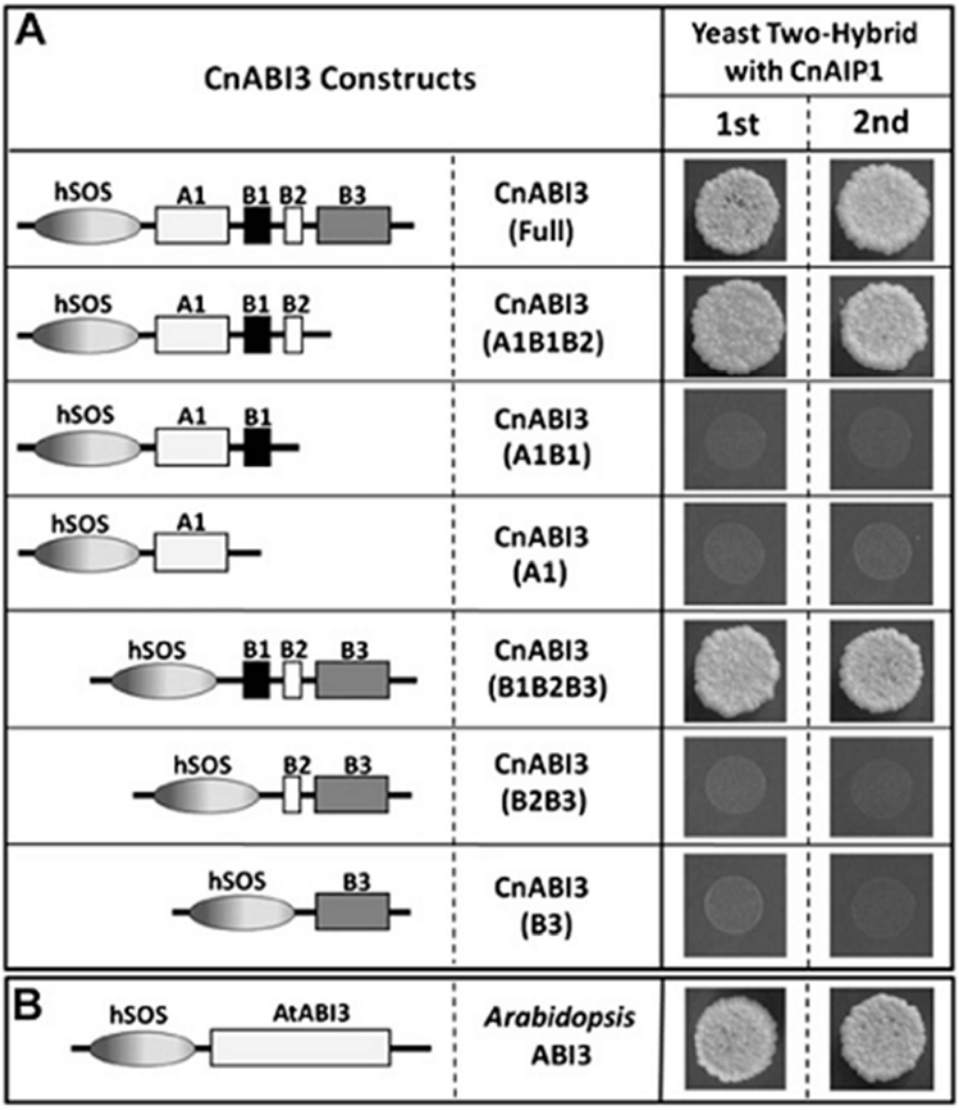

Figure 1-1. Protein-protein interactions of CnAIP1 and CnABI3/AtABI3.

A. Yeast two hybrid screen of domains of CnABI3 that interact with CnAIP1. The four conserved domains in CnABI3 were deleted separately and the resulting protein interaction with full-length CnAIP1 was detected using a yeast two-hybrid approach. B1 and B2 domains of CnABI3 are required for interaction with CnAIP1. B, Yeast-two hybrid approach to determine protein-protein interactions between the Arabidopsis ABI3 protein and the yellow-cedar CnAIP1 protein.

The B1 domain in the Arabidopsis ABI3 is involved in physically interacting with bZIP transcription factors such as ABI5. The domains of ABI5 that interact with ABI3 include two conserved charged domains in the N-terminal region of the protein (Nakamura *et al*., 2001). The interaction of the B1 domain of CnABI3 with CnAIP1 is consistent with previous studies indicating the participation of this domain in protein-

protein interactions. Additionally, the CnABI3-interacting protein CnAIP2 also interacts with the B1 and B2 domains of CnABI3 (Zeng *et al*., 2013). The B2 domain of ABI3 and its orthologs contain a putative nuclear localization signal, ARKKR (Giraudat *et al*., 1992); this domain is further thought to tether ABI3 to the transcriptional complex at promoter sites and to facilitate many protein-protein interactions, including that with ABI5 (Hill *et al*., 1996; Ezcurra *et al*., 2000). All of these previous studies are consistent with the finding of Zeng (2005) that the B2 domain of CnABI3 is critical for interaction with CnAIP1 protein.

1.8. Does CnAIP1 Interact with ABI3 of Arabidopsis?

In addition to its interaction with CnABI3, CnAIP1 also interacts with Arabidopsis ABI3 (AtABI3) (Figure 1-1B). These yeast-two hybrid analyses were performed twice to verify the interaction observed (Zeng, 2005).

1.9. Does the Deduced Amino Acid Sequence of CnAIP1 Share Similarities with Other Proteins?

Comparison of the deduced amino acid sequence of CnAIP1 to similar proteins (putative orthologs) was performed using NCBI blastp and multiple-sequence alignment. The BLAST sequence comparison of CnAIP1 conducted by Zeng (2005) suggests that it has significant homology to AtHIP1, an Arabidopsis protein that is a putative heat shock protein-associated factor with 65% identities and 76% positives between the deduced amino acid sequences of the protein. Hip is one of many cochaperone proteins that mediate Hsp70 chaperone activity (Hohfeld *et al*., 1995; Prapapanich *et al*., 1996a, 1996b; Gebauer *et al*., 1997; Bimston *et al*., 1998). Hsp70 chaperones are found in many organs and tissue types in plants, animals and bacteria (Boorstein *et al*., 1994; Boston *et al*. 1996; Miernyk 1999). Along with their widespread occurrence, plant Hsp70s are found in many subcellular compartments of the cell including the cytosol, endoplasmic reticulum, mitochondria, chloroplasts and peroxisomes (Boston *et al*. 1996; Corpas and Trelease, 1997; Miernyk 1999). They are involved in cellular processes including binding to exposed hydrophobic ends of proteins during protein folding,

preventing non-native protein aggregation, renaturing aggregated proteins and keeping proteins in their proper conformation for import/export to other subcellular compartments (Boston *et al*., 1996, Miernyk 1997). In addition, Hsp70s along with other chaperones and cochaperones, function in signal transduction pathways by forming regulatory complexes (Pratt 1998; Kimmins and McRae, 2000; Smith 2000). The functions of Hsp70-interacting proteins (Hip) and other Hsp70 cochaperones involve assisting and regulating the activities of Hsp70 proteins (Bimston *et al*., 1998; Takayama *et al*., 1999).

1.10. Role of Hip in Hsp70 Regulation

The two major functional domains of Hsp70 proteins include an ATPase domain located in the N-terminal region and a peptide-binding domain in the C-terminal end of the protein (Miernyk 1999). Previous studies report that Hip regulates Hsp70 function by stabilizing Hsp70 in an ADP bound form that allows interaction with peptide substrates. Hsp70s participate in chaperone activities through the constant cycles of peptide substrate binding, ATP hydrolysis and subsequent substrate release (Miernyk 1997). Hsp70 is either in one of two conformations as a chaperone: an ATP-bound form with little substrate activity and an ADP-bound form with increased affinity for substrate activity (Palleros *et al*., 1993). Structural and functional information on Hsp70 proteins comes from the *Escherichia coli* DnaK, the bacterial homologue of Hsp70. There is high conservation of protein sequence among Hsp70 proteins with 50% identical amino acid residues between Hsp70s from eukaryotes and the homologue from *E.coli*, DnaK (Boorstein *et al*., 1994). In bacteria, the cochaperone DnaJ interacts with Hsp70 protein via the ATP-binding/ATPase domain (Greene *et al*., 1998). Substrate binding to the peptide-binding domain and ATP hydrolysis appears to occur due to the presence of DnaJ. Conversely, GrpE, a nucleotide exchange factor, interacts with Hsp70s at the ATP-binding domain and promotes ADP release from DnaK, thereby allowing ATP binding and subsequent lowered affinity for the peptide substrate (Banecki and Zylicz, 1996). In eukaryotes, Hsp40, a DnaJ-like protein, is responsible for hydrolyzing ATP at the ATP-binding domain and Hip subsequently stabilizes Hsp70 in the ADP-bound form by inducing a conformational change which allows for strong substrate binding at the peptide-binding domain (Frydman and Hohfeld, 1997, Hohfeld *et al*., 1995; Prapapanich *et al*., 1996). Prapapanich *et al*. (1996b, 1998) reported that Hip does not perform its

cochaperone activities when mutant Hsp70 is unable to hydrolyze ATP at the ATPase domain. Although there are no GrpE equivalents in eukaryotes, the Bag-1 family of cochaperones perform similar functions to that observed for GrpE (Hohfeld 1998; Sondermann *et al*., 2001). Bag-1 proteins inhibit Hsp70 chaperone activity and therefore act as a competitive antagonist of Hip for binding to the Hsp70 ATPase domain (Gebauer *et al*., 1997; Takayama *et al*., 1997, 1999; Bimston *et al*., 1998).

1.11. A Comparison of Genes Encoding Hip and Hip-like Proteins

CnAIP1 shows significant homology to a HSP70-interacting protein (Hip-1) of Arabidopsis (gene At4g22670) throughout its entire sequence. Both sequences also share significant similarity in the functional domains present. Sequence analysis also revealed similarities to the Hips of grape (*Vitis labrusca*) and humans (p48). The latter protein is known to be involved in mediating the maturation of steroid hormone receptor assembly (Prapapanich et al, 1996a, 1996b, 1998). Sequence similarities between the human and plant Hip proteins include a conserved N-terminal domain, a highly acidic domain, three tetratricopeptide repeats, a highly charged region, a GGMP repeat domain and a carboxy terminal end that has a Sti-like (a cochaperone that mediates interactions between HSPs) domain. Similarities are outlined schematically in Figure 1-2.

1.11.1. Conservation of the Functional Domains of Hip Proteins

The Hip sequences from human and plants are similar in all of the functional domains outlined in Figure 1-2 except that AtHip-1 has a longer GGMP repeat region compared to the other proteins. This conservation of domains among the Hip proteins may suggest that they may play similar roles in both mammalian and plant systems.

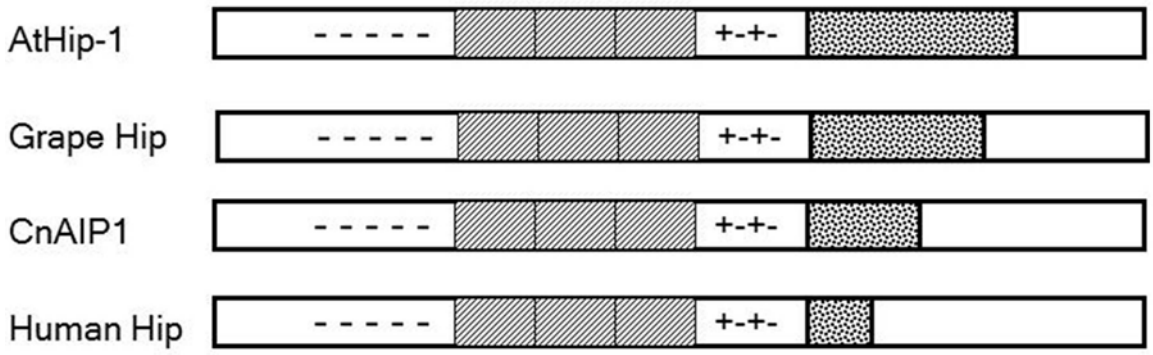

Figure 1-2. Comparison of the structure of Hip proteins found in human and plants.

All Hip proteins have a conserved N-terminal region (not labeled), an acidic domain (dashes), three tetratricopeptide repeat (TPR) regions (light hatched), a highly charged region (positive and negative signs), a variable GGMP degenerate region (dotted) and a Sti-like domain (white) at the C-terminal end

Tetratricopeptide Repeats (TPRs) are Important for Protein-Protein Interactions

Sequence alignment of the HIPs from human, *Arabidopsis*, grape and yellow-cedar reveal that all four have tetratricopeptide repeat (TPRs) regions at the same location as shown in Figure 1-3. Each of the sequences has three TPRs. This suggests that the TPR region and the adjacent charged region are conserved among mammalian and plant systems and could contribute to the general mechanistic abilities of eukaryotic Hips. Additionally as shown in the figure, the TPR2 region has the highest sequence conservation among the different Hip proteins. TPRs are 34-amino acid degenerate repeats that are involved in protein-protein interactions (Blatch and Lassle, 1999). As reported by Webb *et al*. (2001), conserved consensus charged residues in the TPR region and in the adjacent charged region contribute to the overall region of Hip that interacts with the Hsp70 protein. In mammalian Hip, the TPR region and the charged domain are required for Hip to bind to Hsp70 (Hohfeld *et al*. 1995; Prapapanich *et al*., 1996a, 1996b; Irmer and Hohfeld, 1997; Velten *et al*., 2000). In addition to the role of the TPR domain in protein-protein interactions, TPR proteins perform cellular regulatory functions, including cell cycle control, protein folding and transport and mediating multi-protein complex formation (Blatch and Lassle, 1999).

Figure 1-3. Comparison of the TPRs region and the adjacent charged region in the Hip sequences found in human, Arabidopsis, grape and yellow-cedar.

The TPR region in Hip is required for the interaction with the ATPase domain of Hsp70. Alignment illustrates the high degree of primary sequence conservation among animal and plant Hips.

The N-terminal Region and Acidic Domain Contain Specific Amino Acid Residues

The alignment of the N-terminal region of the four Hip sequences is shown in Figure 1-4. The N-terminal region has been reported to be necessary for Hip dimerization, with mutants lacking the N-terminal domain unable to form oligomer complexes (Hohfeld *et al*., 1995; Prapapanich *et al*., 1996b). Despite these findings, Irmer and Hohfeld (1997) have found that oligomerization is not required for Hip interaction with Hsp70 and that an individual Hip subunit can provide binding sites to Hsp70 proteins. Furthermore, they extend this finding to suggest that individual Hip subunits may have a “scaffolding” function by keeping many Hsp70 proteins in close proximity to one another thereby increasing the interaction between Hip and Hsp70s. The N-terminal region contains fairly conserved amino acid residues with many of the residues identical among the four eukaryotic sequences. The level of conservation is higher among the plant sequences. Additionally, an acidic domain which contains glutamic acid (E) and aspartic acid (D) is found in the N-terminal region as well. Although the acidic domains are quite variable in comparison to previous reports, the acidic domain may still contribute to some electrostatic interaction as is seen for the charged region flanking the TPR region which contributes to Hip interacting with Hsp70.

Figure 1-4. Comparison of the N-terminal region and acidic domain of Hip sequences found in human, Arabidopsis, grape and yellow-cedar proteins.

The acidic domain is quite variable but the level of conservation is higher among the plant Hip sequences.

Significance of the Degenerate GGMP Repeat Region

A region of GGMP repeats is found near the C-terminal region of Hip. The four Hip sequences of eukaryotes all contain the GGMP repeat region (Figure 1-5), with AtHip-1 containing an extended repeat region close to 75 residues and human Hip containing the shortest repeat region. The function of the GGMP region is yet unknown.

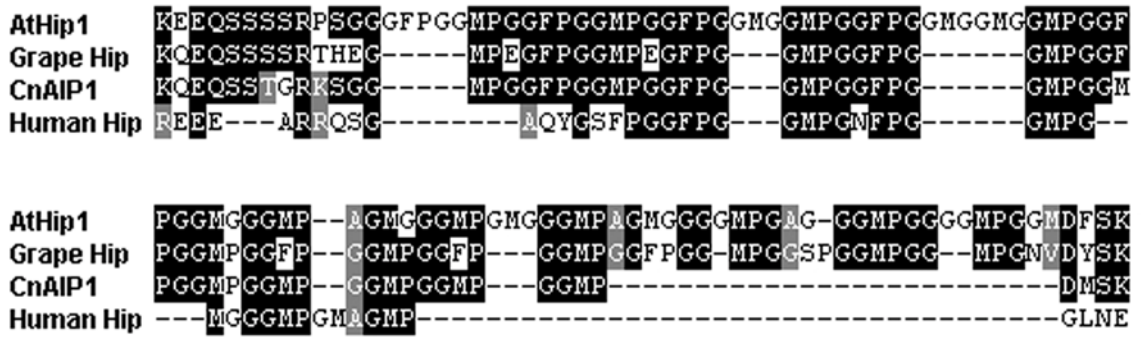

Figure 1-5. Comparison of the GGMP repeat region found in human, Arabidopsis, grape and yellow-cedar.

The repeat region is shorter in CnAIP1 and human hip compared to the other plant Hip proteins.

Sti-like Domain at the C-terminal End

Alignment of the C-terminal end of human Hip and the plant Hip sequences reveals a short Sti-like domain as shown in Figure 1-6. Sti1 is a yeast ortholog of the Hop/p60 protein which is a cochaperone that mediates interactions between Hsp70 and Hsp90 in multichaperone complexes (Webb *et al*., 2001). The Sti1-like domains of each of the four Hip sequences contain at least one conserved DPEV motif and another which is altered to either DPEL or DPEM. The DPEV motif has been shown to be involved in the assembly of mature progesterone receptor in mammals (Prapapanich *et al*. 1998).

Figure 1-6. Comparison of the C-terminal region of Hip sequences found in human, Arabidopsis, grape and yellow-cedar which contains a Sti1-like domain.

Sti1 is the yeast ortholog of Hop/p60 which is a mammalian cochaperone that mediates interaction between Hsp70 and Hsp90 in multichaperone heterocomplexes.

1.12. Possible Roles of Hip in Plants

In mammalian systems, the interaction of Hip with Hsp70 within multichaperone heterocomplexes is involved in regulating signal transduction pathways via the assembly of mature progesterone receptors (Prapapanich *et al*., 1996a, 1996b, 1998). These studies have determined that the Hip-Hsp70 interaction occurs at an intermediate stage of the assembly of the functional receptor to prevent the receptor from interacting with hormone in the cytosol. As mentioned previously, Hsp70 and Hip also interact with Hop and Hsp90 for the final formation of the mature hormone receptor complex (Prapapanich *et al*., 1998; Smith 2000). Nelson *et al*. (2004) further confirmed that the Hip protein is involved in increasing the efficiency of glucocorticoid receptor maturation. Due to the close similarity in sequence and conservation of functional domains between the human Hip and plant Hip sequences, it is possible that Hip plays similar roles in steroid receptor formation in plants. Plant steroids are common and there are conserved biosynthetic enzyme pathways in animals and plants (Li *et al*., 1997). In addition to steroid receptor formation, CnAIP1 and its Hip orthologs in plants may also be involved in regulating signal transduction pathways either alone but most likely through interaction with other chaperones in heterocomplexes. It is likely in this case that CnAIP1 would interact with many proteins including CnABI3, a transcriptional and global regulator to achieve this function. Recent findings suggest that O1 myosin influences protein body biogenesis by affecting ER morphology and motility in maize endosperm. Interestingly, Hip1 acts as an adaptor connecting O1 to the ER and thereby assisting in the process as is expected with chaperones and cochaperones found in the cytosol (Wang *et al*., 2012). Part of my

work has involved abiotic stress treatments, particularly heat and glucose stress, to elucidate a physiological role for CnAIP1 in plant abiotic stress responses.

1.13. Objectives of My Research

My research is geared toward understanding some of the mechanisms underlying seed dormancy and germination. Previous work in the Kermode lab (Lazarova *et al*., 2002) led to the isolation of a conifer ortholog of the ABI3/VPI gene in yellow-cedar. The functions of ABI3/VP1 orthologs are conserved as shown by the rescue of the *Arabidopsis abi3-6* mutant by transgenic expression of the conifer *CnABI3* gene (Zeng and Kermode, 2004). As is observed for ABI3, CnABI3 is thought to interact with other proteins in the regulation of seed development and other processes. The yeast two-hybrid assay which identifies putative protein-protein interactions was used to determine proteins in yellow-cedar that interact with CnABI3. Three proteins showed putative interactions with CnABI3 and were named CnAIP1, CnAIP2 and CnAIP3 (*Cn*ABI3 *I*nteracting *P*rotein) (Zeng 2005).

My focus has been on the functional analysis of the CnAIP1 gene and protein using both yellow-cedar seeds and Arabidopsis as study models. In order to determine the function of CnAIP1, my objectives were: (1) To determine the spatial expression of the native *CnAIP1* gene promoter. (2) To characterize the subcellular localization of the CnAIP1 protein and confirm its physical interaction with CnABI3. (3) To characterize the gene and protein in yellow-cedar and further characterize the physiological and biochemical phenotypes of overexpressing lines of Arabidopsis during seed development, germination and abiotic stress.

2. Functional Analysis of CnAIP1 in Arabidopsis

2.1. Introduction

Determining the underlying mechanisms of seed dormancy and germination in the deeply dormant gymnosperm, yellow-cedar, is crucial for a conifer species that holds high importance in the British Columbia forestry industry. Investigations into proteins that may play a role in dormancy maintenance of yellow-cedar seeds are underway. Lazarova *et al*. (2002) cloned the yellow-cedar gene homolog of ABI3, *CnABI3*. ABI3 and its orthologs play a role in dormancy maintenance of mature imbibed seeds (Jones *et al*., 1997; Fukuhara *et al*., 1999; Nakamura and Toyama, 2001; Carrari *et al*., 2001). CnABI3, like its orthologs has other roles in seed development which include promoting storage reserve accumulation, desiccation tolerance and the prevention of precocious germination. The isolation of CnABI3 was followed by yeast two-hybrid approaches to find protein interactors of CnABI3. One of the main goals of my research was to determine the functions of CnAIP1, a protein found to interact with CnABI3 (Zeng, 2005). The discovery of putative functions of CnAIP1 can reveal additional functional roles for CnABI3.

