Functional Analyses of a Conifer *ABI3* Gene, an ABA Signalling Component Regulating Seed Dormancy and Germination

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Abstract

Seed dormancy is an adaptive trait improving survival of the next generation by controlling germination until favorable conditions ensue. While beneficial to the plant itself, seed dormancy is not always viewed as desirable by agricultural and forest industries, which rely upon the rapid and synchronous germination of seeds. Yellow-cedar *(Chamaecyparis nootkatensis* D. Don Spach), an important conifer species of the Pacific northwest, produces seeds that are deeply dormant at maturity; it is thus an important model species to study mechanisms of dormancy and germination. ABI3 (*Ab*scisic Acid *I*nsensitive*3*) is a transcription factor that regulates ABA-responsive genes during seed development, including those involved in reserve deposition, dormancy inception and acquisition of desiccation tolerance. The yellow-cedar *CnABI3* gene was functionally characterized. *CnABI3* encodes a protein of 794 amino acids and, under normal conditions, its expression is only detected in seeds. A role in dormancy maintenance of seeds of conifer species was established by monitoring mRNA and protein levels before, during and after dormancy termination. Dormancy termination and germination is associated with a down-regulation of the expression of this gene, but posttranscriptional controls are also likely operative. CnABI3 also plays a potential role in oxidative stress responses during early post-germinative growth. CnABI3 is a true orthologue of *ABI3* genes of angiosperms; it shares many common features with other *ABI3* genes, yet displays unique characteristics as well. Like other ABI3 orthologues, ectopic expression of the *CnABI3* gene in tobacco vegetative tissues activates genes driven by seed storage-protein gene promoters and ABA has a synergistic effect on activation. The *CnABI3* gene, when transformed into a severe *Arabidopsis abi3* null mutant (*abi3-6)* functions nearly perfectly. This functional complementation reveals the degree of conservation of ABI3 functions between gymnosperms and angiosperms. Three yellow-cedar proteins that physically interact with CnABI3 were identified using a yeast two-hybrid approach; their biological functions in relation to CnABI3 have been investigated in yellow-cedar, and in *Arabidopsis* using homologous genes. The results suggest a global role for ABI3 in the control of many key transitions in seeds and plants, and a potential role for the protein in the regulation of flowering.

Key words: *Abscisic Acid Insensitive3, Chamaecyparis nootkatensis, Arabidopsis,* germination, seed dormancy, protein-protein interactions, functional complementation

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1.1 A brief introduction to seed dormancy and germination

Seed dormancy is an adaptive trait that enables the next generation to start its new life at the right time, under favourable conditions, thus maximizing survival. After completing its development, the mature seed detaches from its mother plant. When a seed begins its own life journey, it must avoid germinating too quickly at the wrong place and wrong time. Dispersal of a seed can be viewed as escaping in space, while dormancy is the escape in time from unsuitable environmental conditions. Seed dormancy can be defined as the absence of germination of an intact, viable seed under favorable conditions within a specified time lapse (Hilhorst, 1995; Bewley, 1997). Germination includes those events commencing with imbibition or uptake of water by the quiescent dry seed and finishes with the elongation of the radicle (Bewley and Black, 1994; Bewley, 1997). Protrusion of the radicle through the seed structures surrounding the embryo (such as the endosperm, or megagametophyte) is usually used as visible evidence of the completion of germination. Many deeply dormant seeds must undergo specific environmental conditions such as a prolonged cold period which mimics the natural process of passing the winter season, before it is capable of germination. Like a whole plant, a single seed has the ability to perceive environmental conditions and responds accordingly by either germinating or staying dormant. Environmental factors that promote germination include

light of certain wavelengths, temperature, water availability, O_2 in soil and other factors. For example, the seed can perceive information about the depth of soil or the amount of above-ground competition with neighbouring plants through light intensity, and perceive information about time of year through temperature and photoperiod changes. How seeds perceive environmental conditions and regulate their physiological changes accordingly is an extremely complicated process and involves the expression of many genes. The present research attempts to elucidate the role of one of these genes, *CnABI3* (*Chamaecyparis nootkatensis ABscisic acid Insensitive3*), in the control of seed dormancy and germination in conifer species.

Coat-imposed dormancy and embryo dormancy are the two basic types of seed dormancy. Coat-imposed (or coat enhanced) dormancy occurs in seeds in which the tissues surrounding the embryo make it unable to germinate. Tissues like the megagametophyte or endosperm, nucellus and testa contribute, to varying extents, to the degree of whole seed dormancy (Hilhorst, 1995). They may prevent embryo germination by one or more of the following mechanisms: (1) impeding water uptake – e.g. by a thick and rigid seed coat; (2) mechanical restraint – i.e. the tissues physically prevent embryo expansion and radicle protrusion; (3) preventing gas (e.g. O_2) exchange; (4) biosynthesis of germination inhibitors such as ABA and (5) prevention of diffusion of germination inhibitors from the embryo. Inhibitors include ABA, short-chain fatty acids, phenolic acids, inorganic ions, coumarin, catechin and tannins (Bewley and Black, 1994). In conifer species, coat-imposed dormancy is the predominant mechanism, e.g., for the deeply dormant yellow-cedar (*Chamaecyparis nootkatensis*) and western white pine

(*Pinus monticola*) seeds (Ren and Kermode, 1999, 2000; Feurtado *et al.*, 2003; Terskikh *et al.*, 2005).

In the case of embryo dormancy, the embryo excised from the seed and placed on water is unable to germinate (elongate the embryonic axis). Inhibitors and developmental immaturity have been implicated as primary causes. Embryo dormancy is usually more profound than coat imposed dormancy, and the two types of dormancy can simultaneously exist in one species such as in apple seeds (Bewley and Black, 1994). One cause for embryo dormancy could be germination inhibitors such as ABA; another cause could be inhibitory factors present within the cotyledons, because removal of cotyledons often releases embryo dormancy (Bewley and Black, 1994).

The complex processes of dormancy and germination are controlled by many genes and are affected by developmental and environmental factors. It is difficult to fully understand what is happening in the transition from dormancy to germination in seeds because the key events for release of dormancy may take place in only a few cells associated with the embryonic root axis (Bewley, 1997). It would be very interesting to find out whether there is a common event that initiates the release of seed dormancy in different species and if there are molecular markers to distinguish dormant and non-dormant seeds. A number of approaches including molecular (transgenic) studies, mutant analysis, reverse genetics and proteomics have been employed for the investigation of seed dormancy and germination.

While seed dormancy is beneficial to the plant itself, it is often an undesirable trait to agriculture and forest industries, where uniformly and timely germination is very important. Therefore, understanding the mechanisms underlying seed dormancy and

germination, and implementing methods to improve germination performance and dormancy breakage are of significant economical importance.

1.2 Role of ABA in seed developmental processes, dormancy inception and inhibition of germination

Abscisic acid is widely considered the most important hormone regulating seed development, dormancy inception and maintenance, and in the transition from dormancy to germination. Its overall function is to promote and maintain seed developmental processes, and to prevent or inhibit germination. These functions are consistently found in many plant species and numerous reviews have detailed the broad actions of ABA in seeds (for reviews, see Bewley and Black, 1994; Kermode, 1995; Hilhorst, 1995; Bewley, 1997; Leung and Giraudat, 1998; Holdsworth *et al.*, 1999; Rohde *et al.*, 2000b; Kermode and Finch-Savage 2002; Finkelstein et al, 2002b).

Seed development can be roughly described as occurring in three contiguous stages. The first stage, which follows fertilization involves extensive cell division, differentiation and forms the basic body plan of the embryo. During the second stage, cell expansion occurs and there is a high rate of protein and lipid or storage deposition; inception of dormancy also occurs during this stage. The final stage is characterized by maturation drying. This stage is accompanied by a cessation of metabolism and termination of developmental protein synthesis; the seed adjusts to water loss and prepares for germination at this stage. However, these stages cannot be divided clearly and they are confluent processes in a seed (Kermode, 1995). Endogenous ABA content is initially low at the early histodifferentiation stage of seed development, then peaks during

the mid-development when storage reserves are being accumulated; thereafter it returns to low levels especially during the maturation drying stage (Bewley and Black, 1994; Kermode, 1995; Bewley, 1997; Leung and Giraudat, 1998). ABA is thus considered to regulate some essential processes in the developmental stages of the embryo. It controls many events during the final two thirds of seed development, including promoting storage reserve deposition, the acquisition of desiccation tolerance, and the induction/maintenance of seed dormancy. ABA contents may vary greatly for initiating the same process in different species, because of differences in the sensitivity to ABA and the thresholds for ABA action in each species. In the same seed at different development stages, ABA sensitivity and ABA contents also dramatically change (Xu and Bewley, 1991; Parcy *et al.*, 1994; Kermode, 1995).

ABA plays key roles in the inception of seed dormancy at the mid- to late-embryogenesis stages. ABA prevents embryos from germinating while still on the mother plants and maintains seeds in a dormant state when dispersed. There are numerous examples in which an absence of dormancy inception is caused by mutations leading to ABA deficiency or in insensitivity to ABA. For example, seeds of the ABA biosynthetic mutants of *Arabidopsis* (Léon-Kloosterziel *et al.*, 1996a) and tobacco (Marin *et al.*, 1996) fail to become dormant. In maize, ABA-biosynthetic mutants exhibit precocious germination or vivipary while still attached to the mother plant (McCarty, 1995). ABA content and germinability are normally correlated; high levels of ABA inhibit germination while the degradation of ABA in imbibed seeds is usually followed by initiation of germination (Feurtado *et al.*, 2004).

ABA-insensitive mutants of maize (*vp1*) and *Arabidopsis* (such as *abi3*) offer convincing evidence that when sensitivity to ABA changes, it drastically affects the germination behaviour of the mutant seeds. Seeds are non-dormant and can germinate at high concentrations of exogenously applied ABA, up to 300 μM; germination of wild-type seeds is inhibited at 1-2 μM of ABA (Nambara *et al.*, 1994). Because of an insensitivity to ABA, the *abi3-6* mutant also exhibits a deficiency in storage reserve deposition, producing wrinkled seeds that are severely desiccation intolerant (Nambara *et al.*, 1994).

ABA's role in dormancy maintenance and inhibition of germination is also evident in the seeds of yellow-cedar and western white pine. These seeds are deeply dormant at maturity; a prolonged warm and cold treatment is needed to break dormancy. Following a dormancy-breaking treatment, the ABA content of yellow-cedar embryos (but not of the megagametophytes) decreases by approximately 2-fold. In addition, the embryos exhibit a 10-fold lowered sensitivity to (+)-ABA (Schmitz *et al.*, 2000). Both the decline in ABA content and reduced embryo sensitivity to ABA is required to elicit dormancy breakage and high germinability. Furthermore, fluridone (an inhibitor of ABA biosynthesis) is able to relieve dormancy even without a warm/cold stratification treatment (Schmitz *et al.*, 2000, 2001, 2002). In western white pine seeds, effective dormancy breakage is accompanied by a significant decrease in ABA in both the embryo and megagametophyte. The decline of ABA correlates nicely with an increased germination capacity of seeds following their transfer to germination conditions (Feurtado *et al.*, 2004). These findings support the contention that the plant hormone

ABA is indeed a key regulator of the dormancy-to-germination transition in conifer seeds.

1.3 ABI3 is a central regulator of ABA signalling in seeds

ABA has long been associated with many changes in seeds, but how it regulates diverse physiological processes at the molecular level is largely unknown. Attempts to identify an ABA receptor have finally succeeded. For example, FCA, an RNA binding protein involved in flowering time control, appears to be an ABA receptor (Razem *et al.*, 2005). Considerable progress has been made in the characterization of ABA signalling elements and research toward elucidating the mechanisms of ABA action at the molecular level is getting more exciting.

The ABI3/VP1 transcription factor is a key regulator of ABA signal transduction pathways in seeds. *ABI3* stands for *AB*scisic *A*cid *I*nsensitive *3*; *VP1* (*Viviparous 1*) is the orthologue of ABI3 in maize. *VP1* was first cloned from maize by transposon tagging (McCarty *et al.*, 1989, 1991). Later the *ABI3* gene was isolated from *Arabidopsis* by positioning cloning (Giraudat *et al.*, 1992). Orthologues were soon identified in many other plants such as rice, *Phaseolus vulgaris*, wild oat, sorghum, wheat and poplar (Hattori *et al.*, 1994; Bobb *et al.*, 1995; Jones *et al.*, 1997; Chandler and Bartels, 1997; Shiota *et al.*, 1998; Rohde *et al.*, 1998, Fukuhara and Bohnert, 2000; Carrari *et al.*, 2001; Nakamura and Toyama, 2001). The first *ABI3* orthologue of gymnosperms, *CnABI3* (*Chamaecyparis nootkatensis ABI3*), was cloned by our lab from yellow-cedar, a conifer species that produces deeply dormant seeds (Lazarova *et al.*, 2002). In seeds, *ABI3/VP1* genes have multiple roles such as in reserve deposition, dormancy imposition, inhibition

of germination and acquisition of desiccation tolerance (reviewed in Giraudat *et al.* 1994; McCarty 1995; Bonetta and McCourt 1998; Koornneef *et al.* 1998, 2002; Rohde *et al.*, 2000; Kermode and Finch-Savage 2002; Finkelstein et al, 2002). They act as intermediaries in regulating ABA-responsive genes in seeds. More specifically, they mediate the action of ABA in promoting storage reserve (e.g. seed storage-protein) accumulation and synthesis of desiccation/stress protectants (e.g. LEAs, peroxiredoxin-like proteins and small heat shock proteins) (reviewed in Kermode and Finch-Savage, 2002; Finkelstein *et al.*, 2002; Kroj *et al.*, 2003). On the other hand, they repress gene expression, particularly that of post-germinative genes. Mutant seeds, in which *ABI3/VP1* genes are defective, show an altered or premature activation of post-germinative gene expression, such as genes encoding the chlorophyll a/b binding protein and isocitrate lyase in the *Arabidopsis abi3* mutant and genes encoding malate synthase and isocitrate lyase in the maize *vp1* mutant (McCarty, 1995; Paek et al, 1998; Nambara *et al.*, 1995; 2000). VP1 also inhibits the induction of α-amylase gene expression in aleurone layer cells of developing maize seeds (Hoecker *et al.*, 1995).

Severe *abi3* mutants such as *Arabidopsis abi3-6* produce wrinkled, green seeds because of reduced accumulation of storage proteins and an inability to degrade chlorophyll. These seeds are also severely desiccation intolerant, remain non-dormant and highly insensitive to exogenous ABA; in the *abi3-4* mutant, accumulation of various mRNAs of the maturation and late-embryogenesis development stages is greatly reduced (Ooms *et al.*, 1993; Nambara *et al.*, 1994, 1995; Parcy *et al.*, 1994).

ABI3/VP1 genes have great sequence variations at the DNA level among different species but all ABI3/VP1 factors have four conserved regions at the protein level. These

four domains, namely A1, B1, B2 and B3, probably define most of the functions of ABI3/VP1 proteins. A1 is considered as the transcriptional activation domain (McCarty *et al.*, 1991). B1 is essential for the physical interaction between ABI3 and ABI5 (Nakamura *et al.*, 2001). Recently, a second transcription activation domain was found within the B1 region (Monke *et al.*, 2004). The B2 domain is required for ABA-regulated gene expression by enhancing DNA binding at ABREs (ABA Responsive Elements) by diverse DNA binding proteins and is essential for activation of *Em-GUS* in rice cells (Hill *et al.*, 1996, Carson *et al.*, 1997). Genetic analysis of one of the *Arabidopsis* mutants showed that a point mutation within the B2 domain severely affected the accumulation of *Em, At2S1* and *At2S2* gene transcripts (Bies-Etheve *et al.*, 1999). A transactivation study using the *Brassica napus* napin promoter revealed that the interaction between B2 and B3 domains is necessary for transactivation in which a B2 mediates activation through ABRE, the B3 domain interacts with the RY/G-box complex (conserved element in many seed specific gene promoters). Deletion of the ABI3 B3 domain severely impairs the capacity of ABI3 to activate *napA* (Ezcurra *et al.*, 2000). In other instances, the B3 domain has also been confirmed to bind a RY motif to activate seed storage-protein gene promoters (Carranco *et al.*, 2004; Monke *et al.*, 2004). In maize, VP1 interacts with the Sph element of the *C1* promoter by direct binding through its B3 region (Suzuki *et al.*, 1997).

The ability of ABI3/VP1 to transactivate genes driven by seed specific promoters has been verified by a number of methods including transient expression assays, transgenic analysis and mRNA profiles in seeds of mutant plants. Transient expression assays have been routinely used to demonstrate the ability of ABI3/VP1 to activate target

gene expression (McCarty *et al.*, 1991; Hattori *et al.*, 1992, 1994; Bobb *et al.*, 1995; Vasil *et al.*, 1995; Hoecker *et al.*, 1999). My results in stably transformed tobacco plants show that ectopic expression of the conifer ABI3 protein strongly activates the expression of seed specific gene (napin and vicilin) promoters in tobacco leaves (Zeng *et al.*, 2003). Ectopic expression of *Arabidopsis ABI3* also activated the expression of seed-specific genes such as *At2S3* and *AtEm1* in vegetative tissues (Parcy *et al.*, 1997). Comparative analysis between wild type and the *Arabidopsis abi3-4* mutant for mRNA expression at different stages of seed development clearly indicated that *ABI3* participates in the regulation of several seed specific genes such as *At2S3, CRC, PAP10, AtEm6, AtEm1* and *RAB18* (Parcy *et al.*, 1994).

ABI3's role in the acquisition of desiccation tolerance is evident in transgenic carrot non-embryogenic cells expressing *C-ABI3*. Non-transgenic cells do not exhibit desiccation tolerance even after treatment with ABA (Shiota *et al.*, 1998). In the severe *Arabidopsis* mutant *abi3-6,* seeds are totally intolerant of desiccation (Nambara *et al.*, 1994).

ABI3/VP1 transcriptional factors interact with other proteins to realize their broad range of functions. About 15 proteins that interact with ABI3 or VP1 have been identified, all by using yeast two-hybrid assays (Hobo *et al.*, 1999; Jones *et al.*, 2000; Kurup *et al.*, 2000; Nakamura *et al.*, 2001; Lara *et al.*, 2003; Zeng and Kermode, unpublished data). For example, TRAB1, a basic region leucine zipper (bZIP) factor, exhibits bonafide interactions with both VP1 and ABA-responsive elements and participates in VP1's regulation of ABA-induced transcription (Hobo *et al.*, 1999). The ABI3 protein of *Arabidopsis* physically interacts with ABI5, also a bZIP factor and a

homologue of TRAB1 (55% similar) (Nakamura *et al.*, 2001). Four other transcription factors have been identified and show specific *in vivo* and *in vitro* interactions with ABI3 consisting of its B2 and B3 domains (Kurup *et al.*, 2000). At the same time, the same group also isolated three proteins that interact with *Avena fatua* (wild oat) VP1 (Jones *et al.*, 2000). Expression analysis of these interacting proteins in *abi3, lec1, fus3* mutant and wild type embryos led the authors to suggest that these proteins participate in the regulatory network that controls embryo development, dormancy and germination (Jones *et al.*, 2000; Kurup *et al.*, 2000). Recently, two other *Arabidopsis* bZIP proteins, AtbZIP10 and AtbZIP25, were found to interact with ABI3 and regulate seed storage-protein gene expression (Lara *et al.*, 2003). In this study, I have isolated three proteins that interact with yellow-cedar CnABI3 (Chapter 5). Another interesting finding is that GF14, a 14-3-3 protein, interacts with VP1 and EmBP1 and is a part of the VP1 regulatory complex in the Em promoter. GF14 might act as a structural linkage between these transcriptional factors (Schultz *et al.*, 1998).

The roles of *ABI3/VP1* in regulating expression of other genes have been studied extensively. However, it is not understood well how *ABI3* itself is regulated. Genetic analyses using mutants and transgenic plants show that *LEC1, LEC2* and *FUS3* act upstream of *ABI3* and positively induce *ABI3* gene expression (Parcy *et al.*, 1997; Kagaya *et al.*, 2005; Santos Mendoza *et al.*, 2005). On the other hand, *ABI1, ABI2* and *ERA1* negatively regulate *ABI3* expression (Brady *et al.*, 2003). Interestingly, the 5'UTR region of *ABI3* was found to regulate the *ABI3* promoter both quantitatively and spatially in a negative way (Ng *et al.*, 2004). Recently, an ABI3-interacting protein, AIP2, was found to have E3-ligase activity and to polyubiquitinate ABI3 in vitro. AIP2 negatively

regulates ABA signalling by targeting ABI3 for post-translational destruction through the ubiquitin - 26S proteasome pathway (Zhang *et al.*, 2005).

1.4 *ABI3* functions in concert with other factors of ABA signalling in seed development processes

Besides *ABI3*, many other factors of ABA-signalling pathways also play important roles in seeds and they work in concert with *ABI3* to regulate many aspects of seed development processes. Examples of these genes include *ABI1, ABI2, ABI4, ABI5, LEC1, LEC2, FUS3,* and *ERA1.*

ABI1 encodes a protein serine/threonine phosphatase 2C (PP2C). It was cloned independently by two groups at the same time from a dominant mutant *abi1-1* (Leung *et al.*, 1994; Meyer *et al.*, 1994). Recessive mutants of *abi1*, in which the *ABI1* gene contains a missense mutation, express proteins that lack any detectable PP2C activity. The plants show stronger sensitivity to ABA than the wild-type with respect to the inhibition of seed germination and seedling growth. They also have increased seed dormancy and drought adaptive response. These observations indicated that loss of ABI1 PP2C activity resulted in an enhanced responsiveness to ABA, which led the authors to suggest that wild-type ABI1 protein is a negative regulator of ABA signalling (Gosti *et al.*, 1999). This suggestion was also verified in maize protoplasts where constitutive overexpression of *ABI1* inhibited ABA action (Sheen, 1998). *ABI1* participates in various ABA-mediated responses such as maintenance of seed dormancy, inhibition of plant growth and stomatal closure (Leube *et al.*, 1998).

The second gene encoding a PP2C protein of ABA signalling, *ABI2*, was independently cloned by the same two groups, at the same time again, from the *abi2-1* mutant (Leung *et al.*, 1997; Rodriguez *et al.*, 1998). ABI2 shows 80% amino acid sequence identity to ABI1; and they are a pair of highly homologous PP2Cs regulating ABA signalling in *Arabidopsis*. Most of their functions are overlapping, although there are some differences in vegetative tissues in response to ABA and stresses under certain conditions (Vartanian *et al.*, 1994; Soderman *et al.*, 1996). Both *ABI1* and *ABI2* act upstream of *ABI3* and they genetically interact with each other to control ABA responses in plants (Parcy and Giraudat, 1997; Brady *et al.*, 2003).

ABI4 is a transcriptional factor and is a member of the APETALA2 (AP2) domain family (Finkelstein *et al.*, 1998). Mutants of *abi4* have pleiotropic defects in seed development and show lower sensitivity to ABA, salt and osmotic inhibition of germination. An *ABI4* gene was isolated from *Arabidopsis* by positional cloning (Finkelstein *et al.*, 1998). ABI4 protein is expressed throughout seed development, peaking at seed maturity. However, expression is not seed specific; low levels of *ABI4* transcripts are also present in vegetative tissues and the expression is not induced by ABA or stress treatments (Soderman *et al.*, 2000). Genetic interaction analyses among *ABI3, ABI4* and *ABI5* suggest that all the three genes function in a combinational network in the control of seed development (Soderman *et al.*, 2000).

ABI5 is another very important abscisic-acid responsive gene. It encodes a basic leucine zipper (bZIP) transcription factor and regulates a subset of late embryogenesis-abundant genes (e.g. *AtEm1, AtEm6* and *M17* of *Arabidopsis*) in both seeds and vegetative tissues. *ABI5* mutants show pleiotropic defects in ABA response and altered

expression of some ABA-responsive genes (Finkelstein and Lynch, 2000b). Similar to *ABI4, ABI5* is expressed in both seed and vegetative tissues but at much lower levels within the latter. Its expression appears to be controlled by ABA, by other ABA responsive genes such as *ABI3* and *ABI4* or even by itself (Finkelstein and Lynch, 2000b). Unlike *ABI3*, which is expressed at somewhat constant levels throughout the seed development stages, *ABI5* expression peaks at the later stages of embryogenesis. Lopez-Molina *et al.* (2002) reported that *ABI5* transcript levels correlated with an enhanced response to ABA during germination, seedling establishment and subsequent vegetative growth. ABA and *ABI5* are critical players at the post-germinative growth checkpoint. At a narrow developmental window after germination and before vegetative growth, germinated embryos are maintained in a quiescent state by ABI5 (through the interaction with ABI3), during which young seedlings monitor the environmental osmotic status to afford protection from drought (Lopez-Molina *et al.*, 2002). This inhibition of early growth is an adaptive trait to ensure that enough water is present in the environment before a complete transition to vegetative growth occurs. As mentioned before, *ABI3, ABI4* and *ABI5* interact genetically as a network in seed development stages; yeast two-hybrid assays also reveal that ABI3 and ABI5 physically interact with each other (Finkelstein and lynch, 2000b; Nakamura *et al.*, 2001).

Next to *ABI3, FUS3* and *LEC1* are two other key regulatory genes of ABA signalling in seeds. They function during both the early and late embryo development stages, affecting a wide range of ABA-regulated and ABA-independent processes. Their functions are broadly overlapping with *ABI3*, but not identical. Taken together, *ABI3, FUS3* and *LEC1* are the three most crucial regulatory factors of ABA signalling in the

developing seed and they act synergistically to control multiple aspects of seed maturation (Parcy *et al.*, 1997; Wobus and Weber, 1999; Nambara *et al.*, 2000; Raz *et al.*, 2001).

Mutation of the *fus3* gene alters embryo functions; for example, mutant seeds lack dormancy inception, do not acquire the ability to withstand desiccation and exhibit reduced storage protein levels. The mutant embryos have cotyledons with characteristics of leaf primordia and the green embryos directly enter a germinative program before seed desiccation begins (Keith *et al.*, 1994). *Lec1* mutants have similar phenotypes such as striking defects in embryonic maturation and production of viviparous embryos with leafy cotyledons. Immature embryos precociously enter germinative stages and acquire vegetative characteristics (Meinke *et al.*, 1994). These phenotypes clearly indicate that both *FUS3* and *LEC1* are essential regulators of embryogenesis and cotyledon identity (West *et al.*, 1994).

The *FUS3* gene was isolated by map-based cloning and it encodes a transcription factor with a continuous stretch of more than 100 amino acids having significant similarity to the B3 domain of the ABI3/VP1 proteins (Luerssen *et al.*, 1998). Mutant and expression analysis indicate that the B3 domain is essential for FUS3 function. The *LEC1* gene also encodes a transcription factor. Its mRNA is expressed both at early and late embryogenesis in embryo cell types and in endosperm tissue (Lotan *et al.*, 1998). Although *FUS3* and *LEC1* have similar functions in seed development, their genes are structurally unrelated. Later, *LEC2* was cloned from *Arabidopsis* and again, was found to encode a B3 domain transcription factor (Stone *et al.*, 2001), another central embryonic regulator that plays critical roles during both early and late embryo development. *LEC1*

and *LEC2* have similar, complementary and partially redundant functions in embryo development but they are not identical (Stone *et al.*, 2001). Interestingly, a recent study found that the induction of *LEC2* led to the accumulation of storage oil in leaves and triggered the accumulation of seed specific mRNAs. *LEC1, FUS3,* and *ABI3* were also induced by *LEC2* activation (Santos Mendoza *et al.*, 2005).

1.5 Does *ABI3* function outside of seeds?

ABI3/VP1 was initially considered as a seed specific transcription factor (McCarty *et al.*, 1991, Giraudat *et al.*, 1992); later findings revealed that it also functions in vegetative tissues and under certain stress conditions. For example, *ABI3* is expressed in the quiescent apex of *Arabidopsis* seedlings maintained in the dark (Rohde *et al.*, 1999). One of ABI3's targets, the *2S* seed storage-protein gene, was also induced in the arrested apex cells. In addition, the *ABI3* promoter also confers expression in other tissues such as the stipules and the abscission zones of siliques (Rohde *et al.*, 1999). Experiments with dark-grown *abi3* mutant plants indicate that *ABI3* plays a role in plastid differentiation in vegetative tissues and suggests that *ABI3* is at least partly regulated by light (Rhode *et al.*, 2000a). Ectopically expressed *ABI3* in vegetative tissues genetically interacts with *ABI1* and confers to plantlets the ability to accumulate seed specific *At2S3* and *AtEm1* mRNAs in response to ABA. Increased ABA inhibition of root growth is also exhibited by these ABI3-expressing plantlets (Parcy and Giraudat, 1997). Another example of ectopically expressed *ABI3* in *Arabidopsis* vegetative tissues showed increased expression of several ABA/cold/drought-responsive genes such as *RAB18* and *LTI78* and consequently enhanced freezing tolerance of plants in response to ABA and low temperature (Tamminen *et al.*, 2001). When *VP1* is constitutively expressed in an

Arabidopsis abi3 null mutant, an oligomicroarray analysis detected about 250 genes that are affected by both *VP1* and ABA in vegetative tissues (Suzuki *et al.*, 2003). *ABI3* represses the activity of the apical meristem and there is an altered outgrowth of axillary meristems in *abi3* mutant plants (Nambara *et al.*, 1995; Robinson and Hill, 1999). Another interesting result is that *ABI3* is induced by auxin and promotes lateral root development in *Arabidopsis* (Brady *et al.*, 2003). In poplar, *PtABI3* (the *ABI3* orthologue of poplar) is expressed in buds during natural bud set (Rohde *et al.*, 2002).

In western white pine seeds, I found a potential role for *ABI3* in stress sensing during early seedling growth. Exposure of the conifer seed to an oxidative stress leads to a transient re-synthesis of ABI3 protein during early growth and this is accompanied by synthesis of peroxiredoxin, an antioxidant under the control of *ABI3* (Zeng and Kermode, unpublished data). From yellow-cedar seeds, I identified three proteins that interact with the conifer ABI3 protein. The amino acid sequence of one of the interacting proteins shows high similarities to the *FRIGIDA (FRI)* gene family of *Arabidopsis*. *FRI* is a major regulator of flowering time. A role for *ABI3* in the regulation of flowering is of great significance because it extends the importance of this protein to the transition from vegetative growth to reproductive initiation.

Roles of *ABI3* at different stages and different tissues of plants suggest that *ABI3* has broader functions. It probably acts as a general regulator to decide cell fate, to safeguard and maintain the seed/plant at the current developmental stages and to prevent seeds, seedlings, and plants from entering the next stage prematurely.

1.6 Roles of other hormones in the regulation of germination and hormonal cross-talk in seeds

Besides ABA, other hormones such as gibberellins (GAs), ethylene, auxin and brassinosteroids have important roles in seed dormancy and germination processes. Among them, GA probably plays the most important role in promoting germination of seeds. GA is generally regarded as an antagonist of ABA in the control of both dormancy breakage and germination (Karssen and Lacka, 1986; Karssen, 1995). Application of GA together with fluridone to yellow-cedar seeds is able to release dormancy even without the normally required prolonged warm/cold treatments (Schmitz *et al.*, 2001).

Ethylene is another positive germination regulator. Ethylene signaling cascade inhibits ABA signaling in seeds and endogenous ethylene promotes seed germination by decreasing sensitivity to endogenous ABA (Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000). Increased ethylene synthesis in seeds is positively correlated with the capacity of germination (Petruzzelli *et al.*, 2000); and exogenously applied ethylene or ethylene precursor, ACC, promotes germination (Kepcyznski and Kepcyznska, 1997). In white spruce megagametophytes, ethylene increases during and after germination (He and Kermode, unpublished data).

Cross-talk between hormones is fundamentally important and is essential for most processes during plant and seed development. In maize, ABA-synthesis deficient mutants (e.g. *viviparous-5*) exhibit precocious germination while still on the mother plant; if GA deficiency is created in the same mutant, seeds are prevented from germinating and they exhibit desiccation tolerance and storage longevity (White *et al.*, 2000). This implies that ABA antagonizes a positive GA signalling for precocious germination and that the

ABA/GA ratio is more important than the actual amounts of the hormones. In cultured immature maize embryos, exogenous ABA induces quiescence and maturation; inhibition of GA synthesis mimickes the same effects as those induced by ABA. Thus, GA antagonizes ABA signalling in developing embryos and the GA/ABA balance controls the maturation phase (White and Rivin, 2000). In barley aleurone layers, expression of α-amylases is activated by GAMyb, a transcription factor induced by GA (Gubler *et al.*, 1995). GAMyb is suppressed by ABA through an ABA-induced protein kinase, PKABA1 (Gomez-Cadenas *et al.*, 2001; Anderberg and Walker-Simmons, 1992). In *Arabidopsis*, the mutants of ethylene response (*etr*) and ethylene insensitive (*ein2*) are also hypersensitive to ABA, and the ethylene constitutive triple response mutant (*ctr1*) was among the mutants selected as enhancers of the ABA-insensitive mutant *abi1-1* (Beaudoin *et al.*, 2000). These findings offer strong evidence of ABA-ethylene interactions. Another interesting example is that auxin-induced ethylene biosynthesis triggers ABA accumulation and the consequent growth inhibition (Hansen and Grossmann, 2000).

1.7 Objectives of my research

Seed dormancy and germination are critical stages in the life cycle of a plant. Understanding the molecular mechanisms underlying dormancy and germination is fundamentally important to basic research; it is also crucial for economically important species in forestry and agriculture. Forestry is the largest contributor to Canadian economy. Successful reforestation will ultimately depend on seed quality such as high viability, high germinability and the ability to germinate uniformly. Yellow-cedar and western white pine produce deeply dormant seeds and can serve as model species in the

study of dormancy-germination mechanisms. Therefore, the current research attempts to understand some mechanisms underlying seed dormancy and germination at the molecular level. My work focused on the functional analyses of yellow-cedar *CnABI3,* the orthologue of *ABI3/VP1* of angiosperms. Research was carried out in multiple biological systems including conifer species, tobacco, *Arabidopsis* and yeast cells.