Due to the limited methods that can be performed to analyze gene functions and protein-protein interactions in conifer species, *Arabidopsis thaliana* was chosen for most of the functional analyses of CnAIP1. In addition, the lifecycle of conifer species is very long and genetic modifications of tissues are not possible within a regular timespan for a research project. The evolutionary gap between yellow-cedar, a gymnosperm and Arabidopsis, an angiosperm is quite large. However, there are genes between the two that have conserved functions. The functions of ABI3/VP1 orthologs are conserved as shown by the rescue of the *Arabidopsis abi3-6* mutant by transgenic expression of the conifer *CnABI3* gene (Zeng and Kermode, 2004). The complementation of *CnABI3* in the *abi3-6* mutant almost completely restores the mutant phenotypes to wild-type characteristics. Ectopic expression of *CnABI3* gene in transgenic tobacco (*Nicotiana*

tabacum) is able to activate seed storage protein genes (Zeng *et al*., 2003) providing another line of evidence of the similarity between the angiosperm and gymnosperm ABI3 proteins.

Since CnAIP1 interacts with CnABI3, it can be hypothesized that CnAIP1 also plays a role in seed development and dormancy processes. The homologue of CnAIP1 in Arabidopsis, AtHip1 (Accession# *At4g22670*), encodes a heat-shock protein (HSP) associated factor. HSPs have been hypothesized to have a role in adaptation to and/or recovery from stress. Interestingly, sHSPs are expressed during seed development indicating that they play a role in the absence of stress (zur Nieden *et al*., 1995; Waters *et al*., 1996). It is therefore likely that HSP-associated factors or chaperone proteins are also present during development. To uncover these potential functions, experiments are devised to target CnAIP1's potential roles during development and dormancy processes.

A transgenic chimeric system which involves the CnAIP1 native promoter fused to the common reporter protein β-glucuronidase (GUS) can determine where the promoter is being expressed and in which tissues transcription is occurring. Promoter expression can be visualized during seed development and other times during the plant lifecycle with a focus on the spatial and temporal expression patterns.

Transgenic plants can be created which use the cauliflower mosaic virus *35S* promoter to drive the expression of CnAIP1. These constitutive overexpressing lines can be used to determine the role of CnAIP1 in development and dormancy processes. Specifically, these lines can be used to examine whether the overexpression of CnAIP1 affects seed developmental processes, ABA sensitivity, and dormancy in comparison to wild-type seeds.

Comparative analysis of the CnAIP1 protein and its homolog in Arabidopsis may determine similar functions in both species. In our lab, Zeng (2005) and J. Grelet analyzed SALK lines of T-DNA inserted mutants of *At4g22670* (AtHip1), the Arabidopsis homologue of CnAIP1. Heat treatments were conducted (due to its putative role as a heat-shock protein associated factor) during and after germination on the mutant SALK and wild-type lines. Phenotypes of the T-DNA inserted SALK lines that differed from wild-type could elucidate mechanisms in which AtHip1 may play a role. However, none

of the homozygous SALK lines generated were identified as having different characteristics or phenotypes in comparison to wild-type Arabidopsis. In my thesis work, I conducted a more rigorous range of abiotic stress treatments to determine phenotypic differences, including more subtle phenotypes. Throughout my comparisons, a powerful approach was to analyze the SALK line mutant vs. the wild-type vs. my Arabidopsis lines that I generated that were overexpressing CnAIP1.

Thus, the aims of these functional studies in Arabidopsis were as follows: (1) To conduct analyses of the qualitative characteristics of expression of the *CnAIP1* gene by characterization of a CnAIP1 5'-GUS chimeric gene. These included analyzing the temporal and spatial characteristics of expression and induction of expression by hormones/stresses.
(2) To characterize an AtHip1 SALK mutant line (*athip1*) with both the wild-type and the lines over-expressing CnAIP1. This encompassed analyses of proteins expressed during seed development, as well as a characterization of their ABA sensitivity and dormancy. I was particularly interested in mid-maturation stages, when ABI3 regulates the expression of many genes that encode storage reserve proteins as well as proteins that are involved in desiccation tolerance (ie. LEAs, such as dehydrins). Additionally, since CnAIP1 encodes a heat-shock protein-associated factor, this protein might interact with CnABI3 to regulate heat shock protein expression during development. Wehmeyer *et al*. (1996) report that ABI3 activates HSP17.4 transcription during seed development and Wehmeyer and Vierling (2000) also suggest that sHSPs may be essential for desiccation tolerance. These experiments were designed in the hope of elucidating some of the functions of AtHip1 and potentially CnAIP1.

2.2. Materials and Methods

2.2.1. CnAIP1 and CnABI3 Subcellular Localization: Transient Expression Studies and Confocal Microscope Analysis

The stop codon of CnABI3 was removed through PCR amplification to ensure fusion to CFP in the destination vector psite1NB (Chakrabarty *et al*., 2007). CnAIP1 and CnABI3 coding sequences were individually cloned into entry vectors using Invitrogen's

pENTR Directional TOPO Cloning Kits. After amplification in DH5α Ultra-Competent *E.coli* cells, the positively transformed vectors were used in LR recombination reactions with the binary destination vector (CnAIP1-pSITE 3CA YFP, CnABI3-pSITE 1NB CFP). Protein expression was controlled by a duplicated Cauliflower mosaic virus (CaMV) 35S promoter (2x35S) along with a CaMV35S transcriptional terminator (TER). Each recombinant pSITE vector was transformed into *Agrobacterium* GV3101 competent cells by heat shock. Transient expression of the fusion genes (CnAIP1 construct or CnABI3 construct) in *Nicotiana benthamiana* leaves was performed by *Agrobacterium*-mediated infiltration. (Sparkes *et al*., 2006). *Nicotiana benthamiana* plants were grown in the greenhouse at 22°C under a 16-hour photoperiod and were used for *Agrobacterium*-mediated infiltration after 6 weeks. Following 48 hours incubation of infiltrated plants, water mounted sections of leaf tissue were examined using confocal microscopy. All confocal microscopy was performed on the WaveFX spinning disc confocal system by Zeiss and quantifed using Volocity software by PerkinElmer. The Zeiss FL Turret was set for detection of YFP fluorescence (CnAIP1 construct); excitation: 517 nm, emission: 528 nm or CFP fluorescence (CnABI3 construct); excitation: 436 nm, emission: 488 nm.

2.2.2. *Expression of CnAIP1$_{pro}$-GUS in Arabidopsis and fluorometric and histochemical GUS assays following stable expression*

The promoter region of *CnAIP1* was isolated using a genome-walking approach and a 2.0-kb fragment was amplified. The *CnAIP1* promoter sequence was cloned into an entry vector using Invitrogen's pENTR Directional TOPO Cloning Kits. Proper orientation of the promoter insert into the entry vector was verified using a forward primer (5'CACCGAATTCCATCTCTCGAGCAACTCTTGATAGCGACTCAACGC3')
which contains a EcoRI cutting site between CACC and the start of the promoter sequence and (5'AATTTTTTCGGCGTCCATAGCGTCTTCAGGATTCGAACCCG3'), the reverse primer. After amplification in DH5α Ultra-Competent E.coli cells, the positively transformed vectors were used in LR recombination reactions with the binary destination vector pMDC163 (backbone pCambia). The binary vector was transferred into *Agrobacterium* GV3101 competent cells by heat shock. *Arabidopsis* wild-type plants were transformed using the floral-dip method (Clough and Bent, 1998). Putative

transformants were identified by seed germination and growth on hygromycin medium. Transformants were further verified by PCR using *CnAIP1*$_{pro}$ specific primers.

GUS histochemical assays were performed on transformed *Arabidopsis* developing seeds to determine the expression pattern of the *CnAIP1*:*GUS* gene construct during development using the method described by Jefferson (1987). GUS (β-glucuronidase) cleaves the substrate X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid) in the reaction. Additionally, GUS fluorometric assays were carried out on abiotic stress treated young seedlings of Arabidopsis which were stably transformed with the *CnAIP1*$_{pro}$ construct using a method outlined by Jefferson (1987). Seedling samples were GUS extraction buffer (50 mM $NaPO_4$ (pH 7.0), 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine and 0.1% Triton X-100). Following centrifugation for 10 min at 10000 rpm at 4°C, GUS activity of the extract supernatant was determined upon the addition of GUS assay buffer containing MUG (4-methyl umbelliferyl β-D-glucuronide) as a substrate. Reactions were carried out at 37°C. Aliquots were removed at half hour time intervals and 0.2 M Na_2CO_3 was added to terminate the reactions. Fluorescence of the reaction mixture was determined using a Hoefer TKO 100 Fluorometer (Hoefer Scientific Instruments, San Fransicco, CA). GUS activity was reported as pmol MU/min/mg protein.

2.2.3. Generation of 35S-CnAIP1 Transgenic Plants

A previously constructed cDNA library using mRNA isolated from yellow-cedar (*Callitropsis nootkatensis*) seeds was used as the template to clone the full length of the *CnAIP1* gene. The full length sequence was cloned into an entry vector using Invitrogen's pENTR Directional TOPO Cloning Kits. DH5α Ultra-Competent *E.coli* cells were heat shocked and chemically transformed with the entry vector. Following miniprep purification of positive clones, a restriction digestion of the entry vector was performed to further verify the presence of the insert. *AscI* and *NotI* restriction enzymes were used in the restriction digest because both cut close to the insertion site in the entry vector. An LR recombination reaction was performed between the positively transformed entry vector and the destination vector, pSITE 0B using the LR Clonase II Enzyme Mix from Invitrogen. DH5α Ultra-Competent *E.coli* cells were heat shocked and chemically transformed with the destination vector. The newly generated *35S-CnAIP1* plasmid was

transferred into *Agrobacterium tumefaciens* and used to transform *Arabidopsis* using the floral dip method (Clough and Bent, 1998). Putative *CnAIP1* overexpressing (OE) transformants were identified by seed germination and growth on kanamycin medium. Transformants were further verified by PCR using *CnAIP1* specific primers.

2.2.4. Germination Assays

Seed Dormancy and Germination Performance

To investigate the degree of seed dormancy of *CnAIP1* seeds compared to wild-type, freshly harvested mature dry T5 generation *Arabidopsis* seeds were surface sterilized in 40% commercial bleach for 10 min and washed three times with sterilized water (3 x 5 mins). Seeds were sown in Petri dishes containing water and 0.7% agar. Seeds were either immediately placed in germination conditions (21°C, 16-hr light period) in a growth chamber (Conviron E15) or placed in moist chilling conditions (4°C) from 1-4 days prior to transfer to germination conditions in order to release dormancy. Seeds that were counted as germinated were those that exhibited radicle emergence. All tests were carried out in triplicate, with each replicate having 50-100 seeds.

ABA Sensitivity and Germination Performance

To determine any changes in ABA sensitivity with respect to the hormone inhibiting seed germination, *CnAIP1* OE and wild-type seeds were surface sterilized as mentioned above and sown on one-half-strength MS (Murashige and Skoog) medium containing either 1.0 or 2.0 μM (+)-ABA. These seeds were then moist chilled for 4 days prior to transfer to germination conditions (21°C, 16-hr light period in a Conviron E15 growth chamber). Seeds that were counted as germinated were those that exhibited radicle emergence. All tests were carried out in triplicate, with each replicate having 50-100 seeds.

Germination Performance in the Presence of 6% Glucose

CnAIP1, SALK and wild-type mature dry seeds were surface sterilized in 40% commercial bleach for 10 min and washed three times with sterilized water (3 x 5 mins). Seeds were sown on one-half strength MS medium containing 6% glucose. Seeds were placed in moist chilling conditions (4°C) for 4 days prior to transfer to germination

conditions (21°C, 16-hr light period) in a growth chamber (Conviron E15). Seeds that were counted as germinated were those that exhibited radicle emergence. All tests were carried out in triplicate, with each replicate having 50-100 seeds.

2.2.5. Analyses of Seed Maturation Proteins in Arabidopsis

CnAIP1 transgenic, SALK and wild-type plants were grown adjacent to each other under the same growth conditions (21°C, 16-hr light period). Arabidopsis plants were grown until 2 weeks after the onset of flowering, after which opening flowers were tagged to denote the day of pollination. Siliques were removed at 5, 10, 15, 25, 28 days after pollination (DAP). Seeds were removed only at the later stages, and the developing seeds along with mature dry seeds were flash frozen in liquid nitrogen for future protein extraction and western blot analysis. At the younger stages, whole siliques were frozen.

Proteins were extracted from Arabidopsis seeds and siliques at the various timepoints during development by grinding tissues in protein extraction buffer (0.1M potassium phosphate buffer pH 7.0 and Protease Inhibitor Cocktail (for plant cell and tissue extracts – Sigma Aldrich)). A 2X SDS loading buffer was added to the extracts before boiling the samples and centrifugation at room temperature for 10 min at 13000 rpm (Biofuge pico Heraeus). Protein concentration of the supernatant was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin IV (Sigma) as a standard. Following protein quantification, 1M DTT (end concentration 0.1 M) with bromophenol blue was added to each of the samples in preparation for SDS-PAGE loading. Proteins (25 µg) from each sample were separated on 12% SDS-PAGE gels and transferred to HybondECl nitrocellulose protein membrane (GE Healthcare, Amersham Hybond ECl). Membranes were blocked with 5% skim milk powder in 1X PBST (phosphate buffered saline containing 0.05% Tween-20) overnight. Dilutions of antibodies were as follows: α-TIP (1:2000); dehydrin (1:1000); BiP (1:2000); Protein disulphide isomerase (PDI) (1:3000); Peroxiredoxin1 (Per1) (1:3000), 12S cruciferin (1:10000), vicilin (1:1000) and Hsp17.6 (1:1000) in PBST containing 3% skim milk powder for 3 h at room temperature. The membrane was washed three times for 20 min time intervals in 1X PBST at room temperature. Membranes were then incubated for 1 h in goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP). The secondary antibody was diluted 1:3000 in PBST containing 5% skim milk powder.

The membrane was washed three times for 20 min time intervals in 1X PBST at room temperature. Immunodetection was performed using ECL detection (GE Healthcare, Amersham ECL Prime Western Blotting Detection Reagents).

2.3. Results

2.3.1. CnAIP1 Localizes in Nucleus, Cytosol and Plasma Membrane

The yeast two-hybrid approach was used to determine protein interactors of CnABI3, one of which was CnAIP1. Based on online bioinformatics programs, the CnAIP1 protein was predicted to localize to the nucleus (88%). To verify this protein-protein interaction and verify the localization of CnAIP1, I transiently expressed CnAIP1 in *Nicotiana benthamiana* leaf tissue. Transient expression of CnABI3 was also performed to validate the yeast two-hybrid results. Single and double (both CnAIP1 and CnABI3 cotransformed) transformants were generated. Verification of CnABI3-CnAIP1 protein-protein interaction was attempted using FRET analysis. This mechanism involves energy transfer between fluorophores (ie. CFP-donor, YFP-acceptor) that are in close proximity due to an interaction of two proteins. FRET analysis using the Volocity software was conducted on three images obtained for each data point: donor image, acceptor image and FRET image. Bleed-through constants were calculated for each of the donor and acceptor dyes and incorporated into the net FRET calculation to generate normalized FRET images. Although I was able to visualize CnABI3 and CnAIP1 localization in the nucleus of double transformed *Nicotiana benthamiana* leaf tissue, the results were not conclusive because fluorescence in the FRET image was observed in regions not observed for CFP, which is the donor fluorophore for FRET (not shown). Attempts were made to troubleshoot any technical issues regarding microscope setup and/or the associated software but I was unsuccessful in creating a useable FRET image. *Nicotiana benthamiana* leaf tissue that had been transformed with either the CnAIP1 construct or CnABI3 construct (single transformants) were therefore used to determine the subcellular localization of these two proteins. The CnABI3 protein specifically localized to the nucleus as is expected of a transcription factor (Figure 2-1A). The CnAIP1 protein appeared to be present in the extracellular matrix (cell wall), plasma membrane, cytoplasm and to a lesser extent in the nucleus (Figure 2-1B). Bioinformatics

programs have predicted that the protein sequence of CnAIP1 contains a nuclear localization signal (NLS). This signal may direct the protein to the nucleus during some stress or developmental process. I did attempt heat treatment of *Nicotiana benthamiana* leaf tissues, but there was no observed change in the localization of CnAIP1.

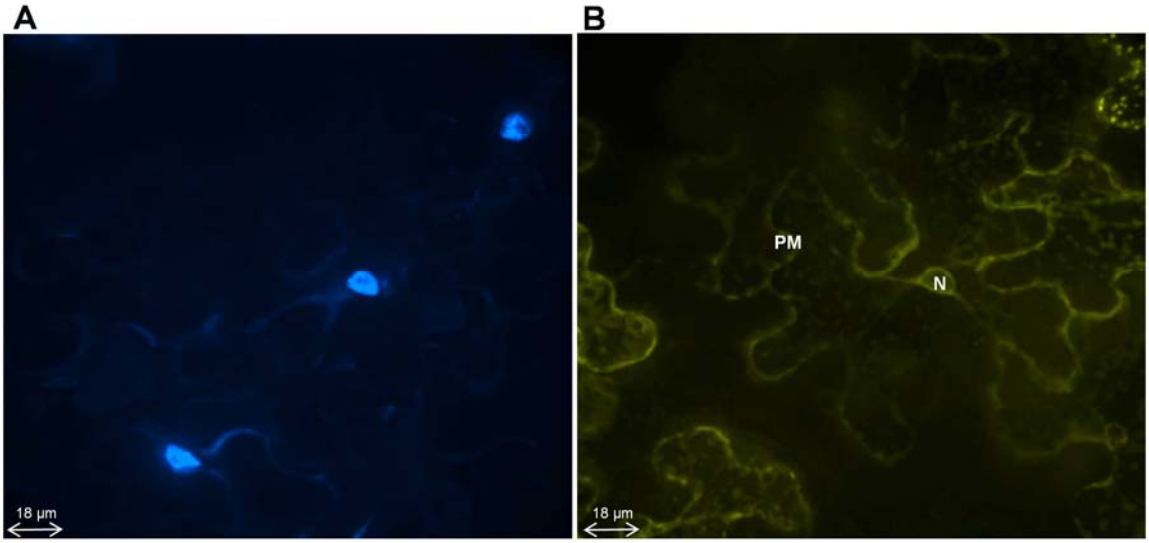

Figure 2-1. Transient expression of CnABI3 and CnAIP1 Nicotiana benthamiana leaf tissue to determine subcellular localization.

A. CnABI3 localized predominantly in the nucleus which was visualized by CFP fluorescence and B. CnAIP1 localization was observed in the cytoplasm, the nucleus and the plasma membrane/cell wall using YFP fluorescence. Both A and B were imaged using confocal microscopy. PM (plasma membrane), N (nucleus)

2.3.2. Expression of CnAIP1$_{pro}$-GUS in Arabidopsis

A 2.0-kb region of the *CnAIP1* gene promoter was isolated using the genomic-walking method. This work was completed in collaboration with a PDF in our lab, Dr. Tiehan Zhao, although I performed most of the downstream gene construction work. To determine the spatial and temporal expression characteristics of the *CnAIP1* promoter, the promoter fragment was fused to the GUS reporter gene coding sequence. The GUS reporter chimeric gene construct was subsequently used to transform Arabidopsis. T2 seeds were analyzed for *CnAIP1* promoter expression. The expression pattern of the *CnAIP1*:*GUS* gene was characterized throughout seed development (Figure 2-2A). Expression was localized to the micropylar region during the first few days after pollination and thereafter expression was evident throughout the embryo (radicle and

cotyledons) from 10 days after pollination onwards (Figure 2-2A). Following germination, CnAIP1 promoter expression was also evident in cotyledons and hypocotyls and in the roots of 3 day-old seedlings (Figure 2-9). Expression in flowers was very high, especially in the anthers and filaments and to a lesser extent in the stigma (Figure 2-2B).

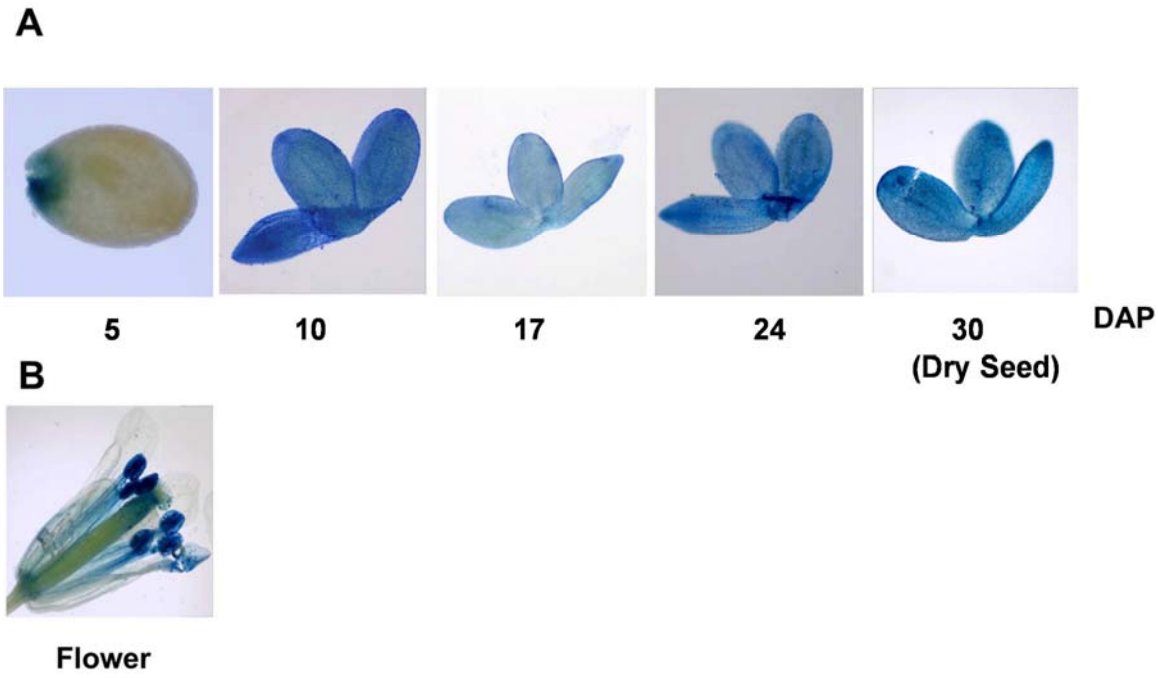

Figure 2-2. Expression patterns of GUS driven by the CnAIP1 promoter at different stages during the Arabidopsis life cycle

A. Histochemical assay showing GUS activities in developing and mature dry *Arabidopsis* seeds transformed with the *CnAIP1*$_{pro}$*::GUS* construct. GUS expression was observed in the micropylar region 5 days after pollination (DAP), thereafter from 10 DAP to 30 DAP (dry seed) expression was observed throughout the embryo, including the cotyledons and radicle. Seed coats of seeds older than 5 DAP were removed before histochemical assays were performed. B. Flower tissues were collected and directly subjected to the histochemical assay. Chlorophyll was removed by subsequent incubation of samples in 95% ethanol. Photographs are cropped at various magnifications.