My objectives are: (1) To understand the basic characteristics of *CnABI3*. (2) To explore its role in the maintenance of dormancy and inhibition of germination in conifer seeds. (3) To demonstrate its functions as a gene-expression activator. (4) To examine if the gymnosperm *CnABI3* functions in angiosperm species. (5) To identify proteins that physically interact with CnABI3. (6) To study CnABI3 functions in relation to the interactions between CnABI3 and its partners.

Chapter 2:
Cloning and Expression of an *ABI3* Gene Orthologue of Yellow-Cedar (*Chamaecyparis nootkatensis*)*

2.1 Introduction

ABI3/VP1 proteins are members of a large group of transcription factors that act as intermediaries in regulating abscisic acid (ABA)-responsive genes during seed development. Processes associated with the later stages of seed development appear to be controlled by ABI3/VP1 proteins; notably these include reserve deposition, dormancy imposition and the acquisition of a tolerance of seed tissues to desiccation (reviewed in Giraudat *et al.* 1994; McCarty 1995; Bonetta and McCourt 1998; Koornneef *et al.* 1998; Kermode and Finch-Savage 2002). They act as transcriptional activators to promote storage reserve (e.g. seed storage-protein) accumulation and synthesis of desiccation/stress protectants (e.g. LEAs, peroxiredoxin-like proteins and small heat shock proteins) (reviewed in Kermode and Finch-Savage, 2002; Finkelstein *et al.*, 2002; Kroj *et al.*, 2003). On the other hand, they repress gene expression, particularly that of post-germinative genes. Mutant seeds in which *ABI3/VP1* genes are defective, show an altered or premature activation of post-germinative gene expression, such as genes encoding the chlorophyll a/b binding protein and isocitrate lyase in the *Arabidopsis abi3*

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mutant and genes encoding malate synthase and isocitrate lyase in the maize *vp1* mutant (McCarty, 1995; Paek et al, 1998; Nambara *et al.*, 1995; 2000). Following the cloning of *VP1* from maize and *ABI3* from *Arabidopsis* (McCarty *et al.*, 1989, 1991; Giraudat *et al.*, 1992), orthologues of ABI3/VP1 were soon isolated from many other plants such as rice, *Phaseolus vulgaris*, wild oat, sorghum, wheat and poplar (Hattori *et al.*, 1994; Bobb *et al.*, 1995; Chandler and Bartels, 1997; Jones *et al.*, 1997; Rohde *et al.*, 1998; Shiota *et al.*, 1998; Fukuhara and Bohnert, 2000; Carrari *et al.*, 2001; Nakamura and Toyama, 2001).

Altered expression of *VP1* genes in sprouting sensitive cultivars of several cereals is suggestive of a role for VP1/ABI3 in germination control (Carrari *et al.*, 2001 and references therein). Following dispersal from the parent tree, seeds of yellow-cedar (*Chamaecyparis nootkatensis* D. Don Spach) are dormant and require several months of moist chilling before they will germinate (Pawuk, 1993). The dormancy mechanism of this conifer species is complex (Ren and Kermode, 1999, 2000; Xia and Kermode, 1999) and ABA has been implicated as a key regulator (Schmitz *et al.*, 2000, 2001). In order to investigate a possible role of ABI3 in the control of dormancy and germination of conifer seeds, the cloning of an *ABI3* gene orthologue from yellow-cedar was carried out by a former PDF, Dr. Galina Lazarova. A cDNA library was constructed using mRNA isolated from yellow-cedar seeds. PCR was used to amplify a partial *ABI3/VP1*-like gene fragment from this cDNA library using degenerate primers based on the sequences of highly conserved regions of ABI3/VP1 proteins from other species. A ^{32}P-labelled probe using this fragment was used for cDNA library screening. One of the 6 positive clones isolated, B11 (designated *CnABI3,* for *Chamaecyparis nootkatensis ABI3*) contained the entire coding region for an ABI3/VP1-like protein, as well as 516 bp of 5'-upstream

sequence and 300 bp of a 3'-flanking region (Lazarova *et al.*, 2002). Here I describe the characteristics of *CnABI3* and some aspects of its expression.

2.2 Materials and methods

2.2.1 Seed materials

Yellow-cedar seeds (clone 108) at mid-maturation were collected from the Mount Newton Seed Orchard (TimberWest, Saanichton, B.C., Canada). Embryos and megagametophytes were excised from the developing seeds, flash frozen in liquid nitrogen and stored at -80 °C prior to use. Some analyses were conducted on leaves and roots of seedlings. For these studies, mature seeds of seedlot 30156 (previously collected from natural stands by MacMillan Bloedel) were obtained from the Tree Seed Centre in Surrey, B.C., Canada. Dormancy of mature seeds was terminated as described in Ren and Kermode (1999); following transfer of seeds to germination conditions, leaves and roots were collected from 15- to 20-cm seedlings. These tissues were immediately ground to a fine power in liquid nitrogen and stored at -80 °C.

2.2.2 RNA extraction and northern blot analysis

Total RNA from yellow-cedar seed and from the leaf and root samples of seedlings was extracted according to the method of Wang *et al.* (2000). RNA samples (10 μg) were fractionated on 1.0 % agarose formaldehyde gels. Following transfer to a nylon membrane (Hybond-N; Amersham, Little Chalfont, Buckinghamshire, U.K.), RNA was fixed onto the membrane by UV-crosslinking. Membranes were hybridized with a ^{32}P-labeled probe consisting of a 2127-bp cDNA fragment. This fragment spanned 89% of

the *CnABI3* coding region and was derived by digesting *CnABI3* with *Eco*RI and *Xba*I. Membranes were briefly rinsed and then washed three times at 65 °C (30 min each) in a buffer containing 40 mM NaH_2PO_4 (pH 7.2) and 1% SDS. In order to verify equal amounts of RNA loading, blots were stripped and re-probed with a Sitka spruce 18S rRNA probe (401 bp).

2.2.3 DNA extraction and Southern blot analysis

Genomic DNA was extracted from leaves of seedlings according to the method of Dellaporta *et al.* (1983) with some modifications. DNA samples (10 µg) were digested overnight with either *Bam*HI, *Eco*RI or *Hind*III and the digests were separated on a 0.75% agarose gel. The separated DNA fragments were then depurinated, denatured and neutralized prior to DNA transfer to a nylon membrane (Hybond-N) according to the manufacturer's instructions (Amersham). Using the same probe as that used for northern blot analysis, the membrane was hybridized at 65 °C in a buffer containing 0.25 M NaH_2PO_4 (pH 7.2), 7% SDS (w/v), 1% BSA and 100 µg/ml denatured salmon sperm DNA. Following hybridization, membranes were washed using high stringency conditions (3 x 20 min in 20 mM NaH_2PO_4 [pH 7.2], 1% SDS and 2 x 20 min in 0.1X SSC, 0.1% SDS at 65 °C).

2.2.4 Protein extraction and western blot analysis

Proteins were extracted from yellow-cedar seeds and from the leaf and root samples of seedlings by grinding tissues in a ground glass homogenizer in chilled buffer (10 mM Tris-HCl, 200 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride [PMSF], 1 mM dithiothreitol, pH 7.5) on ice. Following centrifugation at 10,000 x g for

10 min, protein concentration of the supernatant was determined using the Bio-Rad Dc protein assay (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin fraction V (Bio-Rad) as a standard. Proteins (50 μg) were fractionated on 7.5% SDS-PAGE gels and then electroblotted onto nitrocellulose membranes. Following a brief rinse in PBST (phosphate buffered saline containing 0.05% Tween-20), membranes were blocked overnight with 5% skim milk power in PBST and then incubated with the primary antibody (anti-maize VP1 antiserum [McCarty *et al.*, 1991] diluted 1:2000 in PBST and 3% skim milk powder; kindly provided by Dr. Don McCarty, U Florida) for 2 h at room temperature. The blots were then washed three times in PBST (each 20 min) and incubated in goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). Following three washes (20 min each) in PBST, immunodetection was achieved using NBT and BCIP as substrates.

2.3 Results and discussion

2.3.1 CnABI3 shares several characteristics of other ABI3/VP1 orthologues

CnABI3 encodes an ABI3/VP1-like protein of 794 amino acids with a predicted molecular weight of 88 kDa and isoelectric point of 5.08. It is larger than any of the ABI3/VP1 factors of angiosperm species; for example, the proteins of *Phaseolus vulgaris, Arabidopsis thaliana, Zea mays* and *Daucus carota* are comprised of 752, 720, 691 and 663 amino acids respectively (Bobb *et al.*, 1995; Giraudat *et al.*, 1992; McCarty *et al.*, 1991; Shiota *et al.*, 1998). Like other orthologues, the *CnABI3* gene contains six exons and five introns, the latter having sizes of 105, 113, 110, approx. 1000 and 142 bp, respectively. The estimated size of intron 4 (1 kb) is considerably larger than that found

in other *ABI3/VP1* genes characterized so far, with most ranging between 100 and 200 bp. An exception is intron 3 of the *VP1* gene of *Oryza sativa* that is 449 bp in size. Comparison of the deduced amino acid sequence of CnABI3 to other ABI3/VP1 proteins by multiple-alignment (Fig. 2.1) indicates that the orthologue of yellow-cedar has all four regions that are typically conserved. These include the three highly conserved basic regions: B1 (aa 261 - 327), B2 (aa 459 - 490) and B3 (aa 533 - 651). The core of B2, RKKR, is cited by several authors as a putative nuclear targeting motif and is invariable in all angiosperm orthologues characterized so far. In the yellow-cedar protein, this sequence of the B2 region is RKNR. However, a putative nuclear targeting motif (RKRK) is found in CnABI3 at position 325-328, within the B1 region. In the B3 region, the sequence identity is over 90% within a stretch of 119 amino acids. There is a substitution of glycine (which is conserved in all other orthologues) with valine at position 593. The N-terminal region contains the acidic domain (A1) that plays an important role in the function of these proteins as transcription activators; this region shares a much lower degree of homology, which is typical of other ABI3/VP1 proteins (Fig. 2.1).

2.3.2 A possible gene family for CnABI3 in yellow-cedar?

Southern blot hybridization conducted under high stringency conditions exhibited multiple bands of varying intensities for DNA samples digested with *Bam*HI, *Eco*RI or *Hind*III (Fig. 2.2A). Two *Bam*HI sites occur at 1222 and 1236 bp in B11 (Fig. 2.2B); if CnABI3 is encoded by a single gene, there should be two bands only, instead of the three detected (Fig. 2.2C, predicted numbers of bands on Southern blot, if *CnABI3* is a single copy gene). Similarly, *Eco*RI digestion should yield a single band (due to a site at 687 bp

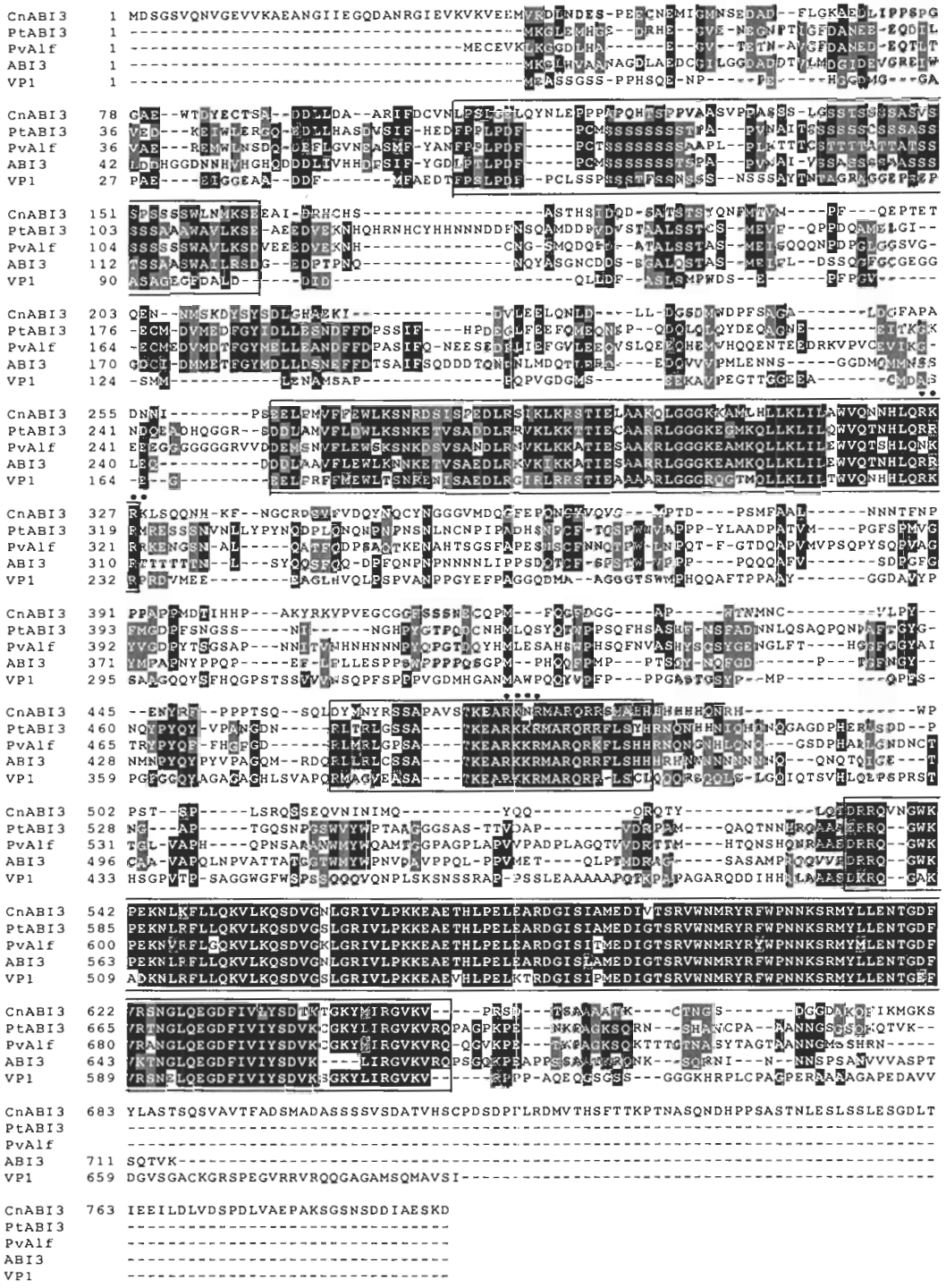
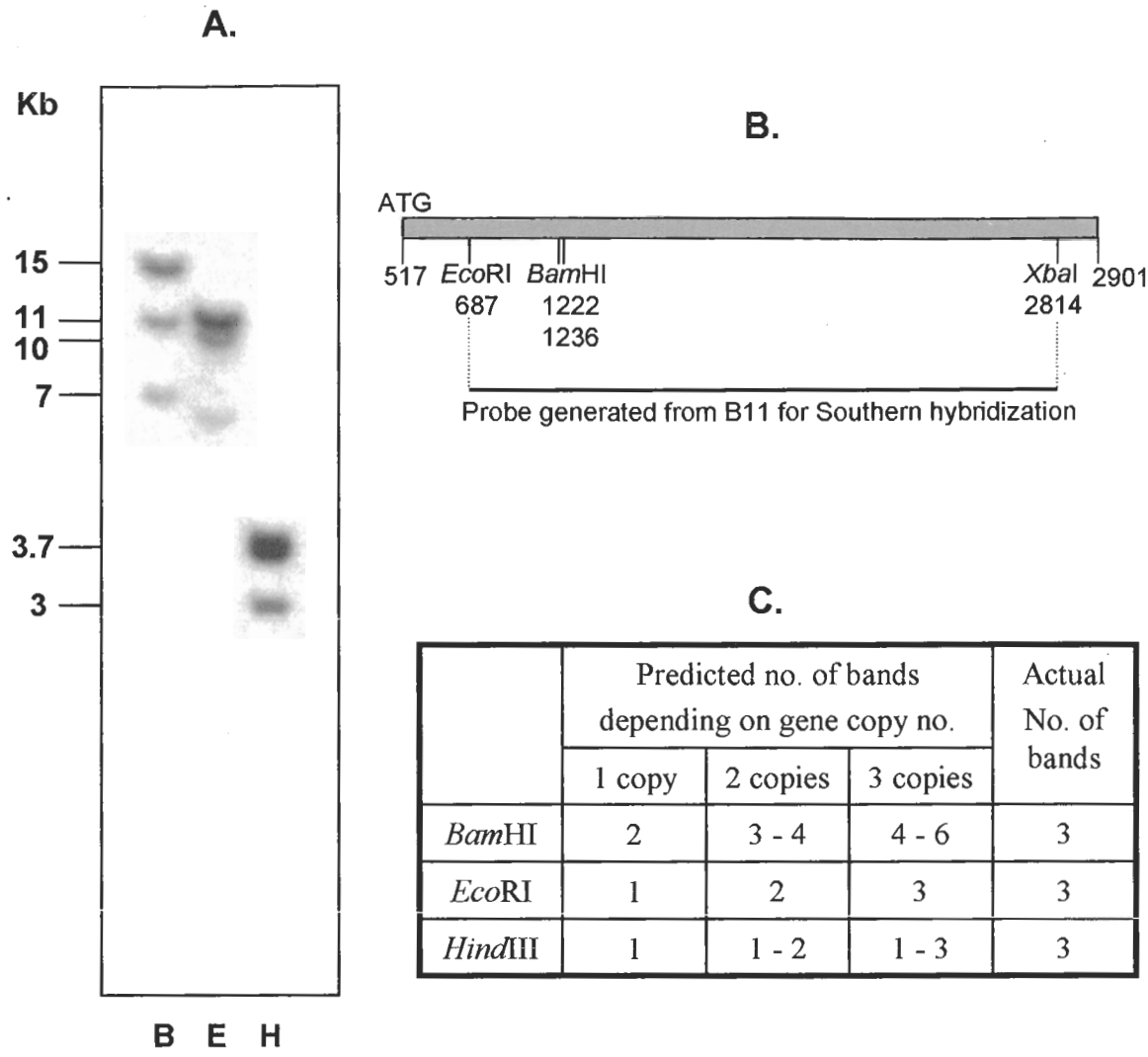


Figure 2.1. Alignment of the deduced amino acid sequence of *Chamaecyparis nootkatensis* ABI3 protein (CnABI3) with that of other VP1/ABI3 proteins (CnABI3 [aj131113]; PtABI3, *Populus trichocarpa* ABI3 [aj003165]; PvAlf, *Phaseolus vulgaris* ABI3-like factor [u28645]; ABI3, *Arabidopsis thaliana* ABI3 [x68141]; VP1, *Zea mays* VP1 [m60214]). The four darkly shaded boxes correspond to the previously described regions of highest sequence homology: the acidic A1 region and the three basic regions, B1, B2 and B3. The putative nuclear targeting signals (RKKR located in the B2 region, and RKRK of CnABI3, located in the B1 region) are indicated by the solid dots above the sequence.