2.3.3. CnAIP1 Seeds have a Higher Dormancy as Compared to Wild-Type Seeds

In order to characterize the overexpression of CnAIP1 as a means of determining some of the functions of CnAIP1, the cauliflower mosaic virus *35S* promoter was used to drive the expression of the *CnAIP1* coding sequence. CnAIP1 transgenic *Arabidopsis* lines exhibited similarities in germination percentage and growth performance compared

to wild-type. The highest expression line, as determined by high protein levels observed through western blot analysis, was used for dormancy experiments. Freshly harvested mature dry seeds from wild-type (Col-0) and CnAIP1 OE plants (T4 seeds) were treated for different durations of moist chilling prior to germination. Germination was defined as visible radicle emergence. *CnAIP1* OE seeds had a germination percentage of 80% on agar plates in the absence of moist chilling (Figure 2-3). *CnAIP1* seeds that were left in moist chilling from 1-4 days also showed a similar capacity for germination. *CnAIP1* seeds took 2 days to reach 80% germination, while wild-type seeds reached 100% after 1 day in germination conditions, regardless of the duration of moist chilling. Compared to wild-type seeds, the *CnAIP1* seeds had a higher degree of dormancy and germinated slower.

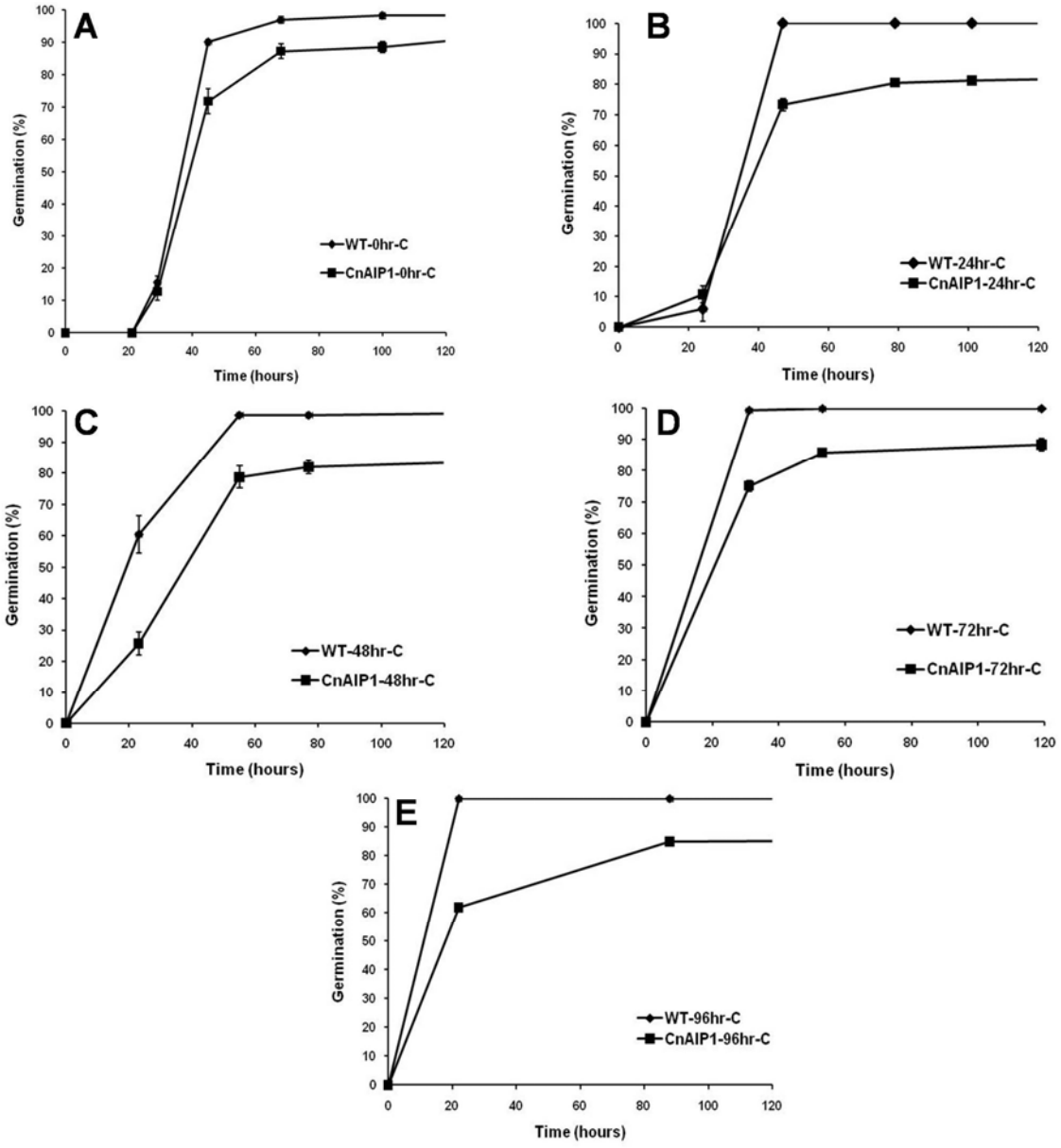

Figure 2-3. Germination performance of wild-type (WT) and CnAIP1 transgenic seeds to determine effect of OE CnAIP1 on depth of dormancy.

Wild-type and CnAIP1 transgenic *Arabidopsis* seeds were treated A. without moist-chilling 0hr, or with B. 24hr, C. 48hr, D. 72hr, E. 96hr moist-chilling treatment prior to transfer of seeds to germination conditions to monitor percent germination. *CnAIP1* seeds had a higher degree of dormancy and germinated slower compared to wild-type seeds. Data represent means ± SE of triplicate tests, with each replicate having 50-100 seeds.

2.3.4. *CnAIP1 Germination is More Sensitive to Inhibition by Exogenous ABA*

The effects of overexpression of CnAIP1 on the ability to sense ABA was studied in terms of germination percent and rate. To determine differences in ABA sensitivity, mature wild-type and *CnAIP1* OE *Arabidopsis* seeds (T4 seeds) were placed on one-half-strength MS medium containing either 1.0 or 2.0 μM ABA and subjected to a 4 day moist-chilling treatment (4°C) to break dormancy. Plates containing seeds were then transferred to germination conditions (21°C, 16-h photoperiod) to monitor percent germination in the presence of exogenous ABA. *CnAIP1* seeds exhibited a greater sensitivity to ABA; these seeds germinated more slowly as compared to wild-type seeds (Figure 2-4). Germination rate of seeds grown without the presence of abscisic acid (control) is shown in Figure 2-5. Interestingly, with increasing concentrations of ABA, the overall germination rate of wild-type and *CnAIP1* seeds increased, but *CnAIP1* seeds germinated slower comparatively which was indicative of their greater sensitivity to ABA as far as the inhibition of germination is concerned. It would be interesting to characterize other ABA physiological responses in the wild-type vs. *CnAIP1* seedlings/plants.

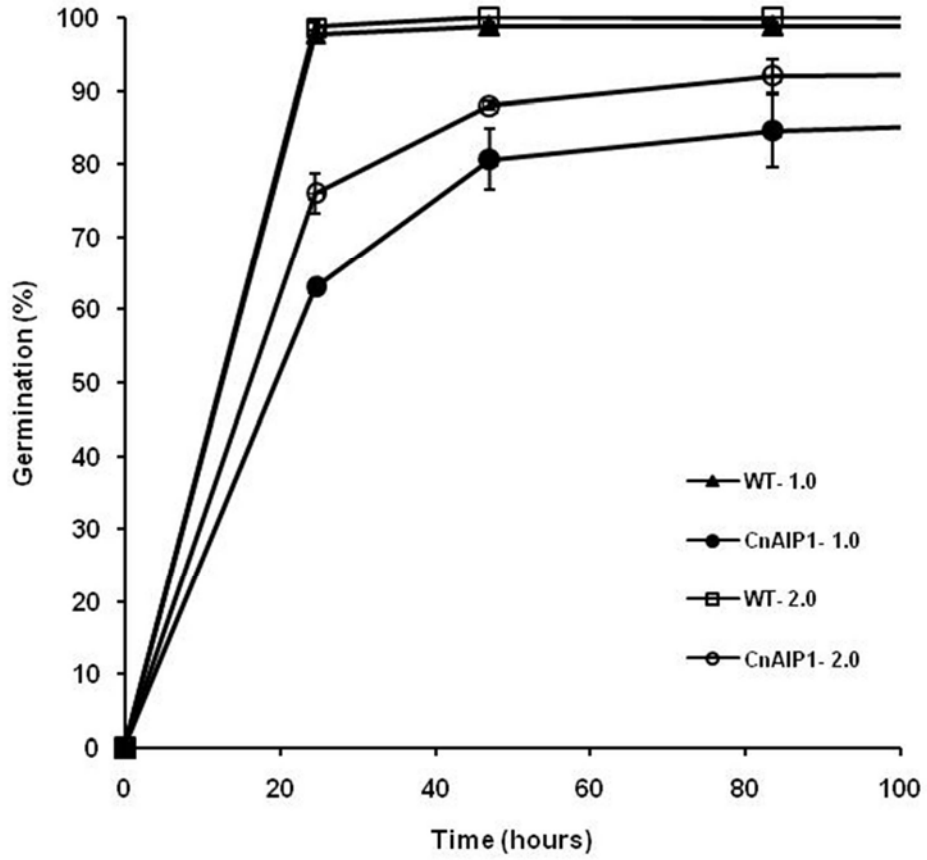

Figure 2-4. Effect of exogenous ABA on germination performance of wild-type (WT) and CnAIP1 transgenic seeds.

Wild-type and CnAIP1 transgenic *Arabidopsis* seeds were sown on plates that contained one-half strength MS medium with either 1.0 or 2.0 µM (+)-ABA. Seeds were subjected to a 4 day moist-chilling treatment at 4°C prior to transfer to germination conditions to monitor percent germination. In the presence of ABA, CnAIP1 transgenic seeds germinated slower compared to wild-type seeds which is indicative of their greater sensitivity to ABA. Data represent means ± SE of triplicate tests, each with 50-100 seeds.

2.3.5. Characterization of a Loss of Function Arabidopsis Mutant for the Homologue of CnAIP1 (AtHip1)

A previous PDF in the Kermode Lab, J. Grelet, noted a phenotypic difference in the *athip1* SALK line – that of sensitivity to glucose. I followed up those initial experiments with more exhaustive testing. My physiological experiments conducted on wild-type and on the mutant AtHIP1 (*athip1* SALK line) revealed differences in germination capacity between the two lines in the presence of 6% glucose with the

mutant line showing less sensitivity to glucose and germinating faster than wild-type (Figure 2-5). There was no difference observed in germination rate in the presence of mannitol, an osmotic control (not shown). This was the first phenotypic difference observed between wild-type and the mutant line. Glucose is known to be a signaling molecule at lower concentrations. By contrast, at the concentrations that I used, it should be considered as a stress treatment. From these results I wanted to explore the inducibility of *CnAIP1* expression in the presence of glucose.

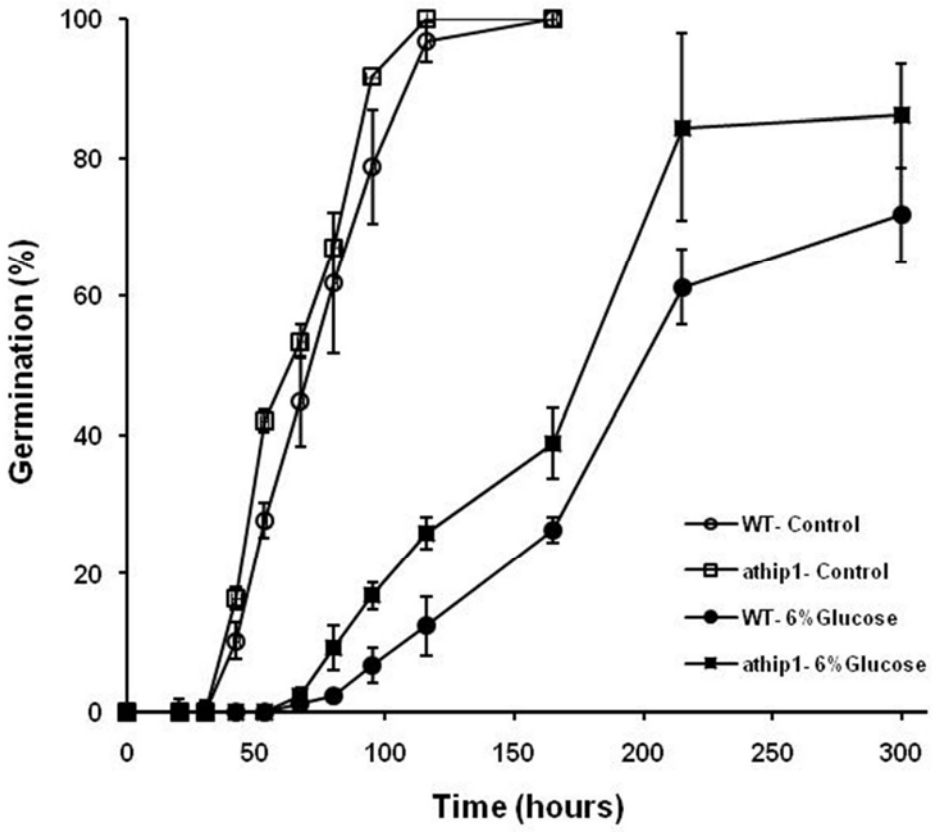

Figure 2-5. Germination performance of the mutant SALK line (athip1) and wild-type (WT) Arabidopsis seeds in the presence of 6% glucose.

Wild-type and *athip1 Arabidopsis* seeds were sown on plates that contained one-half strength MS medium with 6% glucose and subjected to a 4 day moist-chilling treatment at 4°C prior to transfer to germination conditions to monitor percent germination. Control plates did not contain glucose. The mutant SALK line was less sensitive to glucose and germinated faster than wild-type *Arabidopsis* seeds. Data represent means ± SE of triplicate tests, each with 50-100 seeds.

2.3.6. CnAIP1 Seeds are Hypersensitive to Glucose

Based on the germination results with the *athip1* mutant, physiological experiments conducted on wild-type and *CnAIP1* seeds revealed differences in germination capacity between the two lines in the presence of 6% glucose. *CnAIP1* seeds were hypersensitive to glucose, when it was present as a stress (i.e. 6%), and therefore germinated slower compared to the wild-type *Arabidopsis* seeds (Figure 2-6). Wild-type seeds that had been placed on plates containing 6% glucose germinated at a comparable percentage to wild-type seeds that had not been exposed to glucose, but experienced germination delay at higher concentrations of glucose.

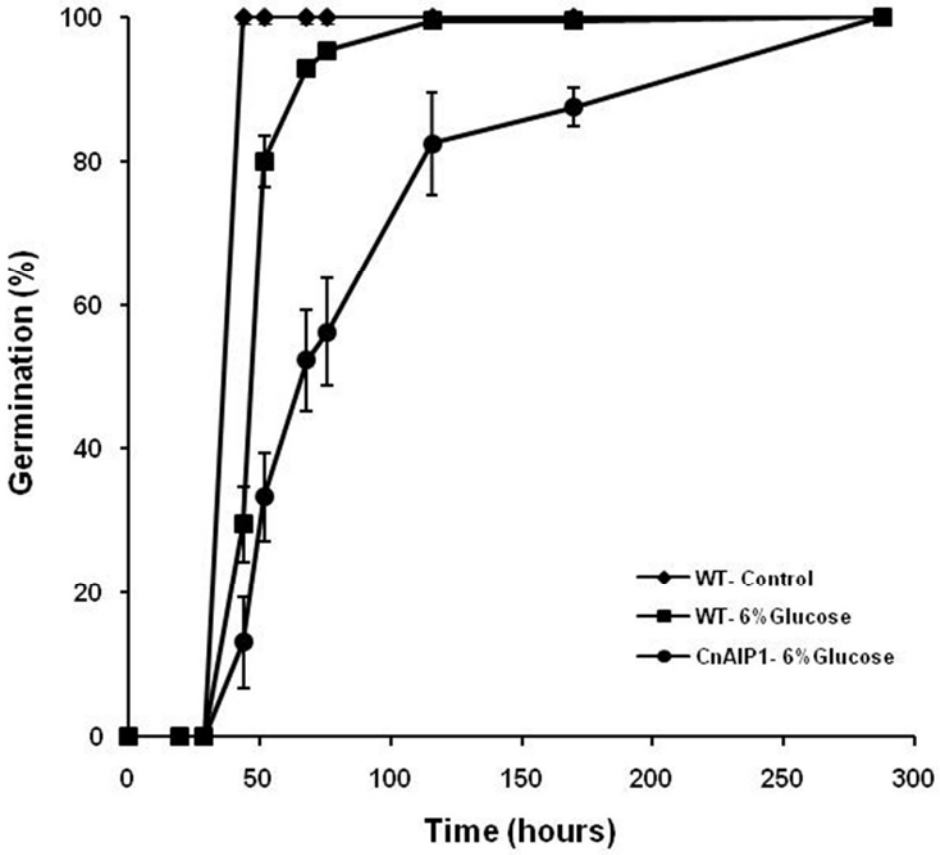

Figure 2-6. Germination performance of the CnAIP1 transgenic and wild-type (WT) seeds in the presence of 6% glucose.

Wild-type and *CnAIP1* transgenic *Arabidopsis* seeds were sown on plates that contained one-half strength MS medium with 6% glucose and subjected to a 4 day moist-chilling treatment at 4°C prior to transfer to germination conditions to monitor percent germination. Control plates did not contain glucose. CnAIP1 transgenic seeds were hypersensitive to 6% glucose and germinated slower compared to wild-type seeds that germinated in the presence of glucose. Data represent means ± SE of triplicate tests, each with 50-100 seeds.

2.3.7. CnAIP1, athip1 (SALK) and Wild-Type Seeds Exhibit Differential Accumulation of Maturation Proteins during Their Development

I investigated whether the overexpression of *CnAIP1* had any effect on the accumulation of various maturation proteins during seed development. In addition, *athip1* (SALK) was used as a comparison to visualize differences in protein accumulation throughout seed development between an over-expression (*CnAIP1* OE)

line, a knock-out (*athip1* SALK) line and wild-type seeds. For all the proteins analyzed (Hsp17.6, Per1, 12S cruciferin, dehydrin, vicilin, PDI, BiP and α-TIP) there was no detectable accumulation during the first 5 days of development in any of the seeds (Figure 2-7). Differences in protein accumulation levels between *CnAIP1* OE, SALK and wild-type were evident in terms of the amount of accumulation as well as in the temporal patterns. For example, differences were clearly evident in the accumulation levels of dehydrins (type II LEA proteins). The accumulation was higher in *CnAIP1* seeds from 15 days after pollination (DAP) to maturity, but was considerably lower in wild-type seeds and very low in the SALK line. The temporal accumulation of Per1 seemed to be altered in the CnAIP1 over-expression (OE) line and Hsp17.6 was clearly lower in the CnAIP1 OE line as compared to the wild-type and SALK lines. It is possible that CnAIP1 regulates dehydrin expression, and possibly some of the other maturation proteins especially during seed development. Loading controls are appended in Figure A1-1.

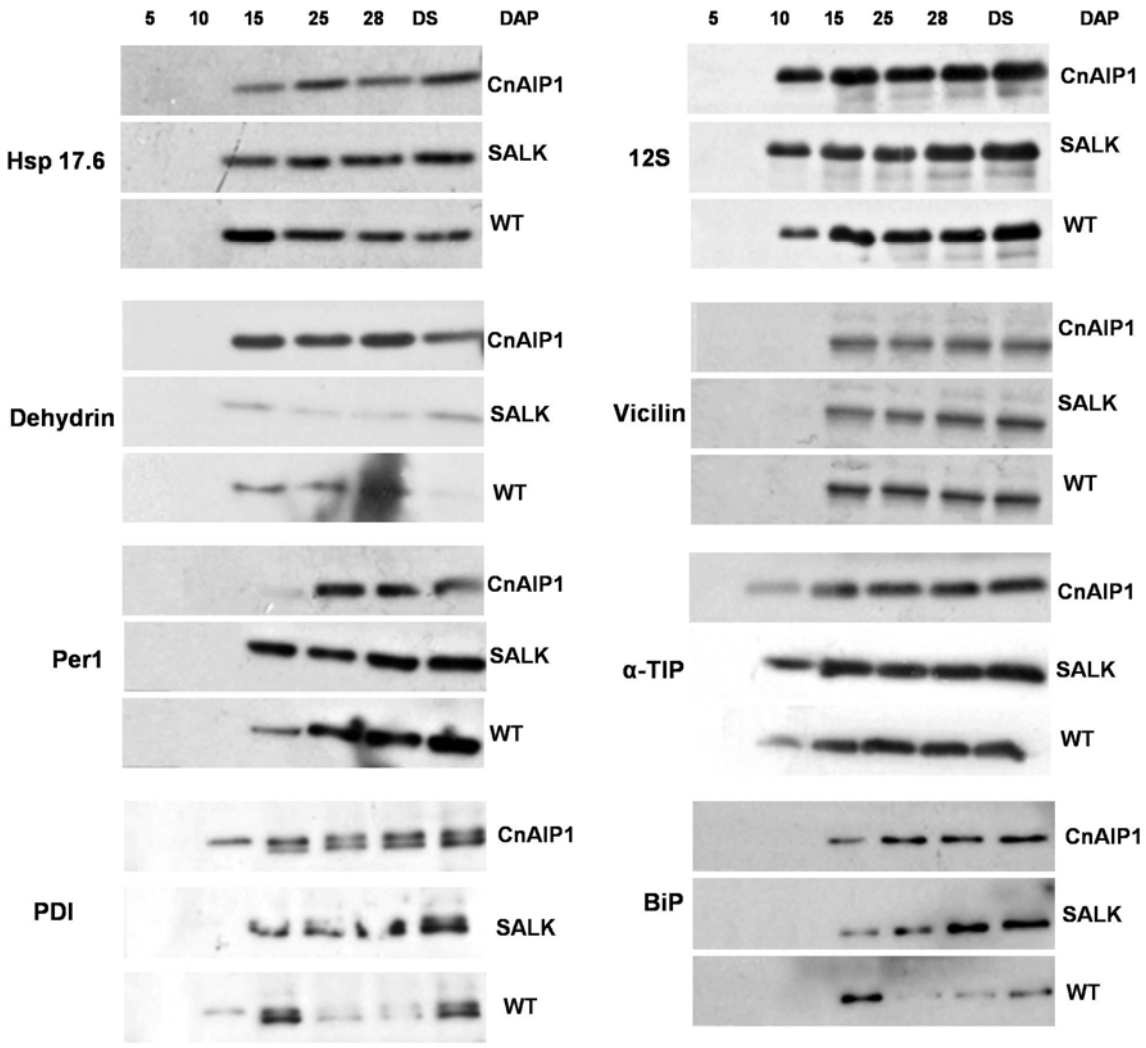

Figure 2-7. Western blot analysis of maturation proteins that accumulate during seed development in CnAIP1 transgenic, SALK (athip1) and wild-type (WT)seeds.