C.

	Predicted no. of bands depending on gene copy no.			Actual No. of bands
	1 copy	2 copies	3 copies	
*Bam*HI	2	3 - 4	4 - 6	3
*Eco*RI	1	2	3	3
*Hind*III	1	1 - 2	1 - 3	3

Figure 2.2. **A.** Southern blot hybridization of yellow-cedar genomic DNA. The ^{32}P-labelled cDNA probe was prepared from clone B11 by *Eco*RI and *Xba*I digestion which released a 2127 bp fragment containing 89% of the *CnABI3* gene coding region. Genomic DNA was digested with *Bam*HI (B), *Eco*RI (E) and *Hind*III (H). **B.** Probe generated from B11 that was used for the Southern blot hybridization showing location of *Bam*HI and *Eco*RI sites. **C.** Predicted numbers of bands on Southern blot, if the *CnABI3* gene of yellow-cedar is present as a single copy or as multiple copies of a single gene. No *Bam*HI, *Eco*RI and *Hind*III sites are present in the introns of the *CnABI3* gene.

in B11). There is no *Hind*III site, therefore only one band is expected instead of the two (or possibly three) that are evident. Figure 2.2C also shows predicted numbers of bands that would be consistent with a gene copy number of two and three copies. Sequence analysis revealed no *Bam*HI, *Eco*RI or *Hind*III sites within the introns of the *CnABI3* gene; the numbers of fragments on the Southern blot are inconsistent with a single gene or multiple gene copies (2 or 3 copies). Although inconclusive, the results are indicative of a gene family for *CnABI3* in this coniferous species, or perhaps some other gene(s) with high homology to *CnABI3* (e.g. *FUSCA3*; Luerssen *et al.*, 1998). This is in contrast to the orthologues of angiosperms, in which ABI3/VP1 is encoded by a single gene. A possible exception may be the orthologue of *Phaseolus vulgaris*, in which an additional band is revealed on Southern blots, although only under lower stringency conditions (Bobb *et al.*, 1995).

2.3.3 CnABI3 of yellow-cedar exhibits seed-specific expression

Under normal growth conditions, expression of the *CnABI3* gene occurs exclusively in seeds of yellow-cedar, with no detectable mRNA (Fig. 2.3) in leaves and roots of young seedlings. Expression in seeds is not confined to mid-maturation (Figs. 2.3), but is also detected in both the embryo and megagametophyte of the mature dry (dormant) seed (Zeng *et al.*, 2003). The role of CnABI3 in maintaining dormancy of yellow-cedar seeds has been examined (Chapter 3).

Synthesis of the CnABI3 protein also occurred only in seeds; no protein was detected in leaves and roots of young seedlings (Fig. 2.4). Western blot analysis using anti-maize VP1 antiserum (McCarty *et al.*, 1991) indicates an apparent size of ~94 kDa,

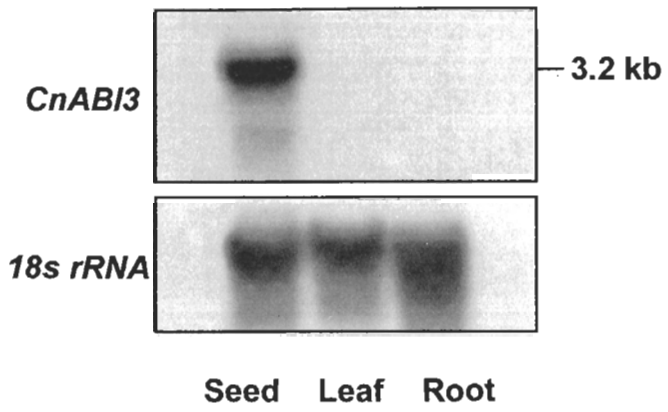


Figure 2.3. Northern blot analysis of total RNA isolated from yellow-cedar seeds (at mid-maturation), and leaves and roots of young seedlings. Ten μg of RNA was loaded on each lane and the ^{32}P-labelled probe was the same as that in the Southern blot hybridization. Ribosomal RNA (18S) was also probed after stripping the same membrane to verify equal amounts of RNA loading.

in contrast to the predicted size of 88 kDa. This is not unexpected since the composition of amino acids of the protein can affect its migration in SDS-PAGE gels, as has been reported for other orthologues (e.g. VP1 of *Zea mays* exhibits an apparent mass of 94 kDa on SDS-PAGE gels, versus its predicted mass of 73.3 kDa; McCarty *et al.*, 1991; ABI3 of *Arabidopsis* exhibits an apparent mass of 116 kDa on SDS-PAGE gels, while its predicted mass is 79.5 kDa; Giraudat *et al.*, 1992). Interestingly, like VP1 of maize, other immunoreactive proteins that have lower molecular masses are detected on the western blot (Fig. 2.4); these occur despite the inclusion of protease inhibitors in the extraction buffer. These lower bands may be products of ABI3 degradation. AIP2, an ABI3-interacting protein from *Arabidopsis*, was recently found to have E3 ligase activity and negatively regulates ABI3 by polyubiquitinating ABI3 for post-translational destruction through the 26S proteasome pathway (Zhang *et al.*, 2005).

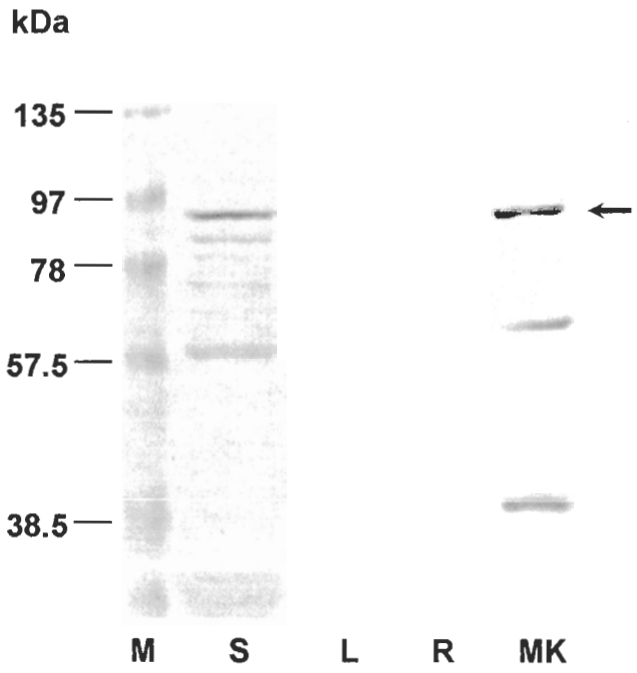


Figure 2.4. Western blot analysis using anti-maize VP1 antiserum to detect the CnABI3 protein in protein extracts of developing yellow-cedar seeds and vegetative tissues of young yellow-cedar seedlings. **M**, prestained molecular mass standards; **S**, developing yellow-cedar seed (isolated from first-year cones); **L** and **R**, leaves and roots (respectively) of young yellow-cedar seedlings; **MK**, maize kernels (positive control). Arrow on the right indicates CnABI3 or VP1 proteins. Numbers on the left indicate the approximate molecular masses (kDa) of the prestained standards.

Chapter 3:
Role of CnABI3 in Dormancy Maintenance of Yellow-Cedar Seeds and in the Activation of Storage Protein Gene Promoters*

3.1 Introduction

ABI3 is a key regulator of seed development. Seeds of severe *abi3* mutants accumulate reduced amounts of storage proteins, fail to degrade chlorophyll, remain non-dormant and display an impaired capacity to express various mRNAs regulated by different temporal programmes during seed development (Ooms *et al.*, 1993; Parcy *et al.*, 1994). ABI3 does not act in isolation to control gene expression central to seed maturation programmes, but rather acts in concert with various other transcription factors such as LEC1, LEC2 and FUS3, which may also control the abundance of the ABI3 protein (Parcy *et al.*, 1997; Harada, 2001; Stone *et al.*, 2001; Santos Mendoza *et al.*, 2005). During the later stages of seed development in an *abi3 fus3* double mutant of *Arabidopsis thaliana*, there is a derepression or precocious activation of genes not normally expressed until the commencement of germination (Nambara *et al.* 2000). Thus, ABI3 acts as a positive regulator of the late embryogenesis processes and simultaneously as a negative regulator of germination programs. The abilities of ABI3 orthologues to transactivate the expression of developmental genes in seeds or embryos have largely

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been demonstrated in different systems by transient gene expression assays; in addition, ectopically expressed ABI3 protein (effected by stable transformation of *Arabidopsis* with a chimeric *35S-ABI3* gene) leads to the re-activated expression of seed-specific genes in vegetative tissues and seedlings (Parcy and Giraudat, 1997).

A number of studies discuss the putative role of the *VP1/ABI3* gene product as a regulator of dormancy in mature seed (Jones *et al.*, 1997; Fukuhara *et al.*, 1999; Carrari *et al.*, 2001; Nakamura and Toyama, 2001). For example, in *Avena fatua*, expression of the *VP1* gene is correlated with the degree of embryo dormancy and may be important for maintaining ABA-controlled metabolism in the imbibed seed (Jones *et al.* 1997). In ice plants, the amount of VP1 mRNA is lower in early- vs. late-germinating seeds that display different degrees of dormancy (Fukuhara and Bohnert, 2000). Expression of the VP1 gene in mature embryos of dormant and non-dormant wheat cultivars is also positively correlated with the level of seed dormancy and embryo sensitivity to ABA (Nakamura and Toyama, 2001).

The dormancy mechanism of yellow-cedar is complex; it is primarily but not exclusively coat-imposed (Ren and Kermode, 1999). Abscisic acid (ABA) plays a major role in maintaining the seed in a dormant state (Schmitz *et al.*, 2000, 2001, 2002). For example, dormancy termination of yellow-cedar seeds is accompanied by a reduced responsiveness of the embryo to ABA as well as changes in the embryo's metabolism of ABA, in which 8' hydroxylation becomes rate limiting. In addition, the megagametophyte plays a role as a mechanical barrier to prevent radicle protrusion, a factor which may also involve regulation by ABA and other hormones such as gibberellins (through regulation of cell wall rigidity) (Ren and Kermode, 1999, 2000).

Western white pine seeds are also deeply dormant at maturity and a 110-d warm and cold treatment is needed to break seed dormancy efficiently. Dormancy termination of western white pine seeds is associated with changes in ABA metabolism; in embryo and megagametophyte, ABA levels decrease significantly during moist chilling, coincident with an increase in the germination capacity of seeds (Feurtado *et al.*, 2004).

In this chapter, I investigated the role of CnABI3 in dormancy maintenance of yellow-cedar seeds by examining the expression of the *CnABI3* gene at the mRNA and protein levels before, during and after dormancy termination. Expression of the ABI3 protein of western white pine was also examined in seeds at various stages. A potential role of ABI3 in response to oxidative stress was revealed during early seedling growth. In addition, transactivation studies were conducted with the *CnABI3* gene to elucidate the specific role of this transcription factor as a regulator of other key developmental genes including those encodng napin and vicilin. The synergistic effects of ABA and CnABI3 on transactivation were determined using stable gene expression in tobacco plants.

3.2 Materials and methods

3.2.1 Seed materials and dormancy-breaking and control treatments

Mature yellow-cedar seeds of seed lot 30156 (previously collected from natural stands by MacMillan Bloedel and obtained from the Tree Seed Centre in Surrey, B.C., Canada) were used. For dormancy termination, seeds were subjected to a 72-h running water imbibition (22 + 1 °C), incubated in near darkness at 25 °C for 4 weeks (warm moist treatment) and then moist chilled at 4 °C for 8 weeks (Ren and Kermode, 1999). After the 87-d dormancy-breaking treatment, seeds were placed in germination

conditions (30 °C days, 20 °C nights with an 8-h photoperiod). A control treatment was carried out in which seeds were subjected to the 3-d soak and a 4-week warm, moist period; however the subsequent 8 weeks of moist chilling was substituted with an equivalent period in warm, moist conditions (25 °C). No germination of the whole seed is elicited by this control treatment (Ren and Kermode, 2000). Megagametophytes and embryos were excised from yellow-cedar seeds at different stages during the dormancy-breaking and control treatments, flash frozen in liquid N_2 and stored at -80 °C prior to northern and western blot analyses.

3.2.2 Western white pine seed materials and dormancy-breaking treatments

Mature seeds (seed lot 08006) of western white pine (*Pinus monticola* Dougl. Ex D. Don) were obtained from the British Columbia Ministry of Forests, Tree Seed Center in Surrey, BC, Canada. To break dormancy, seeds were subjected to a 12-day water soak at room temperature followed by a 98 days of moist chilling at 4 °C in the dark (Feurtado *et al.*, 2004). After sterilization with 3% H_2O_2 (v/v) for 30 min and several rinses in sterilized water, seeds were transferred to germination conditions (25 °C, 8-h photoperiod, 15 °C 16-h dark). Embryos and megagametophytes were excised from western white pine seeds at different stages during the dormancy-breaking treatments and germination/growth, flash frozen in liquid N_2 and stored at -80 °C prior to use.

3.2.3 RNA extraction from yellow-cedar seeds and northern blot analysis

Total RNA was extracted from embryos or megagametophytes according to the method of Wang *et al.* (2000). RNA samples (10 μg) were fractionated on 1.0 % agarose

formaldehyde gels and northern blot analysis was carried out using methods as described in 2.2.2 of Chapter 2.

3.2.4 Protein extraction and western blot analysis

Proteins were extracted from yellow-cedar and western white pine embryos/megagametophytes or transgenic tobacco leaves. Methods used for protein extraction, protein concentration determination and western blot analysis were the same as described in 2.2.4 of Chapter 2. Anti-maize VP1 antiserum (1:2000 dilution) was used to detect ABI3 proteins. Anti-AtPER1 antibodies (1:1000 dilution) (kindly supplied by Dr. Reidunn Aalen, U Oslo, Norway) were also used to detect PER1 proteins in western white pine samples. All blots were repeated at least once with samples at the same stages.

3.2.5 Stable transformation of tobacco

The *CnABI3* gene coding region was expressed under the control of a Cauliflower Mosaic Virus 35S 5' upstream region in which the enhancer element was duplicated (db35S), along with a 5' untranslated region from the alfafa mosaic virus RNA 4 (AMV) (kindly supplied by Dr. Raj Datla, Plant Biotechnology Institute [Datla *et al.*, 1993]). The 3' downstream region was derived from the nopaline synthase (*Nos)* gene. The *db35S-AMV-CnABI3-Nos* fragment was cloned into the binary vector pCambia using *Hind*III and *Eco*RI sites and the resultant construct was transferred into Agrobacterium strain LBA4404 by electroporation in order to carry out stable transformation in tobacco plants.

Stable transformation of tobacco was carried out using the leaf disc method (Higgins *et al.*, 1988; Jiang *et al.*, 1995). Two tobacco lines were used for stable

transformation; these lines had previously been transformed with one of two gene constructs containing promoters of storage protein genes - napin or vicilin - linked to the *GUS* gene coding region (*Nap-GUS-Nos* or *Vic-GUS-Nos*) (Jiang *et al.*, 1995). Positive transformants were screened by resistance of plantlets to both kanamycin and hygromycin and were further verified by Southern blot hybridization.

3.2.6 RT-PCR to detect CnABI3 mRNA in transgenic tobacco leaves

Total RNA was extracted from 50-80 mg of transgenic tobacco leaf tissue using the Qiagen RNeasy Plant Mini Kit (Qiagen Inc., Mississauga, ON, Canada) and from developing yellow-cedar seeds according to the method of Wang *et al.* (2000). Two primers used for RT-PCR were: 5'-ATCTGCAAACAGATCGTCGT-3' (fwd) and 5'-CGAGTCCACCAAGTCTAGAA-3' (rev), with a predicted 731-bp product. Qiagen's One Step RT-PCR kit was used and reactions were carried out according to the kit's instructions using 1 μg total RNA for each reaction. The thermal cycler conditions were 30 min at 50 °C for reverse transcription followed by 15 min at 95 °C for the initial PCR activation, 33 cycles for PCR amplification (50 sec at 94 °C, 50 sec at 56 °C and 1 min at 72 °C) and a 10 min 72 °C final extension. PCR products were separated on a 1.0 % agarose gel. A control PCR reaction was carried out without the reverse transcription step to ensure that RNA samples were free of contaminating DNA.

3.2.7 Fluorometric and histochemical GUS assays following stable gene expression

GUS fluorometric assays were carried out on transgenic tobacco leaves by the method of Jefferson (1987) using MUG (4-methyl umbelliferyl β-D-glucuronide) as a substrate (Sigma Chemical Co., St. Louis, MO, USA). Tissue samples were ground on

ice in GUS extraction buffer (50 mM $NaPO_4$, 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine and 0.1% Triton X-100). Following centrifugation (10,000 x g for 10 min at 4 °C), GUS activities of the supernatants were determined. Reactions were carried out at 37 °C. Aliquots were removed at regular time intervals and following the addition of 0.2 M Na_2CO_3 to terminate the reaction, fluorescence of the reaction mixture was determined with a Hoefer TKO 100 Fluorometer (Hoefer Scientific Instruments, San Francisco, CA). GUS activities were expressed as pmol MU/min/mg protein.

GUS histochemical assays were also conducted to monitor GUS activities in transgenic tobacco plants. The method used was as described in Jefferson (1987) and 5-bromo-4-chloro-3-indoyl-ß-D-glucuronic acid cyclohexylammonium salt (x-gluc; Diagnostic Chemicals Ltd, Charlottetown, PEI) was used as the substrate.

3.3 Results

3.3.1 Generation of transgenic tobacco plants and expression of CnABI3 mRNA and protein

Stable transformation of tobacco was undertaken to determine the ability of the CnABI3 protein to transactivate seed storage-protein gene expression. Two tobacco lines were used for stable transformation using the *db35S-AMV-CnABI3-Nos* construct; these lines had previously been transformed with one of two gene constructs containing promoters of storage protein genes - napin or vicilin - linked to the *GUS* gene coding region (*Nap-GUS-Nos* or *Vic-GUS-Nos*) (Jiang *et al.*, 1995). Positive transformants were

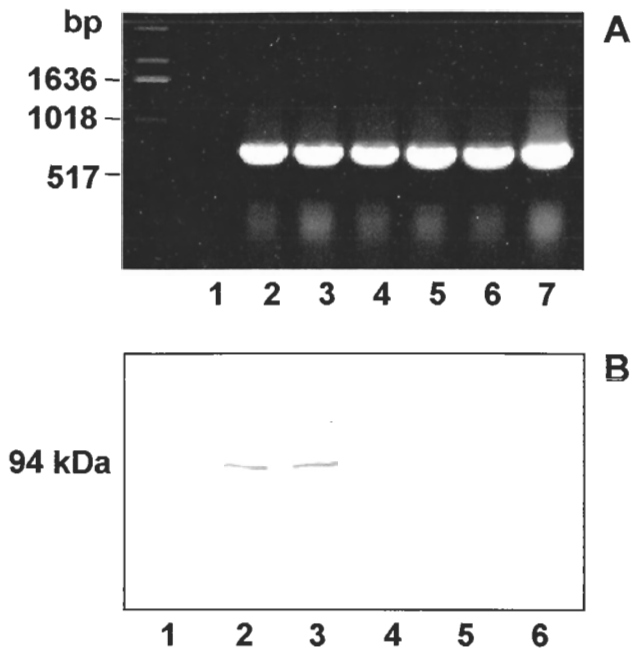

Figure 3.1. **A.** RT-PCR to detect *CnABI3* mRNA in transgenic tobacco leaves with total RNA as a template for reverse transcription. **B.** Western blot analysis with anti-maize VP1 antiserum to detect CnABI3 protein in transgenic tobacco plant leaves. Lane **1**, Control transgenic leaves without *CnABI3* gene; Lanes **2 – 6**, leaves of independent transgenic lines cotransformed with *db35S-AMV-CnABI3-Nos* and *Nap-GUS-Nos* (Lanes 2-4) or *db35S-AMV-CnABI3-Nos* and *Vic-GUS-Nos* (Lanes 5 and 6). Lane 7 (A only), Developing seeds of yellow-cedar at mid-maturation isolated from first-year cones.

screened (2-3 rounds) by resistance of plantlets to both kanamycin and hygromycin and were further verified by Southern blot hybridization (data not shown). In total, 67 positive independent transformants were obtained for further analysis; 42 plants were expressing *db35S-AMV-CnABI3-Nos* with *Nap-GUS-Nos* and 25 plants were expressing *db35S-AMV-CnABI3-Nos* with *Vic-GUS-Nos*.

Expression of the *CnABI3* gene in transgenic tobacco plants was detected at the mRNA level by RT-PCR (Fig. 3.1A). Total RNA was used as the template for reverse transcription. Two primers were designed based on the cDNA sequence of the *CnABI3* gene; a PCR product of 731 bp was predicted. All transgenic plants tested produced *CnABI3* mRNA, and the product generated was the same as that in yellow-cedar developing seeds (Fig. 3.1A). The CnABI3 protein was detected in the transgenic plants by western blot analysis using anti-maize VP1 antiserum (Fig. 3.1B).

3.3.2 Ectopic expression of CnABI3 activates the expression of chimeric genes driven by napin and vicilin seed storage-protein gene promoters

GUS activities in transgenic tobacco leaves (Fig. 3.2) showed that the ectopic expression of the CnABI3 protein strongly activated both the vicilin and napin storage-protein gene promoters. In both cases, GUS activities were substantially higher than those of control plants that had not been transformed with the *CnABI3* construct. The highest GUS activity in plants cotransformed with *db35S-AMV-CnABI3-Nos* and *Vic-GUS-Nos* was more than 1000-fold of that in the control (Fig. 3.2 B). GUS histochemical assays showed expression of GUS driven by the vicilin and napin promoters in co-transformed tobacco leaves (Fig. 3.3), as well as in stems and roots (data not shown).

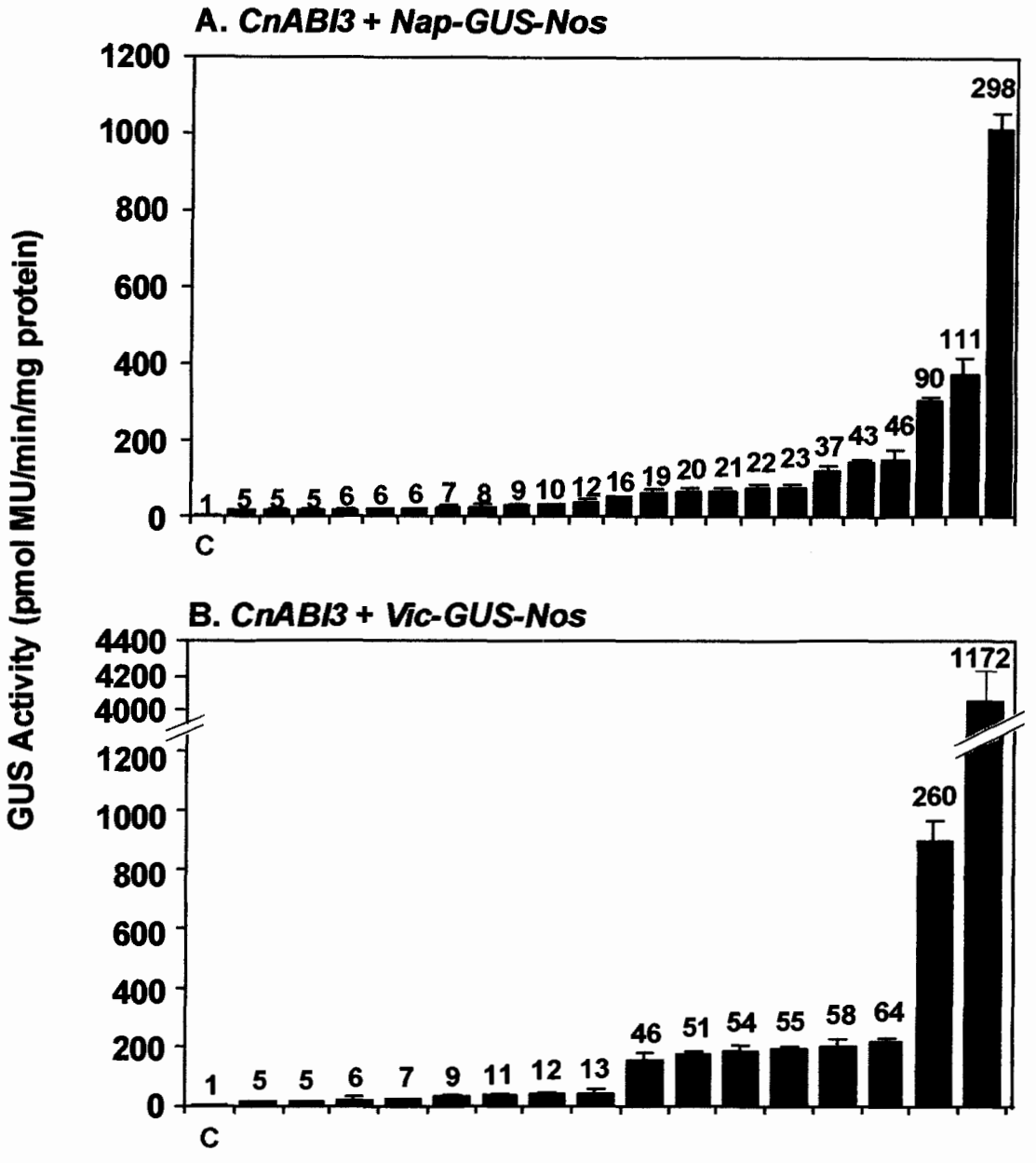

Figure 3.2. GUS activities in leaves of tobacco plants co-transformed with the 35S-driven *CnABI3* gene and the chimeric genes containing storage-protein gene promoters. **A.** Leaves of tobacco plants co-transformed with *db35S-AMV-CnABI3-Nos* and *Nap-GUS-Nos* genes; **B.** Leaves of tobacco plants co-transformed with *db35S-AMV-CnABI3-Nos* and *Vic-GUS-Nos* genes. *C* indicates control plants transformed with *Nap-GUS-Nos* or *Vic-GUS-Nos* genes alone (i.e. without the co-expressed *CnABI3* gene). GUS activities were determined by the fluorometric assay. Each bar represents an independent transformant. Each number on the bar indicates the fold increase in GUS activity compared to the controls (*C*).

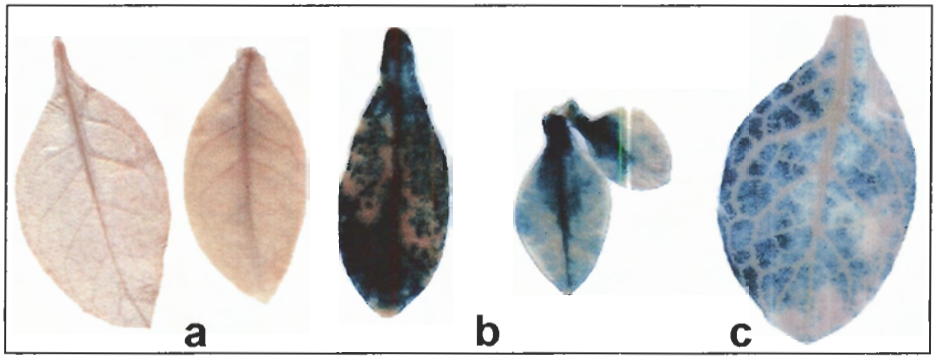

Figure 3.3. Histochemical assay showing GUS activities in leaves of transgenic tobacco plants co-transformed with the *35S*-driven *CnABI3* gene and the chimeric genes containing storage-protein gene promoters. **a**, Control transgenic leaves without the *CnABI3* gene; **b**, leaf co-transformed with *db35S-AMV-CnABI3-Nos* and *Nap-GUS-Nos* genes; **c**, leaf co-transformed with *db35S-AMV-CnABI3-Nos* and *Vic-GUS-Nos* genes. After x-gluc staining (12 h), leaves were treated with ethanol to remove chlorophyll.

3.3.3 ABA and CnABI3 act synergistically to activate gene expression

To examine the effects of ABA on GUS activities of the cotransformed plants, transgenic tobacco leaves were cut into halves along the midrib, one half-leaf was placed in liquid Murashige and Skoog (MS) medium containing 20 μM *S*-(+)-ABA (kindly supplied by Dr. S. Abrams, PBI, SK) and the other was placed in liquid MS medium with no ABA. As compared to the transgenic leaves placed in MS medium without hormone, the GUS activities of leaves treated with *S*-(+)-ABA were increased by up to more than 60-fold (Fig. 3.4). Thus, the ability of CnABI3 to transactivate gene expression was enhanced by ABA, similar to results found with other ABI3/VP1 proteins of angiosperms. The unnatural enantiomer of ABA, *R*-(-)-ABA, was ineffective in its ability to enhance GUS reporter activity (data not shown).

3.3.4 Dormancy breakage of yellow-cedar seeds elicits a decline in CnABI3 mRNA upon subsequent germination

Expression of the *CnABI3* gene occurs in developing seeds of yellow-cedar (Chapter 2). Transcripts were also detected in the embryo and megagametophyte of mature dormant seeds given a 3-d soak. Approximately the same steady-state level of *CnABI3* transcripts was detected in seeds subjected to a full dormancy-breaking treatment; however, transcripts declined markedly in both the embryo and the megagametophyte upon transfer of seeds to germination conditions, particularly in embryos (Fig. 3.5 A and B). (In embryos, a major decline in transcripts occurred after just 2 d in germination conditions; data not shown). In contrast, in mature seeds subjected to a control treatment (12 weeks in warm moist conditions, a treatment ineffective in

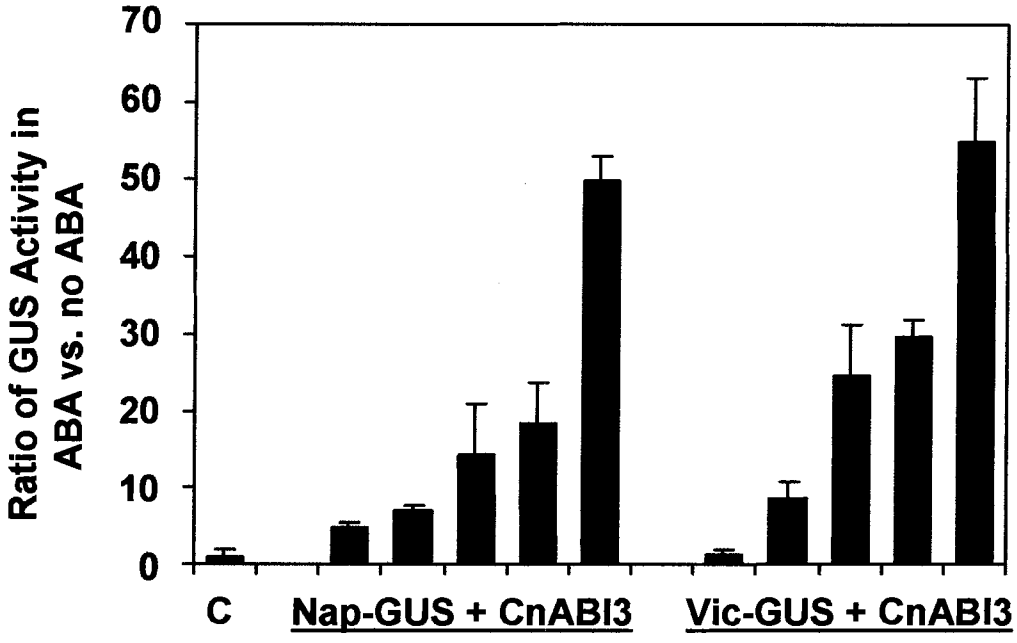

Figure 3.4. Effect of ABA on GUS activities of leaves expressing the *35S*-driven *CnABI3* gene and the chimeric genes containing storage-protein gene promoters. Transgenic tobacco leaves were halved at the midrib and each half was incubated either in liquid MS medium containing 20 μM *S*-(+)-ABA or in liquid MS alone for 5 d under low light, after which the GUS activities were measured by the fluorometric assay. Each bar represents an independent cotransformed line, except C which is a leaf from a control transgenic plant without the *CnABI3* gene.