Accumulation levels of various developmentally expressed proteins were determined 5, 10, 15, 25 and 28 days after pollination (DAP). Protein levels were also observed in dry seed (DS) samples. Differences in protein accumulation levels between wild-type, SALK and *CnAIP1* OE lines were evident in terms of the amount of accumulation as well as in the temporal patterns, especially in regards to dehydrin accumulation with higher accumulation levels observed in the CnAIP1 transgenic line compared to the SALK mutant line.

2.3.8. *CnAIP1 Promoter Expression is Enhanced by Stresses (Glucose, NaCl and Oxidative Stress)*

As noted earlier, a CnAIP1:GUS construct was made and transgenic lines of Arabidopsis were generated. PlantCARE (Lescot *et al*., 2002), a database of plant cis-acting regulatory elements, was used for an initial bioinformatics CnAIP1 promoter motif analysis (Figure 2-8). Based on the motif analysis, hormone and abiotic treatments were applied to 3 day-old CnAIP1pro-GUS transgenic seedlings. For the control treatment, seedlings were placed in half-strength MS media. Various hormone and abiotic stress treatments were applied followed by a visualization of GUS staining. Differences in histochemical GUS staining for treatment vs. control were seen for seedlings treated with glucose, NaCl, H_2O_2 and at 37°C for 16 h (Figure 2-9). These treated seedlings expressed high GUS staining in the cotyledons and roots compared to the control seedlings. A GUS fluorometric activity assay was performed to validate the staining results. Seedlings treated with glucose, NaCl and H_2O_2 and those placed in heat stress exhibited an increase in GUS activity that was almost double of that in the control (non-stressed) seedlings.

Figure 2-8. Motif analysis of the promoter sequence of CnAIP1 using the online bioinformatics tool, Plant Care.

- A-box: cis regulatory element
- AE-box: light response
- Box 4: light responsiveness
- MSA-like: cell cycle regulation
- CAAT-box: promoter and enhancer regions
- CCGTCC-box: meristem specific activation
- GARE-motif: gibberellin-responsive element
- HSE: heat responsiveness
- Skn-1: endosperm expression
- TATA: core promoter element
- TC-rich repeats: defense and stress responsiveness

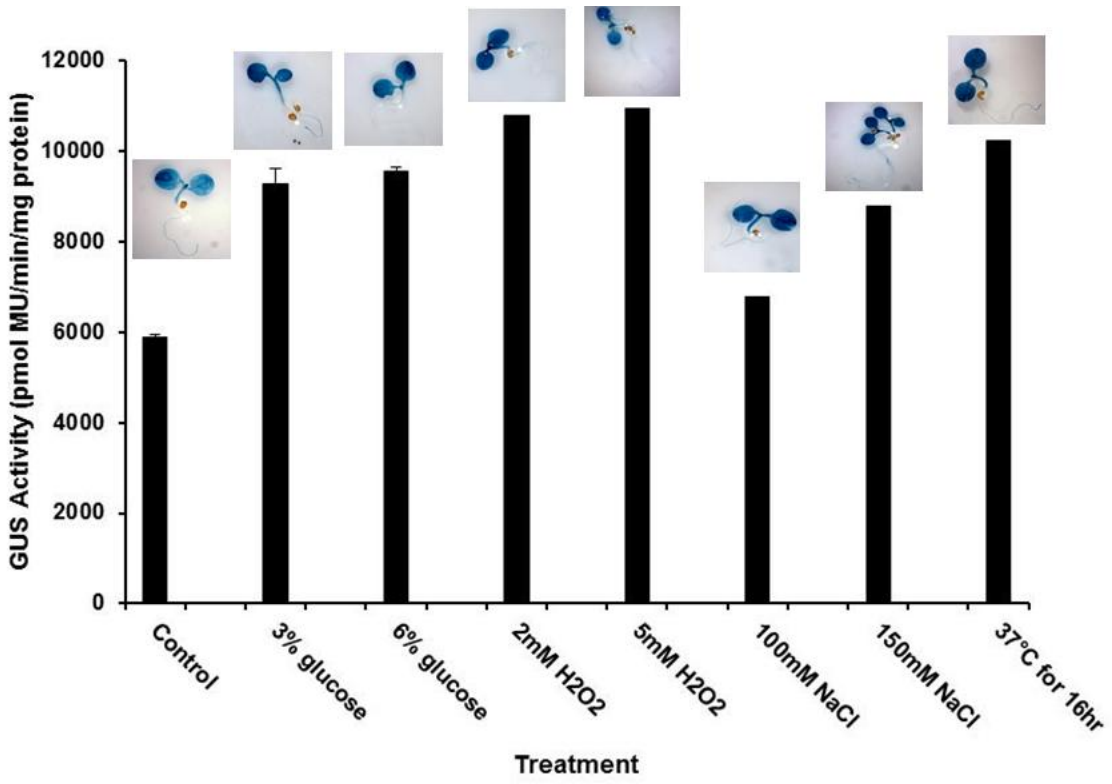

Figure 2-9. Expression characteristics of CnAIP1pro-GUS in 3 day old Arabidopsis seedlings.

Histochemical analysis was conducted on Arabidopsis seedlings expressing *CnAIP1pro-GUS*. Seedlings were treated with various abiotic treatments to visualize changes in CnAIP1 promoter expression levels. GUS activity was determined by a fluorometic assay conducted on 3 day old seedlings incubated overnight (16 hr) in one-half-strength MS liquid medium with different abiotic stresses as indicated. The increase in GUS activity in abiotic stress treated seedlings was almost double of that observed for non-stressed seedlings. Data represents means ± SE of duplicate experiments. MU, Methylumbelliferone.

2.4. Discussion

2.4.1. CnAIP1 may Shuttle between the Cytosol and the Nucleus

CnAIP1 localization was visualized using confocal microscopy. The CnAIP1 protein looks to be constitutive with abundance in the extracellular matrix (cell wall and apoplast), plasma membrane, cytoplasm and to a lesser extent in the nucleus. Bioinformatics programs have predicted that the protein sequence of CnAIP1 contains a nuclear localization signal (NLS). This signal may direct the protein to the nucleus during

stress or during developmental processes. It is therefore likely that CnAIP1 shuttles between nuclear and cytoplasmic compartments. HSPs are known to be abundant throughout the cell with different classes of HSPs found within the cytosol, chloroplast, endoplasmic reticulum and mitochondria (Waters *et al*., 1996). The homolog of CnAIP1, AtHip1 encodes a heat shock protein associated factor. Due to the high sequence similarity between CnAIP1 and AtHip1 (see Introduction), it is likely the two are homologs and share similar functions. As a heat shock protein associated factor or chaperone, it is likely that CnAIP1 would colocalize throughout the cell wherever heat shock protein abundance is found. In this manner, chaperones are able to regulate HSP activity when needed. This is observed for Hip (Hsp70 Interacting Protein) which appears to stabilize Hsp70 in its ADP bound conformation which extends its association with its substrates (Hoefeld *et al*., 1995). Likewise the Hip-Hsp70 interaction aids in the assembly of functional receptor complexes with the Hip-Hsp70 association stabilizing the premature receptor (Prapapanich *et al*., 1998). Conversely, other heat shock protein interacting factors negatively regulate heat shock protein chaperone function. As discussed, in its role as a chaperone, Hsp70 has high affinity for its substrate in the ADP-bound form (Palleros *et al*., 1993) and chaperone function is dependent on ATPase activity. CHIP (carboxyl terminus of Hsc70-interacting protein) is a cytoplasmic protein which inhibits the Hsp40-stimulated ATPase activity of Hsc70 and Hsp70 (Ballinger *et al*., 1999). This implies that CHIP negatively regulates the substrate binding capabilities of Hsc70-Hsp70 and prevents its function. In a separate example, hFAF1 (human Fas-associated Factor 1), negatively regulates the chaperone activity of Hsp70 (Kim *et al*., 2005). In this case, cells which have been heat shocked are unable to recover due to the direct binding of hFAF1 to Hsp70. This interaction prevented Hsp70 from performing its chaperone activities which involves refolding of denatured proteins and preventing protein aggregate formation. It is likely that heat shock protein associated/interacting factors positively or negatively regulate Hsp activity in a situation specific manner. It is possible that some interacting factors may play dual roles in the regulation of Hsp activity. It is likely that CnAIP1 does interact with CnABI3 in the nucleus but this interaction only occurs during certain circumstances such as participation in the activation of particular genes involved in seed developmental processes. It is possible that the NLS of CnAIP1 directs it to the nucleus when ABA levels are increased, but this has not been studied. Hsp70 has been observed to shuttle between the nucleus and

cytoplasm during heat stress. During high-temperature stress Hsp70 localizes primarily to the nucleolus and then redistributes to the cytoplasm during recovery. This has been observed in plants (Milarski *et al*., 1989; Neumann *et al*., 1987; Neumann *et al*., 1989; Nover *et al*., 1986). A 17-amino-acid segment of human Hsp70 can act as a nuclear targeting signal (Dang *et al*., 1989). A change in the binding of Hsp70 to other cellular components/proteins or an alteration of Hsp70 conformation is involved in controlling intracellular localization (Dang *et al*., 1989; Milarski *et al*., 1989). Similarly, CnAIP1 may also alter its conformation or alter its binding partners in the process of shuttling between the cytosol and the nucleus. This will have to be further investigated.

Taking its domain structure into account, it can be assumed that CnAIP1 can interact with heat shock proteins. An additional interaction with CnABI3 would thus imply that CnABI3 is stabilized under certain stress circumstances or its conformation changed by the heat shock protein complexes. In mammals, it has been shown that the Hsp70/Hsp90 complex plays a role in the regulation of steroid receptors by changing their conformation in a way that will expose the steroid binding site (Pratt and Toft, 2003). The complex is thus not merely a repair site for proteins denatured by stress. It has been speculated that the ABI3 ortholog VP1 might have to undergo a conformational change to unmask a cryptic DNA binding domain in the B3 domain, as it would be hidden in the predicted protein structure (Suzuki et al., 1997). In fact the B3 domain confers DNA-binding to the ABA-responsive RY-element *in vitro* when the A1 domain has been removed from ABI3, but not in the full-length protein, which indicates that the conformation of the full protein is unfavorable for DNA-binding of the B3 domain. While there is no experimental evidence yet for a conformational change of ABI3/VP1 proteins, an interaction with a heat shock complex would be a possible mechanism to achieve such a change. CnAIP1 might play a role in this process.

Other AIPs have been identified as being associated with protein folding or turnover in Arabidopsis (Kurup *et al*. 2000): AtAIP2 is an E3-ligase that polyubiquitinates ABI3 and thereby targets it for protein degradation by the proteasome (Zhang *et al*, 2005). Interestingly, AtAIP3 was identified by its homology to AfVIP3 (Kurup *et al*., 2000; Jones *et al*., 2000) and turned out to be part of the prefoldin family (Hill and Hemmingsen, 2001), a group of proteins implicated in protein folding of cytoskeletal elements and in the stress response (Rodriguez-Milla and Salinas, 2009). Like the

AtAIPs that have been identified to date (Kurup et al. 2000), CnAIP1 is expressed throughout development and not restricted to life cycle stages or tissues in which CnABI3 is expressed but CnABI3 can also be induced under stress conditions. It thus seems likely that the interaction is induced under specific circumstances.

2.4.2. CnAIP1 Plays a Role during Seed Development, Dormancy and Germination

My investigation of the temporal and spatial regulation of *CnAIP1* expression during seed development provides insight into *CnAIP1* gene transcription during developmental processes. Expression of the *CnAIP1pro-GUS* fusion gene in Arabidopsis was low initially during the first few days following pollination but promoter expression as indicated by GUS histochemical staining increased in embryos throughout seed development until seed maturity. From day 10 DAP onwards the promoter was highly expressed throughout the embryo. This assay of CnAIP1pro-GUS reporter gene transcription revealed that the expression of CnAIP1 is not tissue specific and is instead spread throughout the embryo during development with all cells being stained up until maturity and desiccation but expression is restricted during very early development. This expression pattern suggests a general protective role for CnAIP1 rather than specialized roles in particular tissues during development. Similar results were observed for sHsps in Arabidopsis (Wehmeyer and Vierling, 2000) and in immunolocalization data for fava bean and pea (zur Nieden *et al*., 1995). There is a localization of CnAIP1 promoter expression in the radicle near the micropylar region of the seed at 5 DAP. At 10 DAP, the expression is not specific and is spread throughout the embryo. Further detailed investigation is required to determine the day by day expression pattern of the CnAIP1 promoter during the first 10 DAP to visualize changes in spatial expression. The temporal expression of CnAIP1pro-GUS activity during development is similar to observations of sHSP protein accumulation (Figure 2.7, Wehmeyer *et al*., 1996). Although protein synthesis may be delayed following transcription, it is interesting to observe the parallel in CnAIP1pro-GUS activity and sHSP protein expression. For a putative heat shock protein associated factor it is likely that its expression patterns will overlap with sHsps, even during developmental processes indicating that a role during development alongside sHsps is likely. The expression of specific sHsps such as

Hsp17.4, 17.6, etc during development occurs during mid-maturation which is when the majority of storage reserves are accumulated and processes underlying dormancy maintenance and desiccation tolerance are initiated. The absence of some Hsps during development suggests that chromatin remodeling and architecture may play a role in determining which *Hsp* genes are expressed during development (Kotak *et al*., 2007). I hypothesized that CnAIP1, like sHsps may play a role in either dormancy maintenance, desiccation tolerance or both during development. *CnAIP1* OE seeds exhibited increased dormancy as compared to wild-type seeds and were hypersensitive to the presence of exogenous ABA as far as an inhibition of their germination is concerned. It could be that CnAIP1 plays a role in the seed developmental and dormancy program as seen from these results. It is possible that CnAIP1 interacts with CnABI3 to aid in keeping systems in a developmental state by delaying the onset of germination. In other words CnAIP1 action supports the actions of CnABI3 as a regulator of seed developmental processes. Despite this, the accumulation of Hsp17.6 and some of the other maturation proteins was not different between *CnAIP1* OE seeds, SALK line mutant and wild-type. Wehmeyer *et al*. (1996) found that mutants with reduced dormancy had wild-type levels of Hsp17.4 indicating that sHsps play a role in seed dormancy although they do not work alone. Although CnAIP1 has a necessary role in seed dormancy it is not sufficient for dormancy maintenance. Other protein factors that interact with CnABI3 and/or CnAIP1 may also be essential for maintaining dormancy. A lack of observable differences is common in large gene families, like the heat shock family which has many members with redundant functions that are able to compensate for mutated proteins when required. The role of CnAIP1 in desiccation tolerance was also investigated although not extensively. The connection of sHsp expression with the initiation of desiccation tolerance during development suggests possible role for sHsps in this process. As a heat shock protein associated factor, CnAIP1 could be hypothesized to play a role in this process as well. Wehmeyer and Vierling (2000) found that desiccant-intolerant mutants had reduced levels levels of Hsp17.4 and were unable to survive desiccation. They emphasized that sHsps were probably involved in the process of desiccation tolerance although other proteins that were expressed during the mid to late maturation stage were likely involved as well. Interestingly, *CnAIP1* OE seeds had an increase in the accumulation of dehydrin proteins (type II LEAs) during developmental profiling of stress protectants in comparison to SALK and wild-type

seeds. LEA genes are upregulated during mid-maturation and have been hypothesized to be important in desiccation tolerance (Parcy *et al*., 1994). The acquisition of desiccation tolerance during mid-maturation occurs many days prior to the actual desiccation of the seed (Meurs *et al*, 1992; Ooms *et al*., 1993; Giraudat *et al*., 1994; Koornneef and Karssen, 1994). LEA transcript levels accumulate during development until seeds are dry and decline after germination occurs (Dure III *et al*., 1989). They are known to protect the dry seed during desiccation. The increase in dehydrin protein accumulation in CnAIP1 OE seeds likely indicates that CnAIP1 plays a role in regulating desiccation tolerance through the upregulation of dehydrins during mid to late maturation. It would be very valuable to do a comparison among the lines of the time during seed development when desiccation tolerance is acquired. Thus, CnAIP1 may be involved in regulating dehydrin expression during the mid-maturation stage of seed development with its partner CnABI3 and may promote an earlier acquisition of desiccation tolerance. This hypothesis needs to be investigated further.

Another protein that is involved in desiccation tolerance is peroxiredoxin1 (Per1). Seed peroxiredoxins protect tissues from reactive oxygen species during desiccation and during the transitional phase to early imbibition and/or are also involved in protection of proteins and membranes during dormancy maintenance when seeds are in the imbibed state (Haslekas *et al*., 1998). Although changes in Per1 protein levels were not observed in *CnAIP1* OE lines compared to SALK mutant line and wild-type, it is interesting to observe that the expression pattern of Per1, dehydrins, sHsps during development coincides with maximal levels of endogenous ABA in developing embryos (Almoguera and Jordano, 1992). This may indicate that ABA is the major regulator of developmental accumulation of protectants during mid- to late-maturation with *CnAIP1* OE seeds showing an increased sensitivity to ABA in regards to the inhibition of germination.

Previous studies hypothesized that cell membranes and proteins are protected during desiccation by a glassy matrix state made of soluble sugars (Bernal-Lugo and Leopold, 1998). Recent studies have shown that sugars play less of a role in desiccation protection but that instead, proteins present during late maturation contribute to the cytoplasmic protective matrix. Wehmeyer and Vierling (2000) suggested that Hsp17.4 may be a good candidate as a protective chaperone to maintain survival in the

desiccation/dry state of seeds. CnAIP1 could be another candidate in this matrix, considering it is likely associated with heat shock factors. This association will have to be investigated further.

The biochemical phenotypes of *CnAIP1* OE seeds, SALK mutant seeds and wild-type seeds were investigated by western blot analyses of various maturation proteins that accumulate during seed development. All of the maturation proteins analyzed (Hsp17.6, Per1, 12S, dehydrins, vicilin, PDI, BiP and α-TIP) started to accumulate at various times after 5 days of development. The transgenic CnAIP1 seeds appeared to accumulate the major storage protein (e.g. 12S globulins and 7S vicilins), at levels that were characteristic of the wild-type seeds. Additionally, the levels of PDI, α-TIP, BiP, Per1 and vicilin were similar in CnAIP1 OE, SALK and wild-type seeds. The SALK line seeds tended to show very similar levels of accumulation of the various maturation proteins as compared to wild-type seeds which is likely a consequence of functional redundancy of HSP interacting factors. It is likely that another protein (i.e. AtHip2) is compensating for the loss of this protein in the SALK line.

There was a slight increase in the accumulation of ER molecular chaperones BiP and PDI in both the *CnAIP1* and SALK lines; but again the temporal differences are most striking amongst the three lines. Both BiP and PDI act as molecular chaperones during and after protein synthesis and their elevation in *abi3-6* mutant seeds may suggest higher protein synthesis levels during the later stages of seed development. The fact that the accumulation levels of these developmentally expressed proteins is not vastly different in *CnAIP1* OE seeds and wild-type seeds is not a surprise. Based on the above results, CnAIP1 may play a role in promoting seed developmental processes, much like the role of ABI3/CnABI3 itself. CnAIP1 may be involved in maintaining normal levels of some of these proteins during development or may control the temporal aspects of their accumulation.

2.4.3. Glucose and Other Abiotic Stresses: Inhibition of Germination

Motif analysis of the promoter sequence of CnAIP1 revealed some interesting regulatory elements. In addition to the expected CAAT-box and TATA elements which

are expected in promoter regions, motif analysis found HSE (heat shock elements) and TC-rich repeats which are involved in defense and stress responsiveness. Heat shock elements and heat stress factors (HSFs) are known to be involved in the regulation of sHsp expression during heat stress (Wu, 1995). Heat shock factors also play a role in developmental regulation of sHsps (Prandl and Schoffl, 1995). The Kotak group (2007) showed that heat stress factors such as HsfA9 regulate the expression of Hsps during development and heat stress, with ABI3 regulating HsfA9 activity. It is likely that HSFs are able to bind to the HSE elements found in the CnAIP1 promoter region and this may be regulated by CnABI3. Interestingly, Hsp70 negatively regulates HSFs binding to HSEs thereby blocking the transcriptional activation of heat shock genes by HSFs (Kim and Schoffl, 2002). Heat stress treatments were tested on *CnAIP1pro-GUS* seedlings to visualize changes in GUS expression and activity. A two-fold increase in CnAIP1 promoter expression was observed in the presence of heat stress treatments. It would be interesting to investigate the GUS activity of the CnAIP1 promoter in an abi3-6 mutant background to determine if the absence of ABI3 has an effect on the activation of the CnAIP1 promoter.