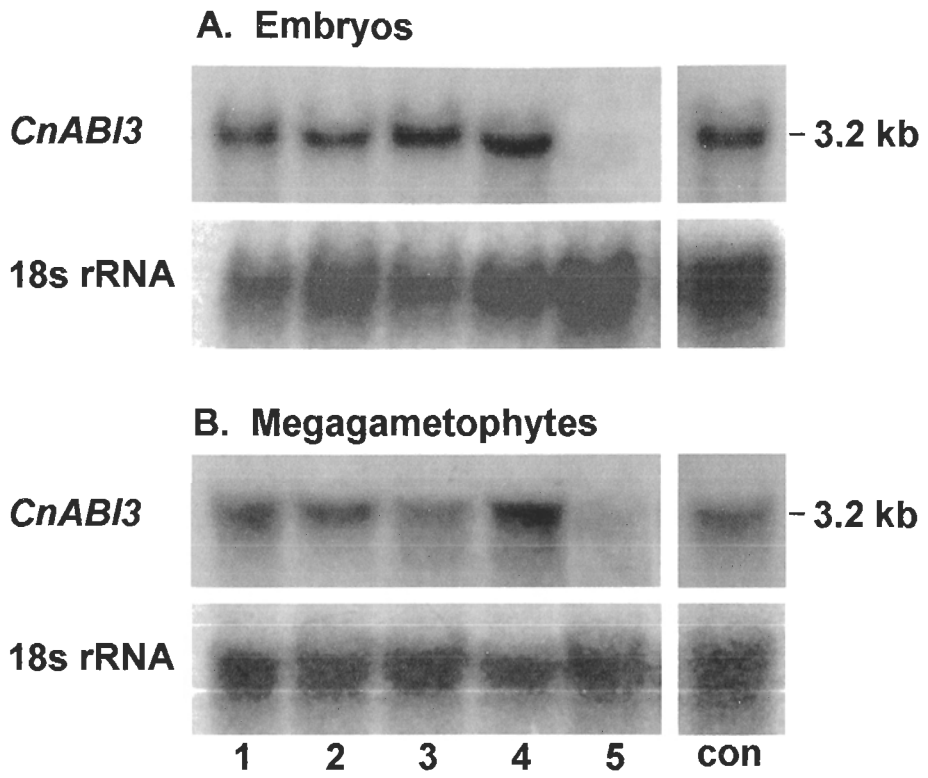

Figure 3.5. Northern blot analysis to detect *CnABI3* transcripts within the embryo (**A**) and megagametophyte (**B**) of yellow-cedar seeds during different stages of dormancy-breaking and control treatments. Ten µg total RNA was loaded in each lane. **1**, Seeds subjected to a 3-d soak; **2**, Seeds subjected to a 3-d soak and four weeks of warm moist conditions; **3**, Seeds subjected to a 3-d soak, 4 weeks of warm moist conditions and 2 weeks of moist chilling; **4**, Seeds subjected to a 3-d soak, 4 weeks of warm moist conditions and 8 weeks of moist chilling (full dormancy-breaking treatment); **5**, Seeds subjected to the full dormancy-breaking treatment followed by 8 d in germination conditions; **Con**, Seeds subjected to a 3-d soak and 12 weeks of warm moist conditions followed by 8 d in germination conditions (control). The same membranes were also probed with 18S rRNA to verify equal amounts of RNA loading.

eliciting dormancy termination), *CnABI3* transcripts remained at high levels (Fig. 3.5 A and B).

3.3.5 Changes in the CnABI3 protein during dormancy termination of yellow-cedar seeds

The CnABI3 protein has a predicted molecular mass of 88 kDa and has an apparent size of ~94 kDa on western blots using anti-maize VP1 antiserum (Chapter 2). Following a full dormancy-breaking treatment, the 94 kDa protein present in dormant mature and warm stratified seeds became undetectable, declining in abundance between 2 and 8 weeks of subsequent moist chilling. In the control (i.e. seeds subjected to warm moist conditions for 12 weeks), the 94 kDa protein was preserved, albeit in lower amount than that in 3-d-soaked dormant seed (Fig. 3.6).

3.3.6 A potential role of ABI3 protein in response to oxidative stress during post-germinative growth of western white pine

The levels of ABI3 protein in western white pine also corresponded well with the dormant state of seeds (Fig. 3.7). A steady amount of ABI3 protein was present in the dormant seed until the end of a full 110-day dormancy breaking treatment. ABI3 protein declined sharply only after 1-day of incubation in germination conditions, becoming undetectable during germination. Some seeds were unable to germinate even after the full dormancy-breaking treatment; in these seeds, the ABI3 protein was preserved at the same level as in dormant seeds (Fig. 3.7, lane 15). Very interestingly, a transient, strong re-synthesis of ABI3 protein occurred during early post-germinative growth (Fig. 3.7). This reveals a potential role of ABI3 in the response to oxidative stress because seeds were

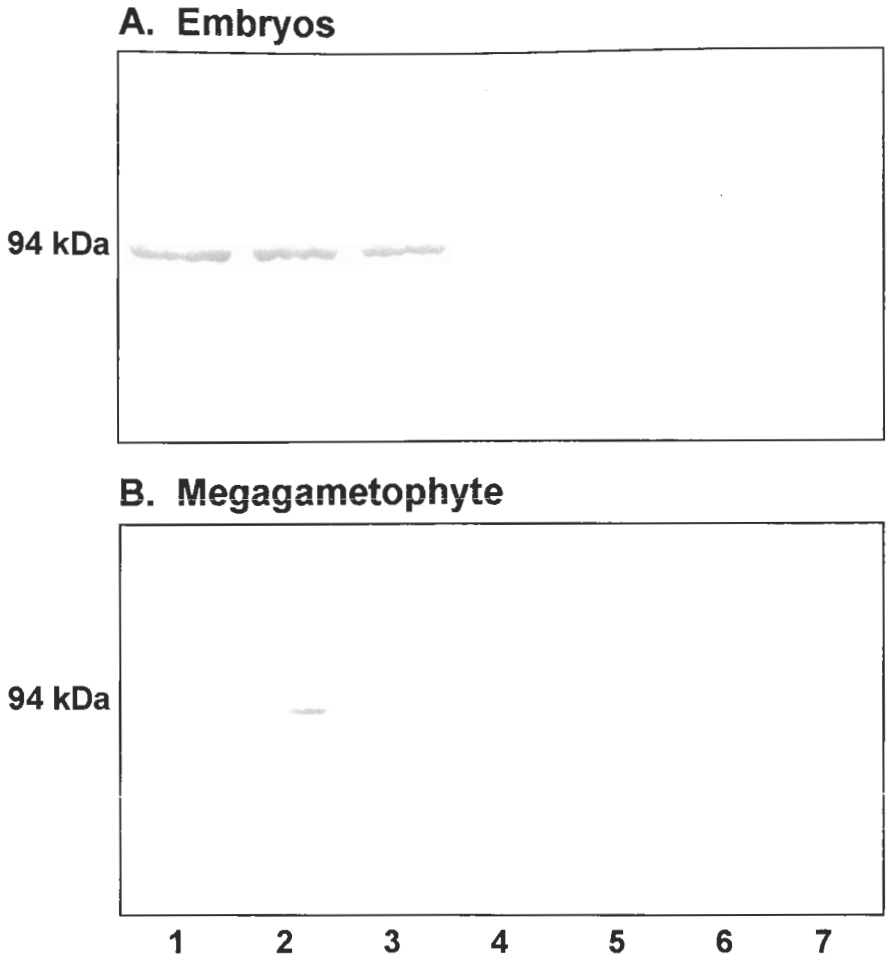

Figure 3.6. Western blot analysis to examine changes in the CnABI3 protein in yellow-cedar embryos (**A**) and megagametophytes (**B**) during different stages of dormancy-breaking and control treatments. **1**, Seeds subjected to a 3-d soak; **2**, Seeds subjected to a 3-d soak and four weeks of warm moist conditions; **3**, Seeds subjected to a 3-d soak, 4 weeks of warm moist conditions and 2 weeks of moist chilling; **4**, Seeds subjected to a 3-d soak, 4 weeks of warm moist conditions and 8 weeks of moist chilling (full dormancy-breaking treatment); **5**, Seeds subjected to the full dormancy-breaking treatment followed by 2 d in germination conditions; **6**, Seeds subjected to the full dormancy-breaking treatment followed by 8 d in germination conditions; **7**, Seeds subjected to a 3-d soak and 12 weeks of warm moist conditions followed by 8 d in germination conditions (control).

surface sterilized with 3% hydrogen peroxide for 30 minutes after the 98-d moist chilling treatment, prior to the transfer of seeds to germination conditions. ABI3 re-induction was not detected in seeds without H_2O_2 surface sterilization (data not shown).

The peroxiredoxin antioxidant gene PER1 is expressed in developing and mature seeds (Aalen, 1999); it is regulated by ABI3 and plays a role in stress sensing during germination/growth (Haslekas *et al.*, 2003). A western blot analysis was carried out with anti-AtPER1 antibodies to examine the abundance of the PER1 protein in western white pine seeds (subjected to surface sterilization after moist chilling). PER1 abundance exhibited an identical pattern with that of ABI3: abundant in dormant seeds, declining during germination and re-synthesized during early post-germinative growth (Fig. 3.8).

3.4 Discussion

3.4.1 Fate of CnABI3 transcripts and protein in conifer seeds during and following dormancy termination

VP1/ABI3 act in concert with other transcription factors (e.g. LEC1, LEC2 and FUS3) to control gene expression central to seed maturation programmes and to prevent the premature activation of germinative and post-germinative genes. A key question addressed by the present study was whether the conifer *ABI3/VP1* protein shares a similar role to its angiosperm counterparts in relation to the control of seed developmental gene expression. I also examined the potential role of CnABI3 in the maintenance of dormancy of yellow-cedar seeds. In particular, the role of ABI3/VP1 proteins in the maintenance of dormancy of imbibed mature seeds needs to be more thoroughly

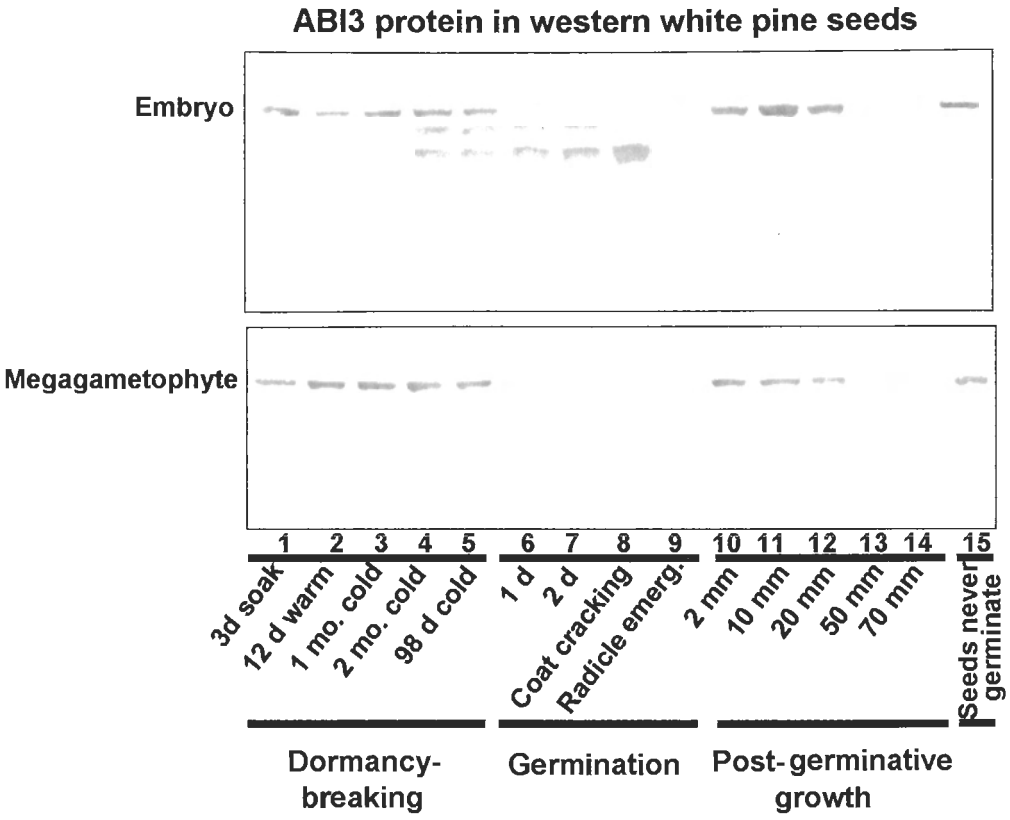

Figure 3.7. Western blot analysis to examine changes in the ABI3 protein in western white pine embryos and megagametophytes during different stages of dormancy-breaking treatments and germination/growth. Fifty μg of proteins was loaded on each lane. **1**, 3-d water soak; **2**, 3-d soak + 9-d warm water soak; **3**, 1 month cold (in addition to the previous warm treatment, same for the following samples); **4**, 2 month cold; **5**, 98-d cold; **6**, 1 d at germination conditions; **7**, 2 d at germination conditions; **8**, seed coat cracking; **9**, radicle emergence; **10**, 2 mm growth; **11**, 10-mm growth; **12**, 20-mm growth; **13**, 50 mm growth; **14**, 70 mm growth and **15**, seeds do not germinate after 30 days. No megagametophyte samples at stages of 50 and 70 mm growth.

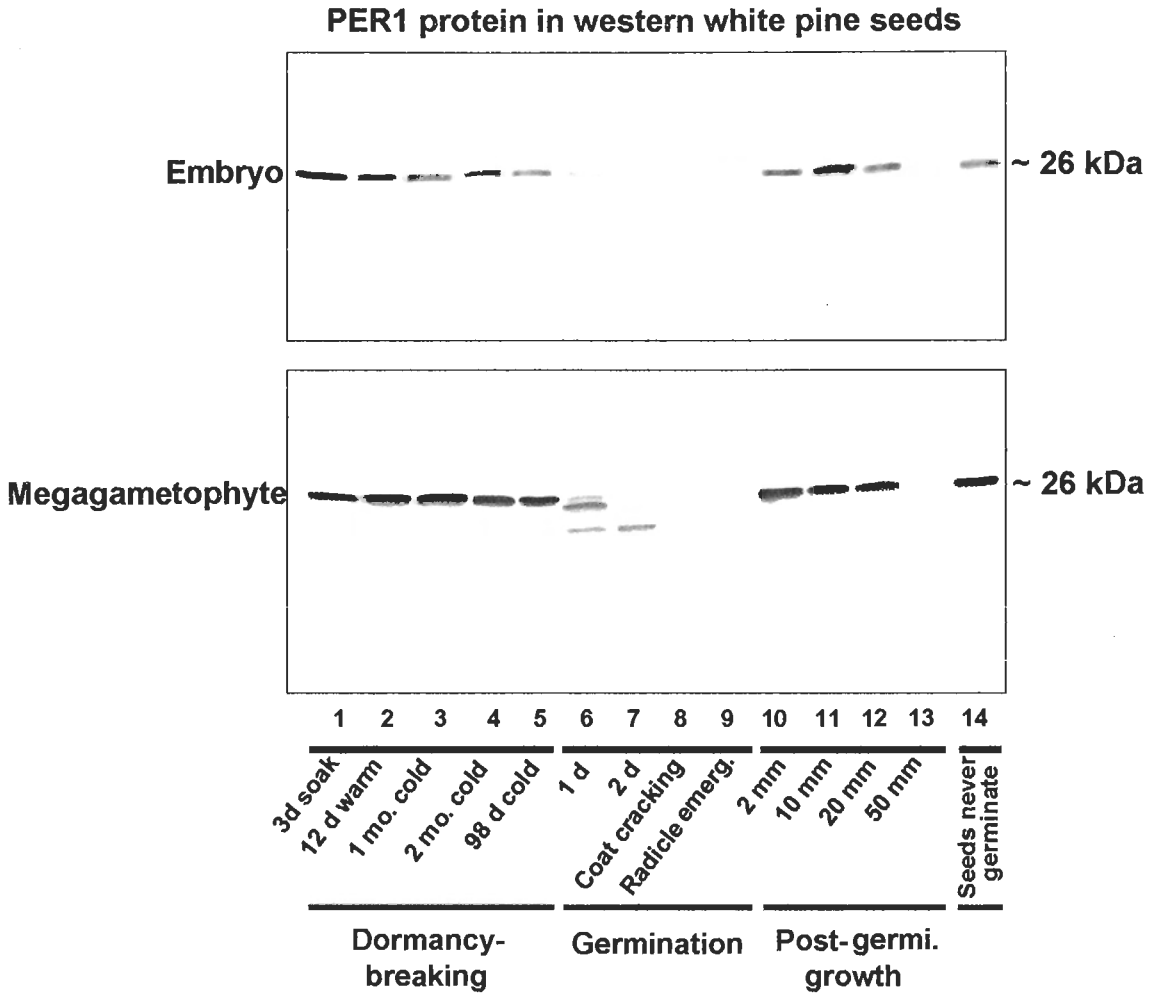

Figure 3.8. Western blot analysis to examine PER1 proteins of western white pine seeds using anti-AtPER1 antibodies. Ten μg of proteins was loaded on each lane and separated by SDS-PAGE (15%). **1**, 3-d water soak; **2**, 3-d soak + 9-d warm water soak; **3**, 1 month cold (in addition to the previous warm treatment, same for the following samples); **4**, 2 month cold; **5**, 98-d cold; **6**, 1 d at germination conditions; **7**, 2 d at germination conditions; **8**, seed coat cracking; **9**, radicle emergence; **10**, 2 mm growth; **11**, 10-mm growth; **12**, 20-mm growth; **13**, 50 mm growth (no megagametophyte samples at this stage). **14**, seeds do not germinate after 30 days of incubation.

addressed in a wider range of species. In the present study I show that down-regulation of *CnABI3* gene expression is positively correlated with dormancy breakage, although for *CnABI3* transcripts, clearly this does not occur during moist chilling itself, but rather during subsequent germination. These results are consistent with that found by others. For example, in embryos of dormant and non-dormant oat (*A. fatua*) seeds transcripts are higher in seeds that are stored at 4 °C and are still dormant, but they decline in seeds that are fully after-ripened at 24 °C (Jones *et al.*, 1997, 2000). A *VP1* gene orthologue cloned from sorghum (*Sorghum bicolor*) has been examined in relation to its potential involvement in pre-harvest sprouting resistance (Carrari *et al.*, 2001). In pre-harvest sprouting, there is germination of physiologically mature grain while still on the parent plant, particularly when maturation of the grain takes place under low temperature and high-humidity conditions (Bewley and Black, 1994). In wheat, barley and sorghum cultivars that are susceptible to pre-harvest sprouting, there is a relative insensitivity of the embryo to ABA as compared to resistant cultivars, generally in the absence of any substantial differences in ABA content, or a higher ABA content in the susceptible cultivars (Walker-Simmons, 1987; Steinbach *et al.*, 1995; Benech-Arnold *et al.*, 1999). Interestingly, the expression pattern of the sorghum *VP1* gene orthologue is different in two cultivars/genotypes exhibiting differential resistance to pre-harvest sprouting at physiological maturity. More specifically, in the embryos of the susceptible cultivar, transcripts encoding VP1 peak at a relatively early stage of grain development (20 days after pollination), while the peak in the resistant cultivar occurs at much later developmental stages when seed maturation is almost complete (Carrari *et al.*, 2001).

Expression of ABI3 in western white pine seeds offered additional evidence that this protein is likely involved in dormancy maintenance of conifer seeds. The ABI3 levels showed dramatic changes immediately following the transfer of seeds to germination conditions and became undetectable during germination. Moreover, in seeds that failed to germinate, the ABI3 protein was abundantly preserved at the same level as in mature seed at the beginning of a dormancy-breaking treatment.

Regulation of CnABI3 (in relation to dormancy of yellow-cedar seeds) is likely not limited to the transcriptional level. Changes in the abundance of CnABI3 protein occurred earlier than those detected at the transcript level. Thus, regulation of the *CnABI3* gene in relation to dormancy and its termination may additionally occur at the level of stability of the CnABI3 protein or involve some other post-translational change. *ABI3* transcripts are rapidly reduced following imbibition of mature *Arabidopsis* seed (Parcy *et al.*, 1994), yet there is an up-regulation of *afVP1* transcripts in dormant *A. fatua* (oat) embryos (Jones *et al.*, 1997). Corresponding changes in ABI3/VP1 proteins at the same stages in these systems (and in the cereal cultivars exhibiting a differential resistance to pre-harvest sprouting) would be worthy of investigation. The nature of control by CnABI3 over dormancy and possible post-translational control of *CnABI3* expression will be the subject of further investigation.

3.4.2 A potential role of ABI3 in sensing oxidative stress

After prolonged stratification (12 days of warm, followed by 98 days of moist chilling), western white pine seeds were surface sterilized with a 3% hydrogen peroxide solution prior to their transfer to germination conditions. This unexpectedly revealed a

potential new role for the ABI3 protein in stress sensing. Exposure of the seeds to oxidative stress led to a transient re-synthesis of ABI3 during early post-germinative growth and this was accompanied by synthesis of peroxiredoxin, an antioxidant under the control of ABI3. The PER1 protein of western white pine showed the same expression pattern as of ABI3 at every stage from seed imbibition to seedling growth. In *Arabidopsis*, the peroxiredoxin antioxidant gene AtPER1 is expressed in the embryo and aleurone layer during maturation and desiccation stages of development, and in the mature seeds (Aalen, 1999; Haslekas *et al.*, 2003a). Subsequently it was revealed that peroxiredoxin antioxidants play a role during inhibition of seed germination by stress (Haslekas *et al.*, 2003b). Peroxiredoxins play roles in protecting DNA, membranes and some enzymes against damage by reactive oxygen species (ROS), and in removing H_2O_2, alkyl hydroperoxides and hydroxyl radicals (reviewed by Wood *et al.*, 2003). Expression of the *AtPER1* gene during development is mediated by ABI3 and its expression is also induced by exogenous ABA and oxidative stress (H_2O_2 and hydroquinone). ABI3-mediated oxidative stress induction of AtPER1 is dependent on the presence of an intact ARE element (antioxidant-responsive promoter element) (Haslekas *et al.*, 2003a). In western white pine seeds, the expression of PER1 protein following oxidative stress treatment and its pattern in relation to ABI3 intimates that both ABI3 and PER1 are involved in stress responses. This is worthy of further investigation in conifer seeds.

3.4.3 CnABI3 transactivates chimeric genes containing promoters of seed storage-protein genes

As noted in the introduction, seeds of the more severe *abi3* mutants of *Arabidopsis* accumulate reduced amounts of storage proteins, remain non-dormant and

display an impaired capacity to express various mRNAs regulated by different temporal programs during seed development (Ooms *et al.*, 1993; Parcy *et al.*, 1994). In the present study I demonstrate that, like its angiosperm counterparts, CnABI3 plays a role in the expression of genes critical for seed developmental processes (e.g. reserve accumulation). Co-expression of the *CnABI3* gene in stably transformed tobacco plants expressing *Nap-GUS-Nos* or *Vic-GUS-Nos* chimeric genes clearly demonstrated the ability of this transcription factor to activate seed storage-protein gene expression, in an ectopic manner. Similar results have been obtained in *Arabidopsis*. ABA-treated transgenic plantlets ectopically expressing ABI3, acquire the ability to accumulate seed-specific mRNAs, including those encoding a 2S albumin (At2S3) and Em1 protein (AtEm1) (Parcy *et al.*, 1994; Parcy and Giraudat, 1997).

ABA had a synergistic effect on CnABI3 function, further enhancing reporter gene expression (GUS activities) driven by storage-protein gene promoters. This contrasts with previous findings in *Arabidopsis* where ABA is absolutely required for the ABI3 induction of cruciferin and napin mRNAs. The tobacco plant may have a higher level of endogenous ABA; alternatively, the use of the GUS reporter may have allowed a more sensitive method of detection.

Ectopically expressed ABI3/CnABI3 proteins are able to effect changes in the transcription of their target (e.g. seed-specific) genes in the absence of the embryo/seed environment. Thus, the other protein factors which physically interact with ABI3/VP1 to effect transcriptional activation/repression (e.g. those isolated by two-hybrid approaches) may not be restricted to seed-specific expression. This is interesting in the context of the functions of the LEC proteins that are transcriptional regulators that appear to establish a

cellular environment sufficient to initiate embryo development (Stone *et al.*, 2001 and references therein). Although the widespread occurrence of VP1/ABI3 proteins suggests they are essential for normal seed development, it has yet to be shown that plants such as ferns that do not undergo embryonic maturation lack these proteins.

On many levels, the CnABI3 protein of yellow-cedar has a role similar to that of ABI3/VP1 proteins of angiosperms. The characterization and functional attributes of proteins that interact with CnABI3 will contribute to an understanding of the functions of ABI3-like proteins in seeds of gymnosperms, particularly in relation to the maintenance of deep dormancy.

Chapter 4:
The Gymnosperm *CnABI3* 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*

4.1 Introduction

All ABI3/VP1 proteins contain four conserved domains: an acidic activation domain and three basic domains, B1, B2 and B3 (Giraudat *et al.,* 1992; McCarty *et al.,* 1991). The transcriptional activation domain (A1) has the least similarities, while the B3 domain exhibits the highest degree of conservation, with about 90% identity between any two given orthologues. The abilities of ABI3/VP1 proteins to transactivate the expression of ABA-responsive developmental genes in seeds have been demonstrated in different systems by transient gene expression assays and by stable transgenic expression studies. ABI3/VP1 likely interacts with other transcription factors to effect gene activation/repression. For example, there appears to be extensive cross-regulation of expression among *ABI3*, *ABI4* and *ABI5* genes (reviewed in Finkelstein *et al.,* 2002; Soderman *et al.,* 2000) and the three transcription factors likely participate in combinatorial control of gene expression, possibly by forming a regulatory complex

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mediating seed-specific and/or ABA-inducible expression. ABI3 and ABI5 interact directly via the B1 domain of ABI3 and two of the conserved charged domains of ABI5 that contain putative phosphorylation residues (Nakamura *et al.,* 2001). ABI5 binding to ABRE elements may tether ABI3 to target promoters and facilitate the interaction of ABI3 with RY elements (a consensus sequence conserved in many seed-specific gene promoters) and transcription complexes (Finkelstein *et al.,* 2002). The B2 domain of ABI3 is required for ABA-regulated gene expression and appears to facilitate the DNA binding capacity of a number of diverse DNA binding proteins (Hill *et al.,* 1996; Carson *et al.,* 1997). Moreover, interactions between the B2 and B3 domains, can mediate activation of target genes by interacting with different cis-acting DNA elements on those genes (Ezcurra *et al.,* 2000). While some studies suggest a direct binding of the B3 domain of ABI3/VP1 to the RY repeat of target genes (Monke *et al.*, 2004), expression of the *Arabidopsis At2S3* storage-protein gene is proposed to be only indirectly regulated by ABI3 with FUS3 and LEC2 binding directly to the two RY elements of the gene and the B3 domain of ABI3 acting as a cofactor in an activation complex (Kroj *et al.,* 2003).

Despite a lack of general conservation between proteins of the ABI3/VP1 family (outside of the four domains), it is assumed that ABI3/VP1 proteins have similar roles in different angiosperm species and that the four domains probably define most of the functions specific to these proteins. This assertion has been borne out by functional complementation studies. For example the *VP1* gene of maize is able to function in *Arabidopsis* seeds with a defective *ABI3* gene (the mutant *abi3-6)* (Suzuki *et al.,* 2001) and most processes are fully restored to the wild-type.

The yellow-cedar CnABI3 protein contains all the four regions that are typically conserved. It shares similar roles to its angiosperm counterparts in relation to the control of seed developmental gene expression and in the maintenance of seed dormancy (Zeng *et al.,* 2003). Although it shares many of the characteristics of other *ABI3/VP1* genes, the conifer gene/protein clearly has unique characteristics as well (Lazazova *et al.,* 2002; Zeng *et al.,* 2003). Because of the distant relationship between yellow-cedar and *Arabidopsis*, a pertinent question is whether the gymnosperm gene is able to functionally complement an *Arabidopsis abi3* null mutant following stable transformation. One severe *Arabidopsis abi3* null mutant is *abi3-6*, in which one third of the *ABI3* gene coding-region is deleted. Seeds of this mutant are green at maturity and are highly insensitive to ABA; they also lack dormancy and are severely intolerant of water loss (Nambara *et al.,* 1994). Due to the severity of the defect in the *ABI3* gene, this mutant serves as an excellent candidate for the analysis of functional complementation of *ABI3* gene between gymnosperms and angiosperms.

In this chapter, the *CnABI3* gene was stably expressed in the *Arabidopsis abi3-6* null mutant as a means of elucidating the degree of conservation of *ABI3* gene functions between gymnosperms and angiosperms. I further examined whether constitutive expression of the *CnABI3* gene in the *Arabidopsis* mutant leads to an enhanced sensitivity of seeds to the inhibitory effects of sugars on post-germinative processes.

4.2 Materials and methods

4.2.1 Generation of transgenic abi3-6 plants expressing CnABI3 gene

Chimeric gene and vector construction was carried out as previously described (3.2.5 of Chapter 3); vector DNA was transferred into *Agrobacterium* strain LBA4404 by electroporation. *Arabidopsis abi3-6* mutant plants (kindly supplied by Dr. Peter McCourt, U Toronto) (Nambara *et al.*, 1994) were transformed using the floral-dip method (Clough and Bent, 1998). Desiccated mature seeds were harvested and the putative transformants identified by seed germination and growth on hygromycin medium. Transformants were verified by PCR using *CnABI3* specific primers (as described in Chapter 3).

4.2.2 Protein extraction for SDS-PAGE and western blot analyses

For SDS-PAGE and western blot analyses, proteins were extracted from *Arabidopsis* seeds or leaves by grinding the tissues in Eppendorf tubes in buffer (10 mM Tris-HCl, 200 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride [PMSF], 1 mM dithiothreitol, 2% SDS, 10% glycerol, pH 7.5). Following centrifugation at 10,000 x g for 10 min, the protein concentration of the supernatant was determined using the Bio-Rad Dc protein assay (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin fraction V (Bio-Rad) as a standard. For western blots using the dehydrin antibody, heat-stable proteins were extracted using methods described in Xia and Kermode (1999).

4.2.3 Quantitative analyses of total, soluble and insoluble proteins of seeds

Quantitative protein analyses were based on three replicates of 40 seeds each. Total seed protein was determined using the procedure noted above with the exception that EDTA, PMSF and DTT were not included in the extraction buffer. Buffer-soluble and insoluble proteins of seeds were separated according to the method of Bates *et al.* (2001). All proteins were quantified using the Bio-Rad Dc protein assay (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin fraction V (Bio-Rad) as a standard.

4.2.4 Protein gels and western blot analyses

For SDS-PAGE profiles of seed and leaf proteins, extracts containing 20 μg total protein were fractionated on 15% gels and the gels stained with Coomassie blue. For western blots, proteins (10 μg) were separated on 7.5% to 15% gels and then electroblotted onto nitrocellulose membranes. Following a brief rinse in PBST (phosphate buffered saline containing 0.05% Tween-20), membranes were blocked overnight with 5% skim milk powder in PBST and then incubated for 1-2 h at room temperature with the primary antibody diluted in PBST and 3% skim milk powder. Dilutions of antibodies were as follows: α-TIP (1:2,000); oleosin (1:10,000); dehydrin (1: 1,000); BiP (1:1000); PDI (1:20,000); 12S cruciferin (1:50,000) and 2S albumin (1:2,000). The western blot procedure was as described in Chapter 2. All blots were repeated at least once with samples at the same stages. (Dr. Timothy J. Close [UC Riverside], Dr. Maarten Chrispeels [UCSD, La Jolla], Dr. Aldo Ceriotti [Instituto Biosintesi Vegetali, Milano], Dr. Maurice Moloney [U Calgary], Dr. Richard A. Dixon

[Samuel Roberts Foundation, Inc., OK] and Dr. Ikuko Hara-Nishimura [Kyoto U, Japan] kindly provided the antibodies against dehydrin, α-TIP, BiP, oleosin, PDI, and 12S/2S storage proteins, respectively).

4.2.5 Quantification of lipids

Mature seeds (three replicates of 50 mg each) were analyzed for crude lipid content (chloroform-soluble compounds) according to the method of Bates *et al.* (2001).

4.2.6 Effects of ABA on seed germination

Seeds were surface sterilized with 75% ethanol (2 min) followed by 10% commercial bleach (v/v, 10 min) and then washed several times with sterile ddH_2O (5 min each). For all treatments, seeds were placed on minimal medium (half-strength Murashige and Skoog salt mixture, Sigma-Aldrich Canada, Mississauga, Ontario, Canada) containing no ABA or natural *S*-(+)-ABA at different concentrations (0.1 to 50 μM) (kindly supplied by Dr. Sue Abrams, Plant Biotechnology Institute, SK) and then subjected to a 4-d moist chilling (4 °C in darkness). Germination and growth were then monitored after transfer of the plates to germination conditions (21 °C with a 16-h photoperiod, maintained in a controlled growth chamber; Conviron Model E15, Winnipeg, Manitoba, Canada). For the ABA treatments, ABA was added at the start of the moist chilling period to avoid any germination of seeds (particularly *abi3-6* seeds) during moist chilling – i.e. prior to their transfer to germination conditions. Three repeats of each of the treatments were carried out. Germination and growth performance were recorded daily.

4.2.7 Effects of ABA and glucose on germination and post-germinative growth

Seeds were surface sterilized and incubated under the same conditions as stated above with the exception that the minimal media contained no ABA or different concentrations of *S*-(+)-ABA (0.2, 0.5 and 1 μM) and/or glucose (0.5%, 1%, 2% and 6%) at the beginning of moist chilling. ABA and glucose were not re-applied thereafter. Germination and growth were observed daily and photographed under a stereomicroscope (Technival 2; Carl Zeiss, Germany).