The presence of TC-rich repeats revealed that the CnAIP1 promoter expression may be enhanced in response to abiotic stress treatments. Many abiotic stress treatments were tested to visualize a SALK line phenotype. Previous characterization of the SALK line at germination into post-germinative growth under abiotic stress conditions did not show any differences from wild-type seeds. It is possible that differences exist at a biochemical level, but are undetectable using the methods described. A difference in phenotype may suggest functional clues about CnAIP1. It was observed that the SALK line could germinate faster in the presence of 6% glucose as compared to wild-type seeds. At such concentrations of glucose, this sugar is no longer a signaling molecule but is sensed as a stress. Seeds of the SALK line, with a deletion insertion in the *AtHip1* gene were insensitive to the high concentration of glucose and were able to germinate faster as compared to wild-type seeds. Consistent with this finding, the *CnAIP1* (OE) seeds were hypersensitive to 6% glucose and germinated slower in comparison to wild-type seeds. Interestingly, over-expression of the *CnABI3* gene in the Arabidopsis abi3-6 background also confers sugar hypersensitivity and enhances the inhibition of post-germinative growth (Zeng and Kermode, 2004). Glucose-induced delay of seed

germination thus seems to be mediated by ABI3. Sugar insensitive mutants, with reduced sensitivity to sugars during germination, are allelic to mutations in ABA biosynthesis or sensitivity (Finkelstein *et al*., 2002). Hormonal and sugar regulation of gene expression are linked especially during the transition from seed maturation to germination (Bradford *et al*., 2003). Sugars have important roles in metabolism and as signaling molecules in the regulation of developmental processes including germination and post-germinative growth (Gazzarrini and McCourt 2001; Finkelstein *et al*., 2002). Sugars generally inhibit germination and post-germinative growth at higher concentrations. The effect of glucose and abiotic stress treatments were tested on *CnAIP1pro-GUS* seedlings to visualize changes in GUS staining and activity. In control seedlings, CnAIP1 promoter expression was observed throughout the seedling, including the cotyledons and roots as indicated by histochemical staining for the GUS reporter. Although not all stress and hormone treatments caused changes in GUS activity, seedlings treated with glucose, NaCl and H_2O_2 exhibited an increase in GUS activity that was almost double of that in the control (non-stressed) seedlings. These results indicate that during these stress conditions, there is an increased expression of the *CnAIP1* gene. An increase of CnAIP1 protein was not observed in yellow-cedar in response to stress condition (Chapter 3). Nonetheless, the results indicate that CnAIP1 plays a prominent role in regulating seeds in a development state and in the responses of seedlings to abiotic stress and glucose treatments, in a manner similar to ABI3/CnABI3. There may be a difference in the regulation pathway governing *CnAIP1* promoter expression during development versus during heat/abiotic stress. Further investigation is required to determine if there are changes in the CnAIP1 promoter temporal and spatial expression during development in response to heat or other abiotic stresses. If this is observed, it would be clear that CnAIP1 promoter expression is differentially regulated in different pathways with CnABI3 as a common key regulatory player.

3. Functional Analysis of CnAIP1 in the conifer, Yellow-Cedar

3.1. Introduction

The conifer species that this work is focused on, yellow-cedar (*Callitropsis nootkatensis*) is a commercially and ecologically important tree species in the BC foresty industry. Its habitat ranges from the California-Oregon border to the coastal regions of Alaska (Hennon *et al*., 2008). It is a slow-growing species that is able to achieve great longevity (Jozsa 1992). Despite its long lifespan, seed production of yellow-cedar is low with natural regeneration rates being quite poor (Hennon *et al*., 2008). Some seeds are produced every year from parent trees, but upon dispersal, seeds are unable to germinate immediately due to low seed quality and deep dormancy imposed during development (Kurz *et al*., 1994; Tillman-Sutela and Kauppi 1998; Jull and Blazich, 2000). After dispersal, most seeds maintain this dormant state and require a prolonged period of time (ie. up to 2 years) to terminate dormancy (Pawuk 1993). The low seed production and prolonged seed dormancy in yellow-cedar is exacerbated by an increased vulnerability of the seedlings to freezing stress-related mortality, especially in the northern regions of its range where a dramatic decline of yellow-cedar trees has been reported.

The dormancy mechanism in yellow-cedar is primarily coat-imposed or coat-enhanced (Ren and Kermode, 1999). The seed coat and megagametophyte are the major inhibitory tissues of the seed. The megagametophyte can act as a mechanical barrier that prevents radicle emergence to complete germination. ABA is involved in the maintenance of dormancy in mature seeds (Schmitz *et al*., 2000, 2001, 2002). Findings suggest that embryos become less responsive to ABA following dormancy-termination induced by moist chilling. In contrast to intact seeds, which are dormant and do not germinate unless moist chilled, Xia *et al*. (2002) report that the isolated embryos are able to germinate when placed in water. This may be mediated through a release of

germination inhibitors such as ABA that are readily leached when embryos are taken from intact seeds.

Research has been focused on developing dormancy-breaking protocols that mimic what occurs in a natural setting albeit in a shorter time span. Protocols for dormancy breakage are crucial not only to enhance germination but also to ensure synchronous seedling emergence. Dormancy-breakage protocols used in an industry setting must ensure optimal seedling growth and successful outplanting in natural forest settings. In our lab, the dormancy breaking protocol has been optimized and consists of a 3-day running water soak at room temperature, followed by 1 month in warm moist conditions at room temperature (in the dark) followed by 2 months in cold moist conditions at 4°C (Ren and Kermode, 1999).

From an ecological and industrial perspective, understanding the mechanisms of seed dormancy and germination in conifer species is critical. Investigations into potential conifer proteins that may play a role in dormancy maintenance of yellow cedar seeds pinpointed CnABI3 as a major candidate in promoting the maintenance of dormancy in mature dispersed (imbibed) seeds (Zeng *et al*., 2003) similar to ABI3/VP1 orthologs of angiosperms (Jones *et al*., 1997; Fukuhara *et al*., 1999; Nakamura and Toyama, 2001; Carrari *et al*., 2001).

In the previous chapter, I conducted various functional analyses of CnAIP1 using Arabidopsis as a model. In this Chapter 3, my aim was to conduct some limited analyses of CnAIP1 in its 'homologous' host – i.e. yellow-cedar.

Due to the long lifecycle of yellow-cedar and the complexities of genetic manipulation of this species, I confined most of my analyses to yellow cedar seeds and seedlings. Functional analyses for *CnAIPl* was carried out in Arabidopsis (Chapter 2), but there is no guarantee that the functions of CnAIP1 in yellow-cedar are the same as those observed in the Arabidopsis host. Likewise, CnAIP1 may have some common as well as unique functions from its presumed Arabidopsis ortholog, AtHip1. Thus while Arabidopsis gives insight into the functions of CnAIP1, eventually the functions of yellow-cedar proteins need to be tested in yellow-cedar itself.

In the conifer work, my objectives were three-fold:

(1) To examine whether CnAIP1 is differentially expressed at the mRNA and protein levels before, during and after dormancy termination as well as during germination, and early seedling growth.

(2) To examine whether CnAIP1 expression is induced by abiotic stress treatments during the dormancy breakage to germination transition.

(3) To analyze whether the levels of two known protein partners that interact with the mammalian HIP1 (Hsp90 and Hsp73) through heteromeric complex formations are changed during heat stress/recovery and/or dormancy maintainence/breakage.

3.2. Materials and Methods

3.2.1. Seed Materials

Yellow-cedar seeds (seed lot 51082) were obtained from the Tree Seed Centre (BC Ministry of Forests, Lands and Range, Surrey, B.C., Canada). Dormancy-breaking protocols consisted of three days running water imbibition at 23°C, one month in the dark under moist conditions at 25°C (warm, moist conditions) and two months of moist chilling at 4°C (Ren and Kermode, 1999). Following the dormancy-breaking treatment, seeds were placed in germination conditions (30°C days, 20°C nights with an 8 hour photoperiod). Seeds were subjected to the 3-d soak and a 12-week warm, moist period (no moist chilling) at 25°C as a control treatment. No germination is observed in seeds subjected to this control treatment.

3.2.2. Protein Extraction for SDS-PAGE and Western Blot Analysis

Proteins were extracted from yellow-cedar seeds (embryos and megagametophytes) and seedlings during various stages of dormancy breakage, germination and growth by grinding tissues in protein extraction buffer (0.1M potassium phosphate buffer (pH 7.0) and Protease Inhibitor Cocktail (for plant cell and tissue extracts – Sigma Aldrich)). A 2X SDS loading buffer was added to the extracts before boiling the samples and centrifugation for 10 min at 13000 rpm (Biofuge pico Heraeus). Protein concentrations of the supernatants were determined using the Bio-Rad protein

assay (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin IV (Sigma) as a standard. Following protein quantification, 1M DTT (end concentration 0.1 M) with bromophenol blue was added to each of the samples in preparation for SDS-PAGE loading. Proteins (25 µg) from each sample were separated on 12% SDS-PAGE gels and transferred to HybondECl nitrocellulose protein membrane (GE Healthcare, Amersham Hybond ECl). Membranes were blocked with 5% skim milk powder in 1X PBST (phosphate buffered saline containing 0.05% Tween-20) overnight and then incubated with the primary antibody (anti-CnAIP1 – produced by genetic immunization at GENEART Gmbh, Germany) diluted 1:2 000 1X PBST for 3 hours at room temperature. The membrane was washed three times for 20 min time intervals in 1X PBST at room temperature. Membranes were then incubated for 1 h in goat anti-mouse secondary antibody conjugated to alkaline phosphatase (Sigma). The secondary antibody was diluted 1:3000 in PBST containing 5% skim milk powder. The membrane was washed three times for 20 min time intervals in 1X PBST at room temperature. Immunodetection of CnAIP1 protein was performed using the NBT/BCIP method.

3.2.3. RNA Extraction and Quantitative RT-PCR

Embryos, megagametophytes and seedlings of yellow-cedar were collected at various stages of dormancy breakage into germination and ground in liquid nitrogen. Total RNA was extracted as described in Chang *et al.* (1993) with the following modifications. After addition of CTAB buffer (2% hexadecyl trimethyl-ammonium bromide [CTAB], 2% polyvinylpyrrolidone [MW=40000/K30], 100 mM Tris-HCl, pH 8.0, 25 mM EDTA, pH 8.0, 2 M NaCl, 2% ß-mercaptoethanol), the extracts were kept at 65°C for 10 min. All chloroform:isoamylalcohol extractions were repeated once. RNA was treated with DNase-I (Fermentas) to remove remaining genomic DNA and the integrity of the RNA was checked on an agarose gel followed by quantity and purity determination using a nanodrop spectrophotometer (ND-2000C) (Thermoscientific). One µg RNA was reverse transcribed using the EasyScript Plus kit (Abmgood) with a mixture of random hexamers and oligo-dT primers. cDNA from three biological replicate RNA samples was used for qPCR.

qRT-PCRs were run in 15 μl reaction volumes on an ABI7900HT machine (Applied Biosystems,) using the PerfeCTa Sybr Green Supermix with ROX (Qanta Biosciences, www.qantabio.com). Primers were designed with the primer3 (Rozen and Skaletsky 2000) tool in Geneious 4.8.5 and based on published sequences. Cn18S rRNA was used as reference gene. The reaction mixture consisted of 150 ng cDNA (RNA equivalent), 7.5 μl supermix and 280 nM (yellow-cedar) or 140 nM (Arabidopsis) of each primer, and the temperature regime was 3 min at 95°C and 40 cycles of 15 s at 95°C/1 min at 60°C. A dissociation curve was run after each qPCR to validate that only one product had been amplified.

The efficiency E of the primer pairs was calculated as the average of the Es of the individual reactions by using raw fluorescence data with the publicly available PCR Miner tool (Zhao and Fernald 2005). The efficiency was then used to calculate transcript abundance for the individual samples as (1 + E)^(-CT). No-template-controls (NTCs) for each primer pair were included to check for contamination of the reagents. Only samples whose corresponding NTC showed no amplification signal were used in the analysis.

3.2.4. Abiotic Stress Treatments During the Dormancy to Germination Transition

Yellow-cedar seeds undergoing dormancy breakage were transferred to stress treatments (3% glucose, 166 mM mannitol, 10 mM H_2O_2) during the final two weeks of the 3-month dormancy breaking treatment and were maintained in those conditions during the germination phase. Samples were collected 6 h after placement in stress conditions, 7 days prior to germination conditions, immediately before transfer to germination conditions, 6 hours in germination conditions, 2 days in germination conditions and at radicle emergence. Control samples that were not subjected to stress treatments were also collected.

Along with studying the inducibility of *CnAIP1* gene expression, CnAIP1 protein accumulation along with Hsp73 and Hsp90 protein levels were also tested in response to heat stress and recovery. Germinating yellow-cedar seeds that had been in germination conditions for 2 days were used for this stress treatment. Seeds were stressed for either

1 or 2 hours at 37°C. Those subjected to 2h at 37°C were then transferred back to recovery conditions – germination conditions at 30°C for either 1, 2 or 24 hours.

Protein extraction and western blot analysis was performed using the methods outlined in 3.2.2. Dilutions of antibodies were as follows: Hsp73 (1:5000) and Hsp90 (1:3000). Hsp90 and Hsp73 were detected as outlined in Section 2.2.4. These proteins were immunodetected using ECL detection (GE Healthcare, Amersham ECL Prime Western Blotting Detection Reagents). RNA extraction and qPCR analysis was performed using the methods outlined in 3.2.3.

3.3. Results

3.3.1. Expression of CnAIP1 in Yellow-Cedar Seeds Before, During and After Dormancy Termination

The role of CnAIP1 in dormancy maintenance of yellow-cedar seeds was examined by monitoring the expression of the *CnAIP1* gene at the mRNA and protein levels before, during and after dormancy termination. This work was performed with Kerstin Mueller. She carried out the RNA work in yellow-cedar while I conducted the protein analyses. The *CnAIP1* gene transcript levels in the embryo were detected in seeds of second year yellow-cedar maturating cones. The levels of transcripts increased during first and second year of development and seed maturity. Once seeds were subjected to the dormancy-breaking protocol, the expression levels steadily increased. Interestingly a strong decline in expression occurred during germination. Coincident with the completion of seed germination (radicle emergence) and early seedling growth there was increased expression of the *CnAIP1* gene (Figure 3-1A). The same pattern of CnAIP1 mRNA expression was observed in megagametophytes.

CnAIP1 protein abundance was present in the embryo throughout all stages of dormancy breakage into germination with a slight increase in protein levels just prior to dormancy breakage (and a slight decrease at the early seedling growth phase) (Figure 3-1B). There was a lower overall accumulation of CnAIP1 protein in the megagametophyte with again, a slight increase in levels prior to dormancy breakage. Any changes in protein abundance are otherwise not visible and western blot analyses

are less amenable to quantification. The protein was preserved in seeds subjected to the control treatment of 12 weeks in warm, moist conditions. Loading controls are appended in Figure A1-2.

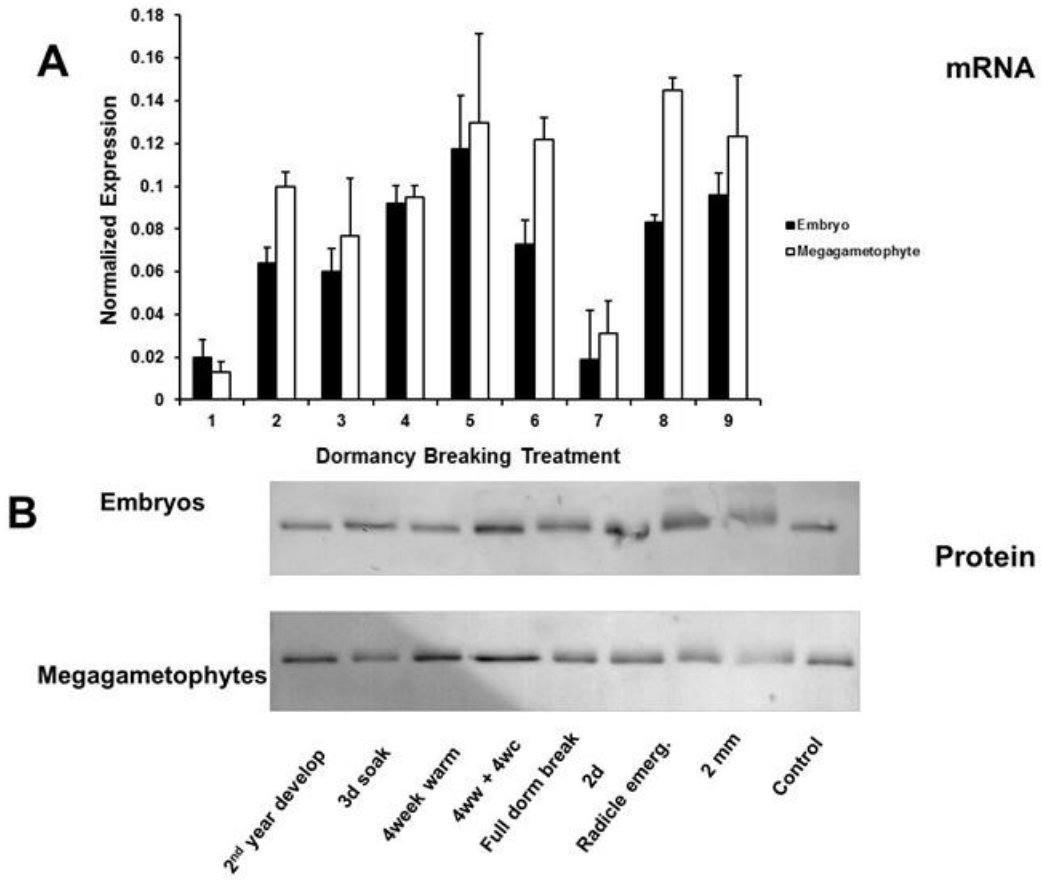

Figure 3-1. Expression of CnAIP1 mRNA and protein in yellow-cedar seed during difference stages of dormancy breakage, germination and post-germinative growth

1: 2nd year developing seeds, 2: dry seed 3: 3-daywater soak, 4: 4 week warm treatment (25°C), 5: 4 week warm treatment (25°C) + 4 week cold treatment (4°C), 6: full dormancy breakage treatment - 4-week warm(25°C) + 8 week cold(4°C), 7: 2-days at germination conditions, 8: radicle emergence, 9: 2mm radicle, 10: Control – 12 weeks warm treatment (25°C) A. quantification of CnAIP1 transcripts by qRT-PCR. Expression was normalized against yellow-cedar 18S rRNA. Averages of three replicates +/- SE are shown.
B. western blot analysis of CnAIP1 protein. 40 µg of total protein were loaded on each lane. CnAIP1 antibody was used to detect CnAIP1 protein expression levels.

3.3.2. *CnAIP1, Hsp73 and Hsp90 Expression in Response to Abiotic Stress*

To study the expression levels of CnAIP1 in the presence of stress treatments, I focused on the transitional period between dormancy and germination. Specifically stress treatments were implemented during the final two weeks of dormancy breakage, during germination, and at the completion of germination (radicle emergence). There was an increase of CnAIP1 transcripts when seeds were subjected to the glucose treatment particularly when first transferred to the stress conditions (6h). Thereafter transcripts decreased during dormancy-breakage and germination, with the lowest transcript levels in seeds that had completed germination (RE, Figure 3-2A). This is in comparison to the control – seeds that were not glucose stressed in which the AIP1 transcript levels do not change as drastically. At the protein level, CnAIP1 did not show much change in response to glucose stress when compared to control seeds (Figure 3-2B). Loading controls are appended in Figure A1-3. The *CnABI3* transcripts were also induced in the presence of glucose, albeit to a lower extent than that of the *CnAIP1* transcripts (Figure 3-2A). Following the introduction of glucose stress, there was a lag before the peak of *CnABI3* transcript levels that occurred just prior to dormancy breakage, after which there was a significant decline. *ABI3* transcript levels did increase once again during germination but never to a level that was observed during dormancy breakage.

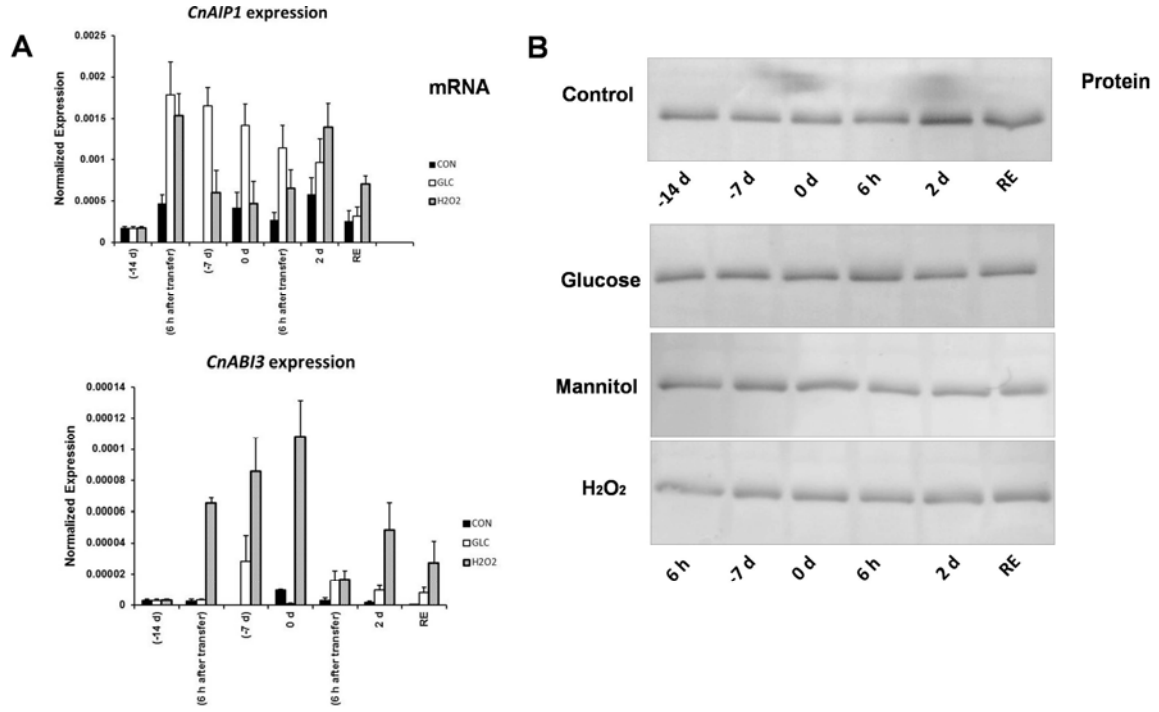

Figure 3-2. Expression of CnAIP1 protein and mRNA in yellow-cedar seed during the dormancy breakage to germination transitional period in the presence of various abiotic stress treatments

A. Quantification of *CnAIP1* and *CnABI3* transcripts in control (no stress present) and abiotic stress (ie. glucose, mannitol, H_2O_2) conditions during the transition period of dormancy breakage into germination. Expression was normalized against yellow-cedar 18S rRNA. Averages of three replicates +/- SE are shown. B. CnAIP1 protein levels during the last 14 days of dormancy breakage, transfer to germination conditions (0 day = 0 d) and in germination conditions up till radical emergence (RE). The first 6 h represent the time at which the stress treatment was introduced during dormancy breakage. Control represents yellow-cedar seeds that were not exposed to a stress treatment. CnAIP1 antibody was used to detect CnAIP1 protein expression levels.

Perhaps the most interesting results were those obtained with oxidative stress (treatments with H_2O_2). Here the changes in *CnABI3* and *CnAIP1* transcripts were highly induced by this stress. Both showed a biphasic pattern of increase. The first peak in expression occurred a few hours following placement of yellow-cedar seeds in the abiotic stress. The second peak in expression corresponded to germination.

CnAIP1 inducibility was also tested in response to mild heat stress and recovery. For this I used germinating yellow-cedar seeds that had been in germination conditions for 2 days. Seeds were stressed for 2 hours at 37°C and then transferred back to recovery conditions after 2 hours of heat stress and were monitored at hours 1, 2 and 24

during recovery. Recovery consisted of returning the yellow-cedar seeds to normal germination conditions for 1, 2 or 24 hours. *CnAIP1* transcript levels increased after 1 hour in heat stress, but no change in protein was detectable on the western blot (Figure 3-3A,B). Loading controls are appended in Figure A1-3. During the recovery phase, the transcript levels were lower than those observed prior to heat stress.

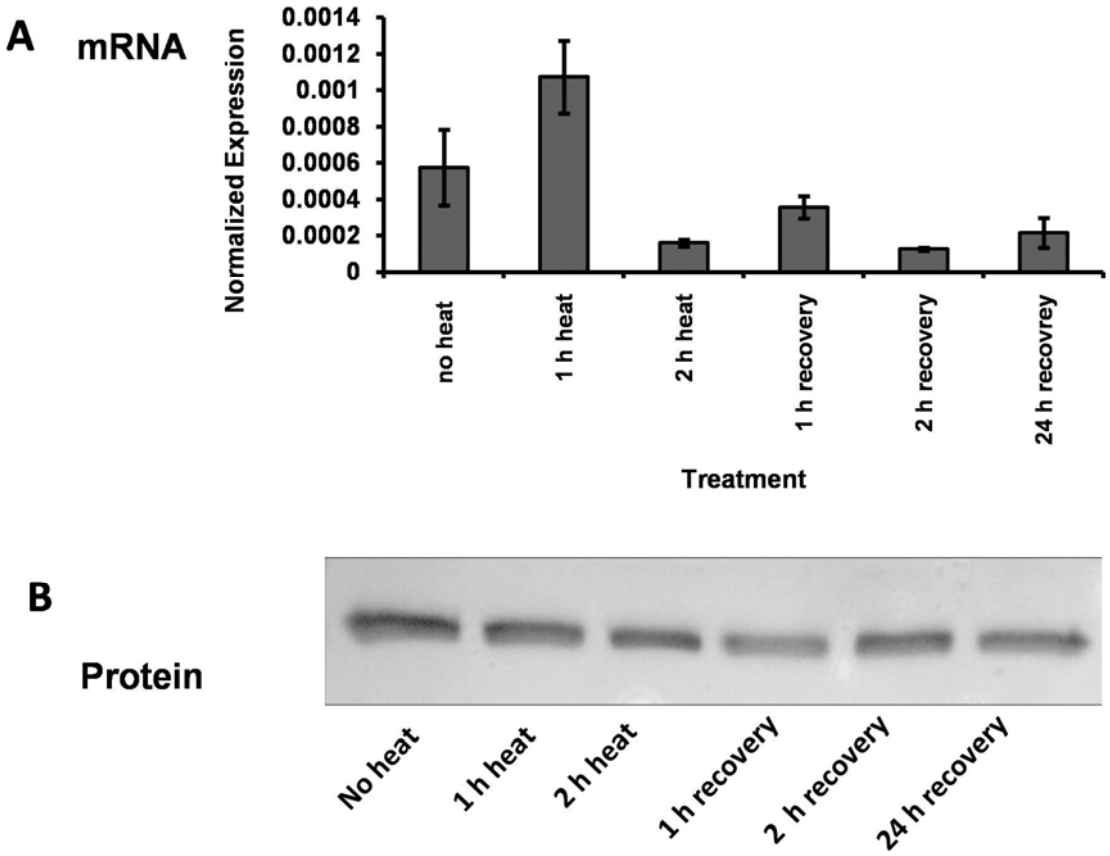

Figure 3-3. Expression of CnAIP1 protein and mRNA in yellow-cedar seeds in response to heat stress.

A. Quantification of *CnAIP1* transcripts during heat stress and recovery. Expression was normalized against yellow-cedar 18S rRNA. Averages of three replicates +/- SE are shown. B. CnAIP1 protein levels in yellow-cedar seeds that had been at germination conditions for 2 days. Seeds were placed at 37°C for 1 and 2 hours prior to the return to germination conditions for recovery. Seeds were transferred to germination conditions for recovery after 2 hours of heat stress. CnAIP1 antibody was used to detect CnAIP1 protein expression levels.

I also sought to determine whether the levels of two known protein partners that interact with the mammalian HIP1 (Hsp90 and Hsp 73) in a heteromeric complex are

changed during heat stress and recovery. Subjecting the germinating seeds to heat stress actually depressed the amounts of these proteins, which could have been due to a general depression of protein synthesis (Figure 3-4, 'heat'). The most startling result was found during the recovery period. At this time, both proteins accumulated. This is likely indicative that these proteins play a role during recovery in which they participate in repairing cellular damage that occurred as a result of the heat stress – especially the repair of any damaged proteins. Loading controls are appended in Figure A1-3.

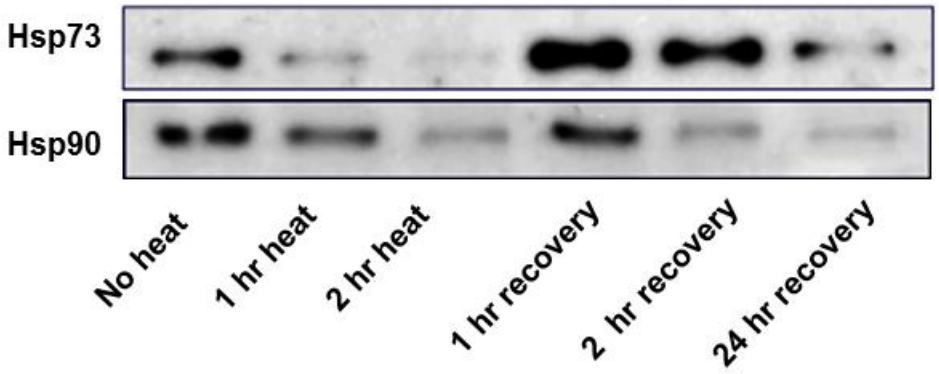

Figure 3-4. Expression levels of Hsp73 and Hsp90 protein in yellow-cedar seeds during heat stress and recovery.

Hsp73 and Hsp90 protein levels in yellow-cedar seeds that had been at germination conditions for 2 days. Seeds were placed at 37°C for 1 and 2 hours prior to the return to germination conditions for recovery. Seeds were transferred to germination conditions for recovery after 2 hours of heat stress. Hsp73 and Hsp90 antibodies were used to detect Hsp73 and Hsp90 protein expression levels respectively.

3.4. Discussion

3.4.1. CnAIP1 Transcripts and Protein Remain Fairly Constant in Yellow-cedar Seeds during and after Dormancy Termination

Yellow-cedar is a deeply dormant conifer species which produces seeds that require 6 to 18 months to undergo moist chilling and terminate dormancy at natural stands (Pawuk, 1993). The mechanism of dormancy in yellow-cedar is primarily coat-imposed (Ren and Kermode, 1999). ABA plays a role in maintaining the seed in a dormant state (Schmitz *et al*., 2000, 2001, 2002). CnABI3 is a global transcriptional regulator that controls the expression of many genes in response to ABA. CnABI3 and

its orthologs have been shown to play a role in development and dormancy maintenance in seeds. The down-regulation of *CnABI3* gene expression correlates with dormancy breakage, even though *CnABI3* transcripts remain until seeds are transferred to germination conditions (Zeng *et al*., 2003). In relation to dormancy maintenance, CnABI3 is also regulated at the protein level. CnABI3 abundance declines during moist chilling leading up to the full dormancy breaking treatment (Zeng *et al*., 2003). The regulation of CnABI3 during dormancy and its termination may involve post-translational control. As discussed in Chapter 2, my results indicate that CnAIP1 plays a role in regulating seeds in a development state and in the responses of seedlings to abiotic stresses. The CnAIP1-CnABI3 proteins likely interact in a synergistic manner during these events.

In yellow-cedar seeds, CnAIP1 protein was present in the embryo and megagametophyte throughout all stages of dormancy breakage into germination with a slight peak just prior to dormancy breakage, which was largely mirrored by the slight increases in transcripts. The levels of CnAIP1 protein in the embryo at the various stages suggest that it has a constitutive protective function rather than a specialized function during dormancy breakage, and germination. Some decline of CnAIP1 protein seemed to take place during early seedling growth. Protein levels in the megagametophyte showed similar temporal changes in abundance. The *CnAIP1* gene transcript levels were very similar in embryo and megagametophyte with a decline taking place at radicle emergence (the completion of germination).

Regulation of CnAIP1 in relation to dormancy and germination may involve control of expression at the posttranscriptional and/or posttranslational levels which may contribute to an increased stability of the CnAIP1 protein. Control at multiple levels could contribute to the changes in abundance of transcripts and protein, and the fact that transcript levels did not consistently correlate with protein levels. Interestingly, another protein found to interact with CnABI3, the CnAIP2 protein, increases after seeds are exposed to moist chilling and its abundance is high during germination and post-germinative growth (Zeng *et al*., 2013). This is in contrast to changes in the abundance of the CnABI3 protein. The nature of control by CnAIP1 in dormancy in relation to CnABI3 and its possible role in the transition to germination will need to be further investigated. Through its interaction with CnABI3, CnAIP1 protein levels may remain rather stable while the levels of CnABI3 or other interacting proteins fluctuate. It is also

likely that although I am not observing drastic changes in CnAIP1 protein levels during dormancy breakage, the subcellular localization of CnAIP1 may vary temporally during dormancy breakage into germination. This hypothesis will need to be explored through immunohistochemical work using yellow-cedar seed tissue at various timepoints during dormancy breakage. I attempted this work, but was unsuccessful with staining appearing in the negative control.

3.4.2. Effects of Abiotic Stress Treatments During Dormancy Breakage on the Expression Levels of CnAIP1 and Some HSPs

The homolog of CnAIP1, AtHip1, encodes a heat shock protein associated factor. Due to the high sequence similarity between CnAIP1 and AtHip1, it is likely the two are homologs that share similar functions. In addition to developmental regulation, HSPs and their associated proteins can be expressed in response to stress conditions such as high concentrations of glucose, osmotic, oxidative and other abiotic stress treatments (Wang *et al*., 2004). To study the expression levels of CnAIP1 in the presence of stress treatments, I focused on a transitional period which included the last two weeks of dormancy breakage into the beginning of germination. Glucose, osmotic and oxidative stresses were applied at a late stage during dormancy breakage (the last 2 weeks of the 3-month dormancy-breaking treatment). CnAIP1 protein levels were relatively stable during glucose, mannitol and H_2O_2 treatments, which may be due to the level of detection permitted by western blot analyses and the limitations of this approach for quantification. It is also likely that there are modifications that have been made at the transcriptional or translational level that contribute to protein stability. Increases in *CnAIP1* and *CnABI3* transcript levels were observed following application of the stress treatment and during germination, during which there is an endogenous increase in the reactive oxygen species (He and Kermode, 2010). Heat stress imposed on yellow-cedar seeds led to a decline in CnAIP1 protein abundance; although an increase in *CnAIP1* transcript levels was observed after 1 hour in 37°C stress conditions (but not after 2 h). Regulation of CnAIP1 in relation to its expression in response to abiotic stresses is not limited to the transcriptional level. Further experiments would need to be conducted to verify CnABI3 abundance in response to stress treatments and whether the protein-protein interaction is influencing protein stability. In addition, more rigorous testing of the

period of time during which stress treatments are applied will need to conducted in terms of a response curve to determine if yellow-cedar seeds are able to express CnAIP1 in response to abiotic stress.

I also looked at the protein abundance of Hsp73 and Hsp90 during the dormancy breakage to germination transition and as a result of heat stress/recovery. Changes in these two proteins were interesting, because the increase in the abundance of Hsp73 and Hsp90 was clearly associated with recovery; this makes sense in terms of a role for these proteins in cellular repair during the recovery phase. Hsp73 and Hsp90 are both involved in non-stressful conditions to facilitate folding of de novo synthesized proteins as well as in the transport and targeting of damaged proteins (Sung *et al*., 2001). Additionally, Hsp90 modulates cellular signal transduction by affecting protein conformation and folding (Nathan *et al*., 1995). Hsp90 is known to mediate stress signal transduction and stress-induced Hsp70 proteins function to prevent aggregation of proteins during stress (Xu *et al*., 2012; Sung *et al*., 2001). However, it makes sense that repair would be largely associated with the recovery phase as many proteins and protein complexes may have become denatured following the heat stress. Hsp70s are known to be involved in facilitating refolding and proteolytic degradation of non-native proteins (Hartl, 1996; Frydman, 2001; Miernyk, 1997).

Experiments to detect changes in subcellular localization or tissue specific expression might elucidate changes in the temporal expression of the CnAIP1 and HSP proteins. Another factor to keep in mind is that the stress treatment was relatively mild, and a future endeavour would be to characterize different heat stress regimes more extensively.

4. Summary/Conclusions and Future Work

4.1. Summary and Conclusions

The work in this thesis focused on the functional analyses of CnAIP1, a protein found to interact with CnABI3, the gymnosperm ortholog of ABI3. Research has been carried out with approaches in both yellow-cedar and in Arabidopsis, as the long life-cycle and the lack of transformation protocols makes it impractical to use yellow-cedar in transgenic or mutant approaches. Through blastp analysis, I found a homologue of CnAIP1 in Arabidopsis (AtHip1). AtHip1 encodes a heat shock protein associated factor. The transcription of heat shock proteins is differentially regulated during seed development and stress responses and is thus connected to two of the main processes in which ABA signaling is involved. The roles of CnAIP1 as an associated or interacting factor of heat shock proteins as well as an interactor of an ABA signaling component was therefore investigated in these processes. CnAIP1 was observed to localize throughout the cytosol and to a lesser extent in the nucleus. Online bioinformatics tools indicate that the amino acid sequence of CnAIP1 contains a nuclear localization signal (NLS). It is possible that the CnAIP1 protein shuttles between the nucleus and cytosol in response to environmental triggers. A role of CnAIP1 in developmental processes including dormancy maintenance and desiccation tolerance was established. CnAIP1 promoter analysis indicated the presence of heat shock elements and motifs that were responsive to abiotic stress. To elucidate the role of CnAIP1 in abiotic stress response, CnAIP1pro-GUS histochemical staining was observed and a flurometric assay was conducted. Results indicated that CnAIP1 promoter activity was almost 2-fold higher in seedlings exposed to abiotic stresses including high concentrations of glucose, NaCl, oxidative stress and heat stress. Arabidopsis seeds of plants overexpressing CnAIP1 in a heterologous system as well as seeds lacking the native AtHIP1 showed altered germination behaviour on glucose. This is further evidence for the involvement of CnAIP1 in the abiotic stress response. The seeds also showed altered dormancy. This

could be due to the changes in seed development, or to the direct effect of CnAIP1 on the ABI3-regulated aspects of dormancy. The developmental versus the abiotic stress regulation of CnAIP1 expression may involve different pathways with CnABI3 as a common factor.

I used the results obtained from work in Arabidopsis to elucidate the roles of CnAIP1 in dormancy maintenance and abiotic stress in yellow cedar seeds and seedlings. CnAIP1 protein levels were stable during dormancy breakage into germination, even though significant changes could be observed on the RNA level. This could indicate that CnAIP1 itself is permanently present in the cell and interacts with differentially regulated proteins such as ABI3. I also determined whether CnAIP1 protein and the levels of two known protein partners that interact with the mammalian HIP1 (Hsp90 and Hsp 73) in a heteromeric complex are changed during heat stress and recovery. While the CnAIP1 protein level was again stable, protein abundance of Hsp73 and Hsp90 increased during recovery following heat stress indicating a role for these proteins in cellular repair during the recovery phase.

From an ecological and industrial perspective, understanding the mechanisms of seed dormancy and germination in conifer species is critical. Particular signals and biosynthetic pathways are responsible for dormancy breakage into germination and for all subsequent lifecycle transitions. CnABI3 is global regulator of many key transitions, especially during seed development into germination. CnABI3 like its ortholog ABI3 contributes towards keeping seeds in a developmental state and prevents germination. My results show that its interactor CnAIP1 plays an important role during seed development and dormancy maintenance. Its functions parallel the functions of CnABI3 in the seed, likely positively supporting CnABI3 in its transcriptional regulatory activity. Thus CnAIP1-CnABI3 protein interactions likely control these important life events, in which the two proteins act in concert, and perhaps in a synergistic manner.

4.2. Future Work

More experiments need to be carried out to further elucidate and verify the functions of CnAIP1 in seed developmental processes. Investigation into the expression

pattern of CnAIP1 promoter between days 5-10 DAP of developing seeds would be essential to track the changes in spatial localization of promoter activity. In addition, changes to promoter expression can be influenced by heat stress applied to embryos during development. Spatial and temporal expression of the CnAIP1 promoter in response to stress may be different from that observed during seed development under optimal conditions. In addition, the developmental accumulation of stress protectants can be monitored on the transcript level where changes in expression may be more obvious. Microarray analysis of a single timepoint (seeds, seedlings, etc) from *CnAIP1* OE lines may indicate genes that are upregulated with respect to maintaining seeds in a developmental state and during heat stress response and downregulate genes that are involved in germination processes.

Further work needs to be conducted in yellow-cedar. Although CnAIP1 is known to interact with CnABI3, it would be interesting to determine interacting/associated factors of CnAIP1 through yeast-two hybrid analysis. These proteins may shed light on additional functions of CnAIP1 and contribute to a further understanding of CnABI3.

5. References

Adkins, S., Loewen, M. and Symons, S. 1986. Variation within pure lines of wild oats (*Avena fatua*) in relation to degree of primary dormancy. Weed Sci 34: 859-864.

Almoguera, C. and Jordano, J. 1992. Developmental and environmental concurrent expression of sunflower dry-seed-stored low-molecular-weight heat-shock protein and Lea mRNAs. Plant Mol Biol 19:781-792.

Arenas-Huertero, F., Arroyo, A., Zhou, L., Sheen, J. and León, P. 2000. Analysis of Arabidopsis glucose insensitive mutants, *gin5* and *gin6*, reveals a central role of the plant hormone ABA in the regulation of plant vegetative development by sugar. Genes Dev 14: 2085-2096.

Audran, C., Borel, C., Frey, A., Sotta, B., Meyer, C., Simonneau, T. and Marion-Poll, A. 1998. Expression studies of the zeaxanthin epoxidase gene in *Nicotiana plumbaginifolia*. Plant Physiol 118: 1021-1028.

Audran, C., Liotenberg, S., Gonneau, M., North, H., Frey, A., Tap-Waksman, K., Vartanian, N. and Marion-Poll, A. 2001. Localisation and expression of zeaxanthin epoxidase mRNA in Arabidopsis in response to drought stress and during seed development. Aust J Plant Physiol 28: 1161-1173.

Ballinger, C.A., Connell, P., Wu, Y., Hu, Z., Thompson, L.J., Yin, L.Y. and Patterson, C. 1999. Identification of CHIP, a novel tetratricopeptide repeat-containing protein that interacts with heat shock proteins and negatively regulates chaperone functions. Mol Cell Biol 19: 4535-4545.

Banecki, B. and Zylicz, M. 1996. Real time kinetics of the DnaK/DnaJ/GrpE molecular chaperone machine action. J Biol Chem 271: 6137-6143.

Barr, H., Hermann, A., Berger, J., Tsai, H., Adie, K., Prokhortchouk, A., Hendrich, B. and Bird, A. 2007. Mbd2 contributes to DNA methylation-directed repression of the *Xist* gene. Mol Cell Biol 27: 3750-3757.

Beaudoin, N., Serizet, C., Gosti, F. and Giraudat, J. 2000. Interactions between abscisic acid and ethylene signaling cascades. Plant Cell 12: 1103-1115.

Bentsink, L. and Koornneef, M. 2008. Seed Dormancy and Germination. In: Somerville, C.R. and Meyerowitz, E.M., eds. The Arabidopsis Book. Rockville, MD, USA: American Society of Plant Biologists, 1-18.

Bernal-Lugo, I. and Carl Leopold, A. 1998. The dynamics of seed mortality. J Exp Bot 49: 1455-1461.

Bewley, J.D. and Black, M. 1994. Seeds: Physiology of Development and Germination. 2nd ed. New York, New York, USA: Plenum Publishing Corporation.

Bewley, J.D. 1997. Seed germination and dormancy. Plant Cell 9: 1055-1066.

Bies-Etheve, N., da Silva Conceicao, A., Giraudat, J., Koornneef, M., Léon-Kloosterziel, K., Valon, C. and Delseny, M. 1999. Importance of the B2 domain of the Arabidopsis ABI3 protein for Em and 2S albumin gene regulation. Plant Mol Biol 40: 1045-1054.

Bimston, D., Song, J., Winchester, D., Takayama, S., Reed, J.C. and Morimoto, R.I. 1998. BAG-1, a negative regulator of Hsp70 chaperone activity, uncouples nucleotide hydrolysis from substrate release. EMBO J 17: 6871-6878.

Blatch, G.L. and Lassie, M. 1999. The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. BioEssays 21: 932- 939.

Boorstein, W.R., Ziegelhoffer, T. and Craig, E.A. 1994. Molecular evolution of the HSP70 multigene family. J Mol Evol 38: 1-17.

Boston, R.S., Viitanen, P.V., Vierling, E. 1996. Molecular chaperones and protein folding in plants. Plant Mol Biol 32: 191-222.

Bradford, K.J., Downie, A.B., Gee, O.H., Alvarado, V., Yang, H. and Dahal, P. 2003. Abscisic acid and gibberellin differentially regulate expression of genes of the SNF1-related kinase complex in tomato seeds. Plant Physiol 132: 1560-1576.

Carrari, F., Perez-Flore, L., Lijavetzky, D., Enciso, S., Sanchez, R., Benech-Arnold, R. and Iusem, N. 2001. Cloning and expression of a sorghum gene with homology to maize vp1 and its potential involvement in pre-harvest sprouting resistance. Plant Mol Biol 45: 631-640.

Carson, C.B., Hattori, T., Rosenkrans, L., Vasil, V., Vasil, I.K., Peterson, P.A. and McCarty, D.R. 1997. The quiescent/colorless alleles of viviparous1 show that the conserved B3 domain of VP1 is not essential for ABA-regulated gene expression in the seed. Plant J 12: 1231-1240.

Chakrabarty, R., Banerjee, R., Chung, S.M., Farman, M., Citovsky, V., Hogenhout, S.A., Tzfira, T. and Goodin, M. 2007. pSITE vectors for stable integration or transient expression of autofluorescent protein fusions in plants: Probing *Nicotiana benthamiana*-virus interactions. Mol Plant-Microbe Interact 20: 740-750.

Chang, S., Puryear, J. and Cairney, J. 1993. A simple and efficient method for isolating RNA from pine trees. Plant Mol Bio Report 11: 113-116.

Clerkx, E.J., Blankestijn-De Vries, H., Ruys, G., Groot, S. and Koornneef, M. 2003. Characterization of *green seed*, an enhancer of *abi3-1* in Arabidopsis that affects seed longevity. Plant Physiol 132: 1077-1084.

Clough, S.J. and Bent, A.F. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16: 735-743.

Corpas, F.J. and Trelease, R.N. 1997. The plant 73kDa peroxisomal membrane protein, PMP73, is immunorelated to molecular chaperones. Eur J Cell Biol 73: 49-57.

Dang, C.V. and Lee, W.M. 1989. Nuclear and nucleolar targeting sequences of c-erb-A, C-myb, N-myc, p53, HSP70, and HIV tat proteins. J Biol Chem 264:18019-18023.

Debeaujon, I., Peeters, A.J., Léon-Kloosterziel, K. and Koornneef, M. 2001. The TRANSPARENT TESTA12 gene of Arabidopsis encodes a multidrug secondary transporter-like protein required for flavonoid sequestration in vacuoles of the seed coat endothelium. Plant Cell 13: 853-871.

Dekkers, B.J., Schuurmans, J.A. and Smeekens, S.C. 2004. Glucose delays seed germination in Arabidopsis thaliana. Planta 218: 579-588.

Dekkers, B.J., Schuurmans, J.A. and Smeekens, S.C. 2008. Interaction between sugar and abscisic acid signalling during early seedling development in Arabidopsis. Plant Mol Biol 67: 151-167.

Dure III, L., Crouch, M., Harada, J., Ho, T.H., Mundy, J., Quatrano, R., Thomas, T. and Sung, Z.R. 1989. Common amino acid sequence domains among the LEA proteins of higher plants. Plant Mol Biol 12: 475-486.

Egerton-Warburton, L. 1998. A smoke-induced alteration of the sub-testa cuticle in seeds of the post-fire recruiter, *Emmenanthe penduliflora* Benth. (Hydrophyllaceae). J Exp Bot 49: 1317-1327.

Ezcurra, I., Wycliffe, P., Nehlin, L., Ellerström, M. and Rask, L. 2000. Transactivation of the Brassica napus napin promoter by ABI3 requires interaction of the conserved B2 and B3 domains of ABI3 with different cis-elements: B2 mediates activation through an ABRE, whereas B3 interacts with an RY/G-box. Plant J 24: 57-66.

Feurtado, J.A. and Kermode, A.R. 2007. A merging of paths: abscisic acid and hormonal cross-talk in the control of seed dormancy maintenance and alleviation. In: Bradford, K.J. and Nonogaki, H., eds. Seed development, dormancy and germination. Oxford,UK: Blackwell, 176-223.

Finkelstein, R. and Lynch, T. 2000. The Arabidopsis abscisic acid response gene ABI5 encodes a basic leucine zipper transcription factor. Plant Cell 12: 599-609.

Finkelstein, R., Gampala, S.S. and Rock, C.D. 2002. Abscisic acid signaling in seeds and seedlings. Plant Cell 14 Suppl: S15-S45.

Frey, A., Audran, C., Marin, E., Sotta, B. and Marion-Poll, A. 1999. Engineering seed dormancy by the modification of zeaxanthin epoxidase gene expression. Plant Mol Biol 39: 1267-1274.

Frey, A., Godin, B., Bonnet, M., Sotta, B. and Marion-Poll, A. 2004. Maternal synthesis of abscisic acid controls seed development and yield in *Nicotiana plumbaginifolia*. Planta 218: 958-964.

Frydman, J. and Hohfeld, J. 1997. Chaperones get in touch: the Hip-Hop connection. Trends Biochem Sci 22: 87-92.

Frydman, J. 2001. Folding of newly translated proteins in vivo: the role of molecular chaperones. Annu Rev Biochem 70: 603–647.

Fukuhara, T., Kirch, H. and Bohnert, H. 1999. Expression of Vp1 and water channel proteins during seed germination. Plant Cell Environ 22: 417-424.

Gazzarrini, S. and McCourt, P. 2001. Genetic interactions between ABA, ethylene and sugar signaling pathways. Curr Opin Plant Biol 4: 387-391.

Gazzarrini, S., Tsuchiya, Y., Lumba, S., Okamoto, M. and McCourt, P. (2004). The transcription factor FUSCA3 controls developmental timing in Arabidopsis through the hormones gibberellin and abscisic acid. Dev Cell 7: 373-385.

Gebauer, M., Matthias, Z. and Gehring, U. 1997. Proteins interacting with the molecular chaperone hsp70/hsc70: physical associations and effects on refolding activity. FEBS Lett 417: 109-113.

Giraudat, J., Hauge, B.M., Valon, C., Smalle, J., Parcy, F. and Goodman, H.M. 1992. Isolation of the Arabidopsis ABI3 gene by positional cloning. Plant Cell 4: 1251-1261.

Giraudat, J., Parcy, F., Bertauche, N., Gosti, F., Leung, J., Morris, P.C., Bouvier-Durand, M. and Vartanian, N. 1994. Current advances in abscisic acid action and signalling. Plant Mol Biol 26: 1557-1577.

Graeber, D., Gelbrecht, J., Pusch, M.T., Anlanger, C. and von Schiller, D. 2012. Agriculture has changed the amount and composition of dissolved organic matter in Central European headwater streams. Sci Total Environ 438: 435-446.

Greene, M., Maskos, K. and Landry, S. 1998. Role of the J-domain in the cooperation of Hsp40 with Hsp70. Natl Acad Sci USA 95:6108-6113.

Hartl, F.U. 1996. Molecular chaperones in cellular protein folding. Nature 381: 571–580.

Haslekås, C., Stacy, R.A., Nygaard, V., Culiáñez-Macià, F. and Aalen, R.B. 1998. The expression of a peroxiredoxin antioxidant gene, AtPer1, in Arabidopsis thaliana is seed-specific and related to dormancy. Plant Mol Biol 36: 833-845.

He, X. and Kermode, A.R. 2010. Programmed cell death of the megagametophyte during post-germinative growth of white spruce (*Picea glauca*) seeds is regulated by reactive oxygen species and the ubiquitin-mediated proteolytic system. Plant Cell Physiol 51: 1707-1720.

Hennon, P.E., D'Amore, D.V., Wittwer, D.T. and Caouette, J.P. 2008. Yellow-cedar decline: conserving a climate-sensitive tree species as Alaska warms. In Integrated restoration of forested ecosystems to achieve multiresource benefits: proceedings of the 2007 national silviculture workshop. Gen Tech Rep PNW-GTR-733. US Department of Agriculture, Forest Service, Pacific Northwest Research Station, Portland, OR, 233-245.

Hill, A., Nantel, A., Rock, C.D. and Quatrano, R.S. 1996. A conserved domain of the viviparous-1 gene product enhances the DNA binding activity of the bZIP protein EmBP-1 and other transcription factors. J Biol Chem 271: 3366-3374.

Hill, J.E. and Hemmingsen, S.M. 2001. *Arabidopsis thaliana* type I and II chaperonins. Cell Stress Chaperones 6: 190-200.

Höhfeld, J., Minami, Y. and Hartl, F.U. 1995. Hip, a new cochaperone involved in the eukaryotic hsc70/hsp40 reaction cycle. Cell 83: 589-598.

Höhfeld, J. 1998. Regulation of the heat shock cognate Hsc70 in the mammalian cell: the characterization of the anti-apoptotic protein BAG-1 provides novel insights. Biol Chem 379: 269-274.

Holdsworth, M., Kurup, S. and McKibbin, R. 1999. Molecular and genetic mechanisms regulating the transition from embryo development to germination. Trends Plant Sci 4: 275-280.

Holdsworth, M.J., Bentsink, L. and Soppe, W.J. 2008. Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination. New Phytol 179: 33-54.

Homrichhausen, T.M., Hewitt, J.R. and Nonogaki, H. 2003. Endo-β-mannanase activity is associated with the completion of embryogenesis in imbibed carrot (*Daucus carota* L.) seeds. Seed Sci Res 13**:** 219-227.

Irmer, H. and Höhfeld, J. 1997. Characterization of functional domains of the eukaryotic co-chaperone Hip. J Biol Chem 272: 2230-2235.

Jefferson, R.A. 1987. Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Report 5: 387-405.

Jiang, L., Abrams, S.R. and Kermode, A.R. 1996. Vicilin and napin storage-protein gene promoters are responsive to abscisic acid in developing transgenic tobacco seed but lose sensitivity following premature desiccation. Plant Physiol 110: 1135-1144.

Jones, H.D., Peters, N.C. and Holdsworth, M.J. 1997. Genotype and environment interact to control dormancy and differential expression of the *Viviparous 1* homologue in embryos of *Avena fatua*. Plant J 12: 911-920.

Jones, H.D., Kurup, S., Peters, N.C. and Holdsworth, M.J. 2000. Identification and analysis of proteins that interact with the *Avena fatua* homologue of the maize transcription factor VIVIPAROUS 1. Plant J 21: 133-142.

Jozsa, L.A. 1992. Yellow cypress trivia. In: Yellow Cypress: Can we grow it? Can we sell it? FRDA Report 171: 13-15.

Jull, L.G. and Blazich, F.A. 2000. Seed germination of selected provenances of Atlantic white-cedar as influenced by stratification, temperature, and light. HortScience 35: 132-135.

Kagaya, Y., Toyoshima, R., Okuda, R., Usui, H., Yamamoto, A. and Hattori, T. 2005. LEAFY COTYLEDON1 controls seed storage protein genes through its regulation of FUSCA3 and ABSCISIC ACID INSENSITIVE3. Plant Cell Physiol 46: 399-406.

Karssen, C.M., Brinkhorst-van der Swan, D.L., Breekland, A.E. and Koornneef, M. 1983. Induction of dormancy during seed development by endogenous abscisic acid: studies on abscisic-acid deficient genotypes of *Arabidopsis thaliana* (L.) Heynh. Planta 157: 158-165.

Karssen, C.M. and Lacka, E. 1986. A revision of the hormone balance theory of seed dormancy: studies on gibberellin and/or abscisic acid deficient mutants of *Arabidopsis thaliana*. In: Bopp, M.,ed. Plant growth substances. Berlin, Germany: Springer-Verlag, 315-323.

Karssen, C.M. 1995. Hormonal regulation of seed development, dormancy, and germination studied by genetic control. In: Kigel, J. and Galili, G., eds. Seed development and germination. New York, NY, USA: Marcel Dekker Inc., 333-350.

Keith, K., Kraml, M., Dengler, N.G. and McCourt, P. 1994. fusca 3: a heterochronic mutation affecting late embryo development in Arabidopsis. Plant Cell 6:589-600.

Kermode, A.R. 1990. Regulatory mechanisms involved in the transition from seed development to germination. Crit Rev Plant Sci 9: 155-195.

Kermode, A.R. 1995. Regulatory mechanisms in the transition from seed development to germination: interactions between the embryo and the seed environment. In: Kigel, J. and Galili, G., eds. Seed development and germination. New York, NY, USA: Marcel Dekker Inc., 273-332.

Kermode, A.R. and Finch-Savage, W. 2002. Desiccation sensitivity in orthodox and recalcitrant seeds in relation to development. In: Black, M. and Pritchard, H., eds. Desiccation and plant survival. Oxford, UK: CABI, 149-184.

Kermode, A.R. 2005. Role of abscisic acid in seed dormancy. J Plant Growth Regul 24: 319-344.

Kim, B.H. and Schoffl, F. 2002. Interaction between Arabidopsis heat shock transcription factor 1 and 70 kDa heat shock proteins. J Exp Bot 53: 371–375.

Kim, H.J., Song, E.J., Lee, Y.S., Kim, E. and Lee, K.J. 2005. Human Fas-associated factor 1 interacts with heat shock protein 70 and negatively regulates chaperone activity. J Biol Chem 280: 8125–8133.

Kimmins, S. and MacRae, T.H. 2000. Maturation of steroid receptors: an example of functional cooperation among molecular chaperones and their associated proteins. Cell Stress Chaperones 5: 76-86.

Koornneef, M. and Karssen, C.M. 1994. Seed dormancy and germination. In: Meyerowitz, E.M. and Somerville, C., eds. Arabidopsis. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press, 313-334.

Koornneef, M., Bentsink, L. and Hilhorst, H. 2002. Seed dormancy and germination. Curr Opin Plant Biol 5: 33-36.

Kotak, S., Vierling, E., Bäumlein, H. and von Koskull-Döring, P. 2007. A novel transcriptional cascade regulating expression of heat stress proteins during seed development of Arabidopsis. Plant Cell 19: 182-195.

Kroj, T., Savino, G., Valon, C., Giraudat, J. and Parcy, F. 2003. Regulation of storage protein gene expression in Arabidopsis. Development 130: 6065-6073.

Kucera, B., Cohn, M.A. and Leubner-Metzger, G. 2005. Plant hormone interactions during seed dormancy release and germination. Seed Sci Res 15: 281-307.

Kurup, S., Jones, H.D. and Holdsworth, M.J. 2000. Interactions of the developmental regulator ABI3 with proteins identified from developing Arabidopsis seeds. Plant J 21: 143-155.

Kurz, M., Roberts, D.R., Flinn, B.S. and Vidaver, W.E. 1994. A morphological and biochemical analysis of embryo maturation during yellow-cypress seed development. Can J For Res 24: 431-441.

Lara, P., Oñate-Sánchez, L., Abraham, Z., Ferrándiz, C., Díaz, I., Carbonero, P. and Vicente-Carbajosa, J. 2003. Synergistic activation of seed storage protein gene expression in Arabidopsis by ABI3 and two bZIPs related to OPAQUE2. J Biol Chem 278: 21003-21011.

Lazarova, G., Zeng, Y. and Kermode, A.R. 2002. Cloning and expression of an ABSCISIC ACID-INSENSITIVE 3 (ABI3) gene homologue of yellow-cedar (*Chamaecyparis nootkatensis*). J Exp Bot 53: 1219-1221.

Lee, S.C. and Luan, S. 2012. ABA signal transduction at the crossroad of biotic and abiotic stress responses. Plant Cell Environ 35: 53-60.

Lefebvre, B., Furt, F., Hartmann, M., Michaelson, L.V., Carde, J., Sargueil-Boiron, F., Rossignol, M., Napier, J.A., Cullimore, J., Bessoule, J. and Mongrand, S. 2007. Characterization of lipid rafts from *Medicago truncatula* root plasma membranes: A proteomic study reveals the presence of a raft-associated redox system. Plant Physiol 144: 402-418.

Lescot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, Y., Rouzé, P. and Rombauts, S. 2002. PlantCARE, a database of plant *cis*-acting regulatory elements and a portal to tools for *in silico* analysis of promoter sequences.

Leubner-Metzger, G., Petruzzelli, L., Waldvogel, R., Vogeli-Lange, R. and Meins, F. 1998. Ethylene-responsive element binding protein (EREBP) expression and the transcriptional regulation of class I beta-1,3-glucanase during tobacco seed germination. Plant Mol Biol 38: 785-795.

Li, J., Biswas, M.G., Chao, A., Russel, D.W. and Chory, J. 1997. Conservation of function between mammalian and plant steroid 5α-reductases Proc Natl Acad Sci USA 94: 3554–3559.

Lin, Z., Griffith, M.E., Li, X., Zhu, Z., Tan, L., Fu, Y., Zhang, W., Wang, X., Xie, D. and Sun, C. 2007. Origin of seed shattering in rice (*Oryza sativa* L.). Planta 226: 11-20.

Marcotte, W.R., Russell, S.H. and Quatrano, R.S. 1989. Abscisic acid-responsive sequences from the Em gene of wheat. Plant Cell 1: 969-976.

Marin, E., Nussaume, L., Quesada, A., Gonneau, M., Sotta, B., Hugueney, P., Frey, A. and Marion-Poll, A. 1996. Molecular identification of zeaxanthin epoxidase of *Nicotiana plumbaginifolia*, a gene involved in abscisic acid biosynthesis and corresponding to the ABA locus of *Arabidopsis thaliana*. EMBO J 15: 2331-2342.

McCarty, D.R., Hattori, T., Carson, C.B., Vasil, V., Lazar, M. and Vasil, I. 1991. The *viviparous-1* developmental gene of maize encodes a novel transcriptional activator. Cell 66: 895-905.

McKibbin, R., Wilkinson, M.D., Bailey, P.C., Flintham, J.E., Andrew, L.M., Lazzeri, P., Gale, M.D., Lenton, J.R. and Holdsworth, M.J. 2002. Transcripts of *Vp-1* homeologues are misspliced in modern wheat and ancestral species. Proc Natl Acad Sci U S A 99: 10203-10208.

Meinke, D.W., Franzmann, L.H., Nickle, T.C. and Yeung, E.C. 1994. Leafy cotyledon mutants of Arabidopsis. Plant Cell 6: 1049-1064.

Meinke, D.W. 1995. Molecular genetics of plant embryogenesis. Annu Rev Plant Physiol Plant Mol Biol 46: 369-394.

Meurs, C., Basra, A.S., Karssen, C.M. and van Loon, L.C. 1992. Role of abscisic acid in the induction of desiccation tolerance in developing seeds of *Arabidopsis thaliana.* Plant Physiol 98: 1484-1493.

Miernyk, J.A. 1997. The 70 kDa stress-related proteins as molecular chaperones. Trends Plant Sci 2: 180-187.

Miernyk, J.A. 1999. Protein folding in the plant cell. Plant Physiol 121: 695-703.

Milarski, K.L. and Morimoto, R.I. 1989. Mutational analysis of the human HSP70 protein: distinct domains for nucleolar localization and adenosine triphosphate binding. J. Cell Biol 109: 1947-1962.

Nakamura, S. and Toyama, T. 2001. Isolation of a VP1 homologue from wheat and analysis of its expression in embryos of dormant and non-dormant cultivars. J Exp Bot 52: 875-876.

Nakamura, S., Lynch, T.J. and Finkelstein, R.R. 2001. Physical interactions between ABA response loci of Arabidopsis. Plant J 26:627–635.

Nambara, E., Keith, K., McCourt, P. and Naito, S. 1994. Isolation of an internal deletion mutant of the *Arabidopsis thaliana ABI3* gene. Plant and Cell Physiol 35: 509-513.

Nambara, E., Hayama, R., Tsuchiya, Y., Nishimura, M., Kawaide, H., Kamiya, Y. and Naito, S. 2000. The role of *ABI3* and *FUS3* loci in *Arabidopsis thaliana* on phase transition from late embryo development to germination. Dev Biol 220: 412-423.

Nambara, E. and Marion-Poll, A. 2003. ABA action and interactions in seeds. Trends Plant Sci 8: 213-217.

Nambara, E. and Marion-Poll, A. 2005. Abscisic acid biosynthesis and catabolism. Annu Rev Plant Biol 56: 165-185.

Nelson, G.M., Prapapanich, V., Carrigan, P.E., Roberts, P.J., Riggs, D.L. and Smith, D.F. 2004. The heat shock protein 70 cochaperone hip enhances functional maturation of glucocorticoid receptor. Mol Endocrinol 18: 1620-1630.

Neumann, D., zur Nieden, U., Manteuffel, R., Walter, G., Scharf, K.-D. Nover, L. 1987. Intracellular localization of heat -shock proteins in tomato cell cultures. Eur. J. Cell. Bioi. 43:71-81.

Neumann, D., Nover, L., Parthier, B., Rieger, R., Scharf, K.D., Wollgiehn, R. and zur Nieden, U. 1989. Heat shock and other stress response systems of plants. Results Probl Cell Differ 16: 1-155.

Nover, L., Munsche, D., Neumann, D., Ohme, K., Scharf, K.-D. 1986. Control of ribosome biosynthesis in plant cell cultures under heat-shock conditions. Eur J Biochem 160: 297-304.

Ooms, J., Léon-Kloosterziel, K.M., Bartels, D., Koornneef, M. and Karssen, C.M. 1993. Acquisition of desiccation tolerance and longevity in seeds of *Arabidopsis thaliana* (A comparative study using abscisic acid-insensitive *abi3* mutants). Plant Physiol 102: 1185-1191.

Palleros, D.R., Reid, K.L., Shi, L., Welch, W.J. and Fink, A.L. 1993. ATP-induced protein-Hsp70 complex dissociation requires K+ but not ATP hydrolysis. Nature 365: 664-666.

Parcy, F., Valon, C., Kohara, A., Miséra, S. and Giraudat, J. 1997. The ABSCISIC ACID-INSENSITIVE3, FUSCA3, and LEAFY COTYLEDON1 loci act in concert to control multiple aspects of Arabidopsis seed development. Plant Cell 9: 1265-1277.

Parcy, F. and Giraudat, J. 1997. Interactions between the *ABI1* and the ectopically expressed *AB13* genes in controlling abscisic acid responses in *Arabidopsis* vegetative tissues. Plant J 11: 693-702.

Pawuk, W.H. 1993. Germination of Alaska cedar seed. Tree Planters' Notes 44: 21-24.

Prändl, R., Kloske, E. and Schöffl, F. 1995. Developmental regulation and tissue-specific differences of heat-shock gene expression in transgenic tobacco and Arabidopsis plants. Plant Mol Biol 28:73-82.

Prapapanich, V., Chen, S., Nair, S.C., Rimerman, R.A. and Smith, D.F. 1996a. Molecular cloning of human p48, a transient component of progesterone receptor complexes and an hsp70-binding protein. Mol Endocrinol 10: 420-431.

Prapapanich, V., Chen, S., Toran, E.J., Rimerman, R.A. and Smith, D.F. 1996b. Mutational analysis of the hsp70-interacting protein Hip. Mol Cell Biol 16: 6200-6207.

Prapapanich, V., Chen, S. and Smith, D.E. 1998. Mutation of Hip's carboxy-terminal region inhibits a transitional stage of progesterone receptor assembly. Mol Cell Biol 18: 944-952.

Pratt, W.B. 1998. The hsp90-based chaperone system: involvement in signal transduction from a variety of hormone and growth factor receptors. Proc Soc Exp Biol Med 217: 420-434.

Pratt, W.B. and Toft, D.O. 2003. Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. Exp Biol Med (Maywood) 228: 111-133.

Price, J., Li, T.C., Kang, S.G., Na, J.K. and Jang, J.C. 2003. Mechanisms of glucose signaling during germination of Arabidopsis. Plant Physiol 132: 1424-1438.

Raz, V., Bergervoet, J. and Koornneef, M. 2001. Sequential steps for developmental arrest in Arabidopsis seeds. Development 128: 243-252.

Ren, C. and Kermode, A.R. 1999. Analyses to determine the role of the megagametophyte and other seed tissues in dormancy maintenance of yellow cedar (*Chamaecyparis nootkatensis*) seeds; morphological, cellular and physiological changes following moist chilling and during germination. J Exp Bot 50: 1403-1419.

Ren, C. and Kermode, A.R. 2000. An increase in pectin methyl esterase activity accompanies dormancy breakage and germination of yellow cedar seeds. Plant Physiol 124: 231-242.

Robinson, C.K. and Hill, S.A. 1999. Altered resource allocation during seed development in Arabidopsis caused by the *abi3* mutation. Plant Cell Environ 22: 117-123.

Rodríguez-Gacio Mdel, C., Matilla-Vázquez, M.A. and Matilla, A.J. 2009. Seed dormancy and ABA signaling: the breakthrough goes on. Plant Signal Behav 4: 1035-1048.

Rodríguez-Milla, M. A. and Salinas, J. 2009. Prefoldins 3 and 5 play an essential role in Arabidopsis tolerance to salt stress. Mol Plant 2: 526-534.

Rohde, A., Van Montagu, M. and Boerjan, W. 1999. The ABSCISIC ACID-INSENSITIVE 3 (ABI3) gene is expressed during vegetative quiescence processes in *Arabidopsis*. Plant Cell Environ 22: 261-270.

Rohde, A., Kurup, S. and Holdsworth, M. 2000. ABI3 emerges from the seed. Trends Plant Sci 5: 418-419.

Rolland, F., Moore, B. and Sheen, J. 2002. Sugar sensing and signaling in plants. Plant Cell 14 Suppl: S185-205.

Saito, S., Hirai, N., Matsumoto, C., Ohigashi, H., Ohta, D., Sakata, K. and Mizutani, M. 2004. Arabidopsis CYP707As encode (+)-abscisic acid 8'-hydoxylase, a key enzyme in the oxidative catabolism of abscisic acid. Plant Physiol 134:1439-1449.

Santos Mendoza, M., Dubreucq, B., Miquel, M., Caboche, M., & Lepiniec, L. 2005. LEAFY COTYLEDON 2 activation is sufficient to trigger the accumulation of oil and seed specific mRNAs in Arabidopsis leaves. FEBS Lett 579: 4666-4670.

Schmitz, N., Abrams, S.R. and Kermode, A.R. 2000. Changes in abscisic acid content and embryo sensitivity to (+)-abscisic acid during the termination of dormancy of yellow cedar seeds. J Exp Bot 51: 1159-1162.

Schmitz, N., Xia, J.H. and Kermode, A.R. 2001. Dormancy of yellow-cedar seeds is terminated by gibberellic acid in combination with fluridone or with osmotic priming and moist chilling. Seed Sci Technol 29: 331-346.

Schmitz, N., Abrams, S.R. and Kermode, A.R. 2002. Changes in ABA turnover and sensitivity that accompany dormancy termination of yellow-cedar (*Chamaecyparis nootkatensis*) seeds. J Exp Bot 53: 89-101. doi:10.1093/jexbot/53.366.89

Schultz, T.F., Medina, J., Hill, A. and Quatrano, R. 1998. 14-3-3 proteins are part of an abscisic acid-VIVIPAROUS1 (VP1) response complex in the *Em* promoter and interact with VP1 and EmBP1. Plant Cell 10: 837-847.

Seo, M. and Koshiba, T. 2002. Complex regulation of ABA biosynthesis in plants. Trends Plant Sci 7: 41-48.

Seung, D., Pablo, J., Risopatron, M., Jones, B.J. and Marc, J. 2012. Circadian clock-dependent gating in ABA signalling networks. Protoplasma 249: 445-457.

Smith, D.F. 2000. Chaperones in progesterone receptor complexes. Cell Dev Biol 11: 45-52.

Söderman, E.M., Brocard, I.M., Lynch, T.J. and Finkelstein, R.R. 2000. Regulation and function of the Arabidopsis *ABA-insensitive4* gene in seed and abscisic acid response signaling networks. Plant Physiol 124: 1752-1765.

Sondermann, H., Scheufler, C., Schneider, C., Höhfeld, J., Hartl, F-U. and Moarefi, I. 2001. Structure of a Bag/Hsc70 complex: convergent evolution of Hsp70 nucleotide exchange factors. Science 291: 1553-1557.

Sparkes, I.A., Runions, J., Kearns, A. and Hawes, C. 2006. Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants Nat Protoc 1: 2019-2025.

Steber, C. and McCourt, P. 2001. A role for brassinosteroids in germination in Arabidopsis. Plant Physiol 125: 763-769.

Stone, S.L., Kwong, L.W., Yee, K.M., Pelletier, J., Lepiniec, L., Fischer, R.L., Goldberg, R.B. and Harada, J.J. 2001. *LEAFY COTYLEDON2* encodes a B3 domain transcription factor that induces embryo development. Proc Natl Acad Sci U S A 98: 11806-11811.

Sung, D.Y., Vierling, E. and Guy, C.L. 2001. Comprehensive expression profile analysis of the Arabidopsis Hsp70 gene family. Plant Physiol 126: 789-800.

Suzuki, M., Kao, C.Y. and McCarty, D.R. 1997. The conserved B3 domain of VIVIPAROUS1 has a cooperative DNA binding activity. Plant Cell 9: 799-807.

Suzuki, M., Kao, C.Y., Cocciolone, S. and McCarty, D.R. 2001. Maize VP1 complements Arabidopsis *abi3* and confers a novel ABA/auxin interaction in roots. Plant J 28: 409-418.

Takayama, S., Bimston, D.N., Matsuzawa, S., Freeman, B.C., Aime-Sempe, C., Xie, Z., Morimoto, R.I. and Reed, J.C. 1997. BAG-1 modulates the chaperone activity of Hsp70/Hsc70. EMBO J 16: 4887-4896.

Takayama, S., Zhihua, X. and Reed, J.C. 1999. An evolutionarily conserved family of Hsp70/Hsc70 molecular chaperone regulators. J Biol Chem 274: 781-786.

Tan, B.C., Schwartz, S.H., Zeevaart, J.A. and McCarty, D.R. 1997. Genetic control of abscisic acid biosynthesis in maize. Proc Natl Acad Sci U S A 94: 12235-12240.

Tan, B.C., Joseph, L.M., Deng, W.T., Liu, L., Li, Q.B., Cline, K. and McCarty, D.R. 2003. Molecular characterization of the Arabidopsis 9-cis epoxycarotenoid dioxygenase gene family. Plant J 35: 44-56.

Tillman-Sutela, E. and Kauppi, A. 1998. Structures restricting passage of water in the mature seeds of yellow-cedar (Chamaecyparis nootkatensis). Can J Bot 76: 1458-1466.

To, J., Reiter, W-D. and Gibson, S.I. 2002. Mobilization of seed storage lipid by Arabidopsis seedlings is retarded in the presence of exogenous sugars. BMC Plant Biol 2: 4.

Velten, M., Villoutreix, B.O. and Ladjimi, M.M. 2000. Quaternary structure of the hsc70 cochaperone Hip. Biochemistry 39: 307-315.

Wang, W., Vinocur, B., Shoseyov, O. and Altman, A. 2004. Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends Plant Sci 9: 244-252.

Wang, G., Wang, F., Wang, G., Wang, F., Zhang, X., Zhong, M., Zhang, J., Lin, D., Tang, Y., Xu, Z. and Song, R. 2012. *Opaque1* encodes a myosin XI motor protein that is required for endoplasmic reticulum motility and protein body formation in maize endosperm. Plant Cell 24: 3447-3462.

Waters, E.R., Lee, G.J. and Vierling, E. 1996. Evolution, structure and function of the small heat shock proteins in plants. J Exp Bot 47: 325-338.

Webb, M.A., Cavaletto, J.M., Klanrit, P. and Thompson, G.A. 2001. Orthologs in *Arabidopsis thaliana* of the Hsp70 interacting protein Hip. Cell Stress Chaperones 6: 247-255.

Wehmeyer, N., Hernandez, L., Finkelstein, R. and Vierling, E. 1996. Synthesis of small heat-shock proteins is part of the developmental program of late seed maturation. Plant Physiol 112: 747-757.

Wehmeyer, N. and Vierling, E. 2000. The expression of small heat shock proteins in seeds responds to discrete developmental signals and suggests a general protective role in desiccation tolerance. Plant Physiol 122: 1099-1108.

West, M.A.L., Yee, K.M., Danao, J., Zimmerman, J.L., Fisher, R.L., Goldberg, R.B. and Harada, J.J. 1994. *LEAFY COTYLEDON1* is an essential regulator of late embryogenesis and cotyledon identity in Arabidopsis. Plant Cell 6: 1731-1745.

White, C.N. and Riven, C.J. 2000. Gibberellins and seed development in maize. II. Gibberellin synthesis inhibition enhances abscisic acid signaling in cultured embryos. Plant Physiol 122: 1089-1097.

White, C.N., Proebsting, W.M., Hedden, P. and Rivin, C.J. 2000. Gibberellins and seed development in maize. I. evidence that gibberellin/abscisic acid balance governs germination versus maturation pathways. Plant Physiol 122: 1081-1088.

Wu, C. 1995. Heat shock transcription factors: structure and regulation. Annu Rev Cell Dev Biol 11: 441-469.

Xia, J.H. and Kermode, A.R. 1999. Analyses to determine the role of embryo immaturity in dormancy maintenance of yellow cedar (*Chamaecyparis nootkatensis*) seeds: synthesis and accumulation of storage proteins and proteins implicated in desiccation tolerance. J Exp Bot 50: 107-118.

Xia J.-H., Stewart D. and Kermode, A.R. 2002. Modified moist chilling treatments that promote germination and post-germinative reserve mobilization of different seed lots of yellow-cedar (*Chamaecyparis nootkatensis* [D. Don] Spach). Seed Sci Technol 30: 263-277.

Xu, N. and Bewley, J.D. 1991. Sensitivity to abscisic acid and osmoticum changes during embryogenesis of alfalfa (*Medicago sativa*). J Exp Bot 42: 821-826.

Yamaguchi, S., Kamiya, Y. and Sun. T.P. 2001. Distinct cell-specific expression patterns of early and late gibberellins biosynthetic genes during *Arabidopsis* seed germination. Plant J 28: 443-453.

Yuan, K. and Wysocka-Diller, J. 2006. Phytohormone signalling pathways interact with sugars during seed germination and seedling development. J Exp Bot 57: 3359-3367.

Zaharia, L.I., Walker-Simmon, M.K., Rodríguez, C.N. and Abrams, S.R. 2005. Chemistry of abscisic acid, abscisic acid catabolites and analogs. J Plant Growth Regul 24: 274-284.

Zeng, Y., Raimondi, N. and Kermode, A.R. 2003. Role of an *ABI3* homologue in dormancy maintenance of yellow-cedar seeds and in the activation of storage protein and *Em* gene promoters. Plant Mol Biol 51: 39-49.

Zeng, Y. and Kermode, A.R. 2004. A gymnosperm *ABI3* gene functions in a severe abscisic acid-insensitive mutant of *Arabidopsis* (*abi3-6*) to restore the wild-type phenotype and demonstrates a strong synergistic effect with sugar in the inhibition of post-germinative growth. Plant Mol Biol 56: 731-746.

Zeng, Y. 2005. Ph.D Thesis: Functional analyses of a conifer *AB13* gene, an ABA signalling component regulating seed dormancy and germination.

Zeng, Y., Zhao, T. and Kermode, A.R. 2013. A conifer ABI3-interacting protein plays important roles during key transitions of the plant life cycle. Plant Physiol 161: 179-195.

Zhang, X., Garreton, V. and Chua, N.H. 2005. The AIP2 E3 ligase acts as a novel negative regulator of ABA signaling by promoting ABI3 degradation. Genes Dev 19: 1532-1543.

Zhou, L., Jang, J.C., Jones, T.L. and Sheen, J. 1998. Glucose and ethylene signal transduction crosstalk revealed by an *Arabidopsis* glucose-insensitive mutant. Proc Natl Acad Sci U S A 95: 10294-10299.

zur Nieden, U., Neumann, D., Bucka, A. and Nover, L. 1995. Tissue-specific localization of heat-stress proteins during embryo development. Planta 196: 530-538.

Appendices

Appendix A.

Supplemental Figures

A1. Loading controls for western blots – Coomassie Blue protein stain

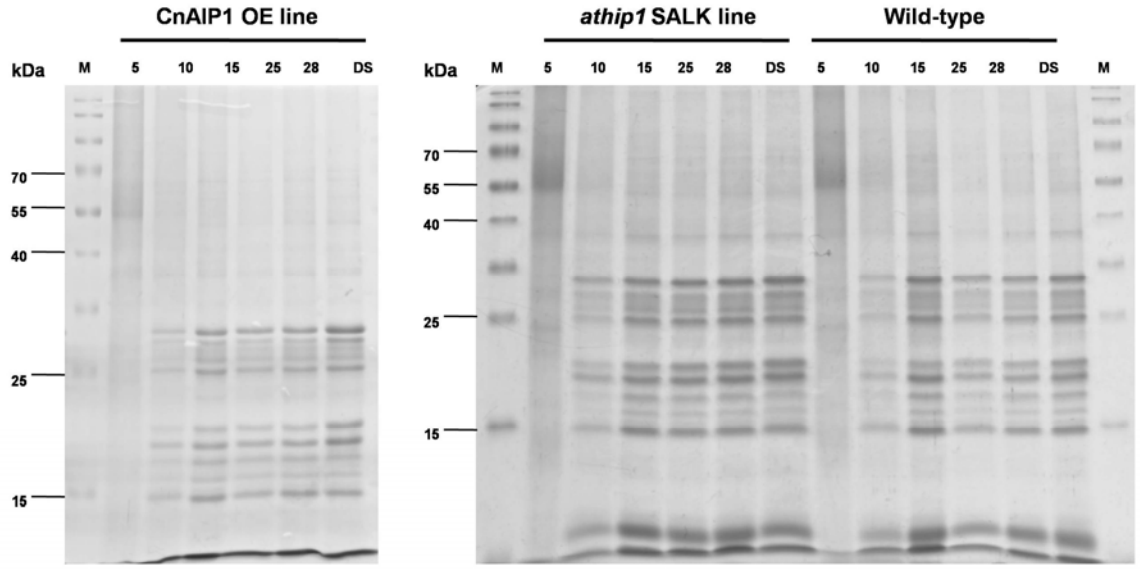

Figure A1-1. Loading controls for Western blot analysis of maturation proteins that accumulate during seed development in CnAIP1 transgenic, SALK (athip1) and wild-type seeds.

Proteins stained with Coomassie Blue as a loading control. 5-28 indicate days after pollination at which samples were collected from each line. Molecular mass standards (kDa) are from Fermentas.

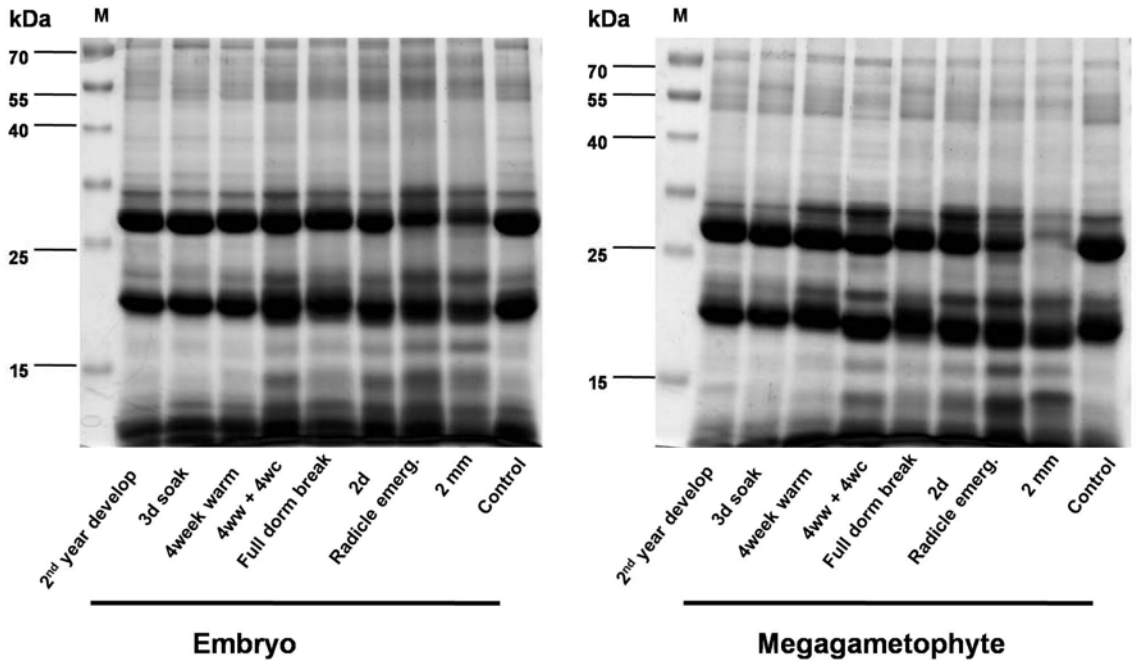

Figure A1-2. Loading controls for Western blot analysis of CnAIP1 protein expression in yellow-cedar seeds during different stages of dormancy breakage, germination and post-germinative growth.

Proteins stained with Coomassie Blue as a loading control in yellow-cedar embryo and megagametophyte samples. Molecular mass standards (kDa) are from Fermentas.

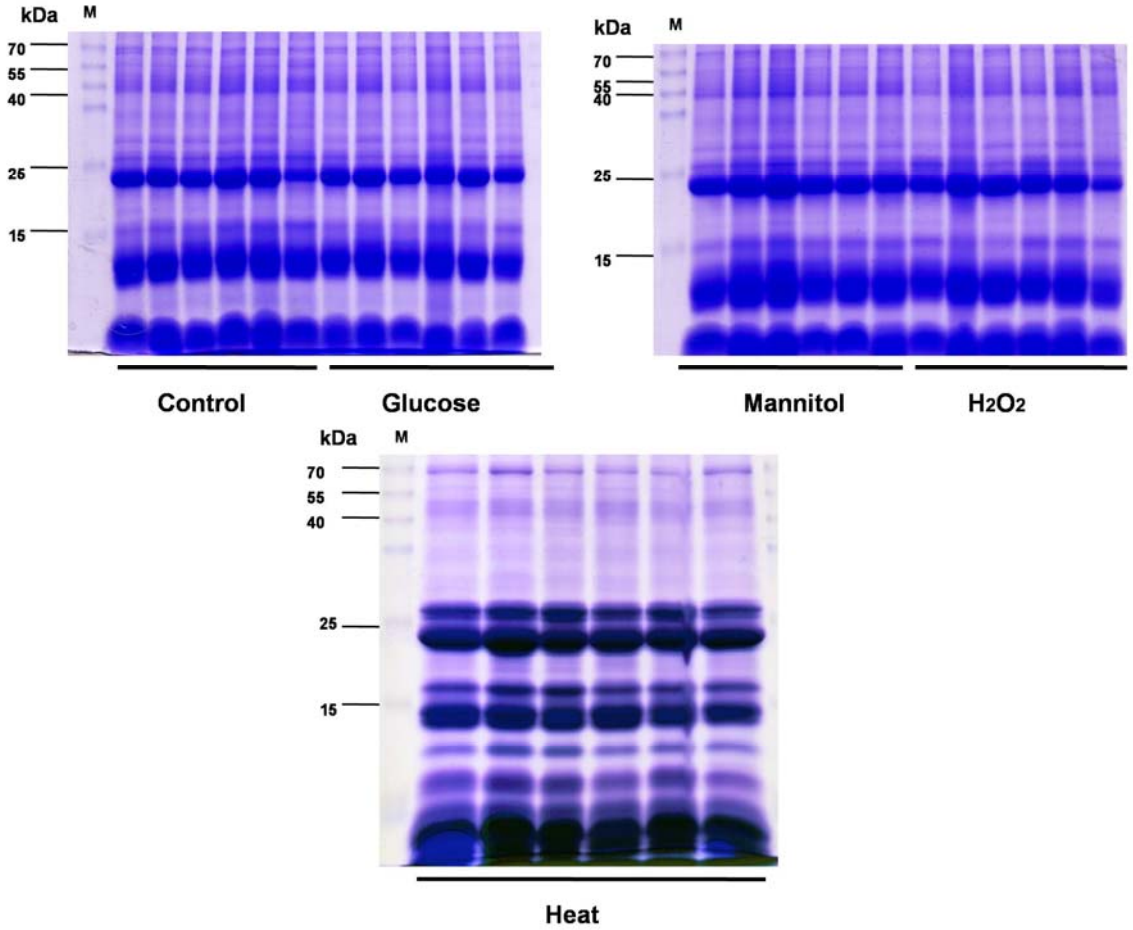

Figure A1-3. Loading controls for Western blot analysis of CnAIP1 and Hsp protein expression in yellow-cedar seeds in the presence of abiotic stress treatments.

Details of the timepoints at which yellow-cedar seeds were sampled during dormancy breakage into germination for each of the abiotic stress treatments are outlined in Figure 3-2. Heat stress was imposed on yellow-cedar seeds that had been at germination conditions for 2 days. Details of the heat stress, duration and recovery are outlined in Figures 3-3 and 3-4. Proteins stained with Coomassie Blue as a loading control in yellow-cedar seed samples. Molecular mass standards (kDa) are from Fermentas.