4.2.8 Light Microscopy

Mature dry seeds of wild-type, *abi3-6* and transgenic lines were fixed overnight in PBS containing 10% formaldehyde, 5% acetic acid and 50% ethanol and subsequently dehydrated in a graded ethanol series (10%, 30%, 50%, 70%, 80%, 90% and twice in 100%; 1 h each step). Seeds were then infiltrated with graded concentrations of LR White acrylic resin (Sigma Co., St Louis, MO, USA) at 25%, 50% and 75% (diluted in 100% ethanol), each for a minimum of 3 h followed by two changes of 100% resin (each for 16 h). Seeds were placed in fresh 100% LR White acrylic resin in an embedding capsule and polymerized at 60 °C for 24 h. Sections were cut using glass knives on an OmU3 ultramicrotome (Reichert, Austria) to a thickness of ~ 2 μm, mounted on glass slides, stained (amido black 10B for protein and nucleic acids and basic fuchsin for general structures) (Sigma Co., St Louis, MO, USA)' and then observed under a light microscope (Nikon Eclipse E600, Japan).

4.3 Results

4.3.1 Generation of transgenic lines and visible phenotypes of seeds and plants

The *CnABI3* gene of yellow-cedar, whose expression was driven by an enhanced 35S promoter, was introduced into *Arabidopsis abi3-6* plants using the floral-dip method (Clough and Bent, 1998). In total, 77 transgenic lines were verified by hygromycin resistance and PCR analysis using *CnABI3* specific primers (data not shown). Seeds that expressed a functional *CnABI3* gene at sufficient levels were readily and conveniently identified because they exhibited wild-type-like phenotypes at maturity (yellow-brown, desiccation-tolerant), in marked contrast to seeds derived from untransformed *abi3-6* plants, which were green, desiccation-intolerant and unable to germinate in the dry state (0% germination). Although the expression levels for the different transgenic lines were not directly determined, the degree of complementation obviously varied. Of the 77 transgenic lines that contained the *CnABI3* gene, 49 produced mature seeds that were visibly indistinguishable from those of wild-type plants. The remaining transgenic lines produced green, wrinkled T2 seeds similar to those of *abi3-6* plants. Four transgenic lines (7, 18, 34 and 62) were randomly selected for further analysis in this study; as shown in Figure 4.1, all produced mature seeds that were yellow-brown and full, in contrast to the green wrinkled seeds characteristic of the *abi3-6* mutant plants. In addition, mature desiccated seeds from all 4 lines exhibited virtually 100% germination after a 4-d moist-chilling period.

Early flowering is a phenotype of *abi3-6* plants (Suzuki et al., 2001); the four transgenic lines expressing the *CnABI3* gene exhibited flowering times that were somewhat intermediate of those characteristic of wild-type and *abi3-6* plants (Fig. 4.2).

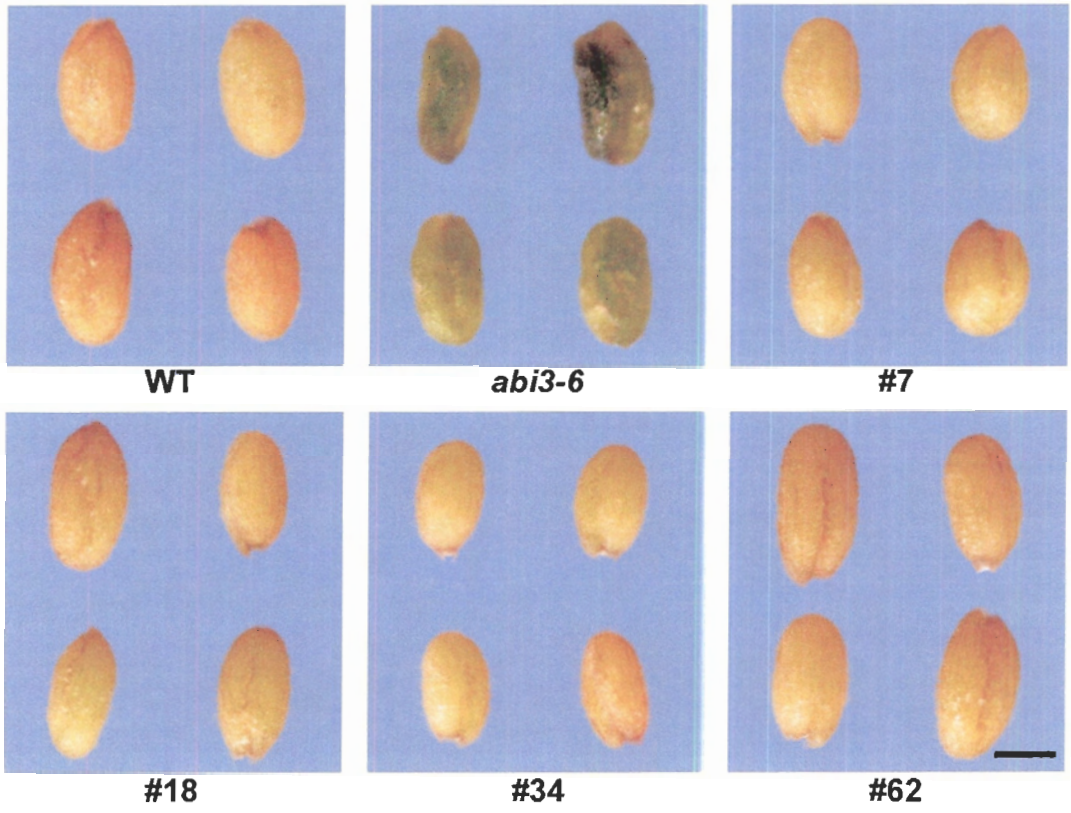


Figure 4.1. Mature desiccated seeds of the wild-type (WT), *abi3-6*, and *35S-CnABI3* transgenic plants (4 lines). All images are at the same magnification. Bar = 0.25 mm.

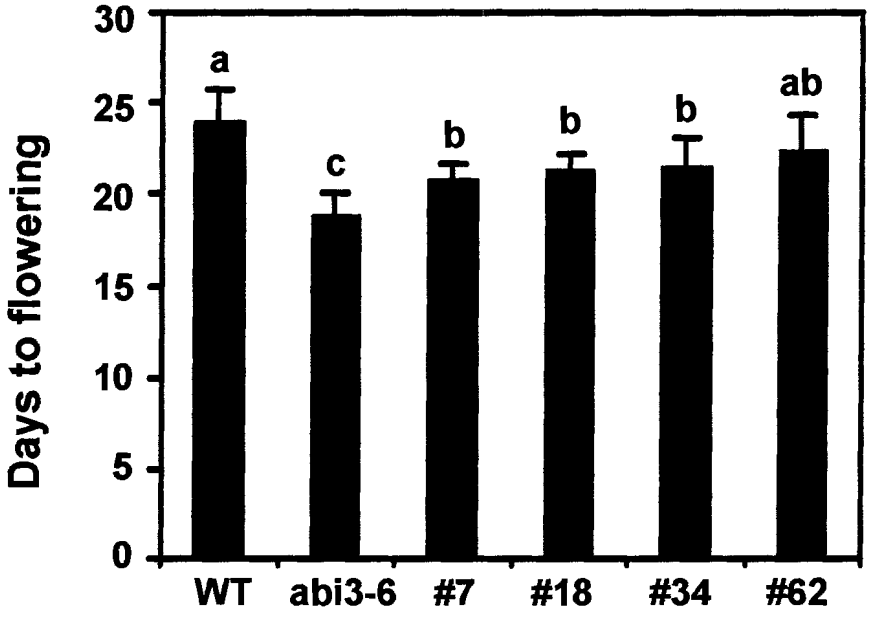


Figure 4.2. Flowering times of the wild-type (WT), *abi3-6*, and *35S-CnABI3* transgenic plants (four lines). Seeds were germinated on plates and 5 days after germination, seedlings with similar growth performance from each line were transferred to soil and incubated in a growth chamber at 21 °C with a 16 h light period. Days to flowering was measured from the day seedlings were transferred to soil to the day of emergence of the first open flower. Different letters over bars indicate significant differences ($p < 0.05$).

Consistent with the *abi3-6* background, trichomes were absent from the leaves of all of the transgenic plants, ruling out any possibility of cross-pollination of *abi3-6* plants by wild-type plants (data not shown).

4.3.2 Protein and lipid accumulation in seeds of transgenic plants expressing the CnABI3 gene

There was a marked contrast between the total protein profiles of seeds derived from the transgenic plants expressing the *CnABI3* gene as compared to the untransformed *abi3-6* seeds (Fig. 4.3A). The transgenic lines appeared to have the full complement of the major storage proteins characteristic of wild-type *Arabidopsis* seeds (e.g. 12S globulins and 2S albumins). The amounts of both of these storage proteins were much reduced in *abi3-6* seeds (Fig. 4.3 A, B). The total protein profiles of leaves derived from the wild-type, *abi3-6* and transgenic plants were nearly identical; however, a protein of ~ 20 kDa of unknown identity was detected exclusively in the leaves of the transgenic plants (data not shown).

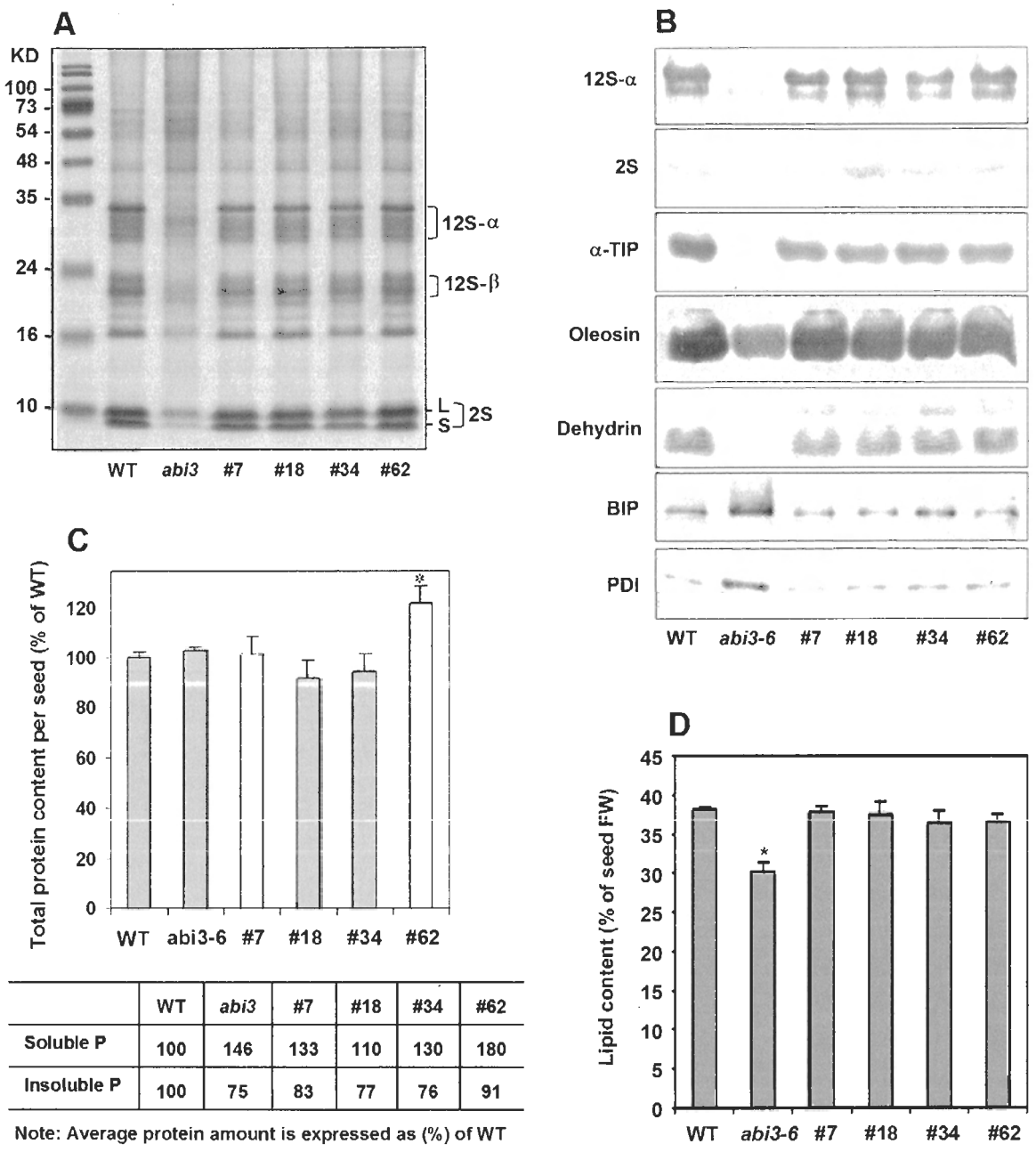
The functional complementation of the mutant extended to the accumulation of several specific developmental proteins as shown by western blot analyses. The levels of several seed proteins, which were considerably reduced in mature *abi3-6* seeds, were restored to wild-type levels by expression of the *CnABI3* gene (Fig. 4.3B); this included α-tonoplast intrinsic protein, oleosin and dehydrin-related polypeptides. Surprisingly, levels of the ER-resident molecular chaperones Binding Protein (BiP) and protein disulphide isomerase (PDI) were elevated in mutant seeds; expression of the *CnABI3* gene decreased the accumulation of these proteins to levels characteristic of the wild-type (Fig. 4.3B). The elevated levels of these molecular chaperones in mutant seeds may be

indicative of ER stress; despite reduced amounts of specific proteins in mature seeds, the *abi3* seeds may remain synthetically active for a longer developmental period than do wild-type *Arabidopsis* seeds (see discussion).

Because of the marked differences in the amounts of specific proteins accumulated by mutant versus transgenic seeds, quantitative protein analyses were conducted (Fig. 4.3C). While there were no differences between the total protein contents of wild-type and mutant seeds, interestingly, the soluble protein fraction of *abi3-6* mutant seeds and of seeds of most of the transgenic lines was markedly elevated as compared to that of wild-type seeds. The reverse was true for the insoluble protein fractions of these seeds, which were reduced in the mutant and in the transgenic lines as compared to wild-type. We cannot rule out the possibility that free amino acids (which may accumulate to greater levels in the *abi3-6* and transgenic seeds) interfered with the quantitative measurement of the soluble protein fraction. In any event, the transgenic expression of the *CnABI3* gene generally did not reverse the effects of the mutation on the ratio of soluble to insoluble protein.

There was a reduced accumulation of lipids in *abi3-6* mutant seeds; expression of the *CnABI3* gene led to full restoration of lipid quantity in all of the transgenic lines to levels characteristic of wild-type seeds (Fig. 4.3D).

Seeds of wild-type-, *abi3-6* mutant- and transgenic plants were examined by light microscopy to determine any differences in storage parenchyma and aleurone layer cells at the subcellular level (e.g. in protein storage vacuole morphology). Differences were particularly evident within cells of wild-type and mutant *abi3-6* seeds (Fig. 4.4). Seeds of the transgenic lines expressing the *CnABI3* gene (images of line #7 are shown as



	WT	*abi3*	#7	#18	#34	#62
Soluble P	100	146	133	110	130	180
Insoluble P	100	75	83	77	76	91

Note: Average protein amount is expressed as (%) of WT

Figure 4.3. Analyses of proteins and lipids in the mature dry seeds of the wild-type (WT), *abi3-6*, and *35S-CnABI3* transgenic plants (four lines). **A**. Stained gel showing the major (12S and 2S) storage proteins of seed. **B**. Western blots using different antibodies to detect specific proteins in total protein extracts or heat soluble extracts (dehydrin-related proteins only). **C**. Comparisons of total seed protein and the soluble and insoluble protein fractions. Data represent the average $\pm$ S.E. of three individual determinations. **D**. Crude lipid content (% of seed fresh weight). In C and D, "*" over bar indicates a significant difference ($p < 0.01$).

representatives for transgenic lines) exhibited subcellular features that were characteristic of wild-type seeds (Fig. 4.4). Three aspects are noteworthy. First, both protein storage vacuole morphology and number were drastically different in the storage parenchyma cells of the *abi3-6* seeds as compared to the wild-type and transgenic seeds. Second, the single layer of cells comprising the aleurone layer of *abi3-6* seeds was almost undetectable; the cells comprising this layer were significantly thinner and contained fewer protein storage vacuoles. This was in marked contrast to the aleurone layer cells of the wild-type and transgenic seeds, which were relatively large and filled with multiple protein storage vacuoles (more numerous than within the storage parenchyma cells of the embryos). Third, the cell walls of the storage parenchyma cells of the wild-type and transgenic embryos were much thicker compared to those of the mutant embryo (This is particularly evident in the enlarged images; right panel of Fig. 4.4).

4.3.3 ABA sensitivities of seeds expressing the CnABI3 gene

Following 4 d of moist chilling, all seeds on media with no ABA showed an equal capacity to germinate (95-100%; Fig. 4.5A, 0). The effects of exogenous ABA on seed germination and growth were tested. Seeds of the *abi3-6* mutant were highly insensitive to ABA and exhibited 100% germination in all of the ABA concentrations tested (Fig. 4.5A). Seeds of wild-type plants were the most sensitive to ABA and their germination was increasingly inhibited by ABA at concentrations of 1 to 25 μM and greater (Fig. 4.5A). None of the wild-type seeds was able to expand cotyledons and develop into normal vigorous seedlings at ABA concentrations greater than 1 μM (Fig. 4.5 B-D). Interestingly, seeds of all four transgenic lines expressing the *CnABI3* gene showed ABA sensitivities intermediate between those of the wild-type and the *abi3-6* mutant. The

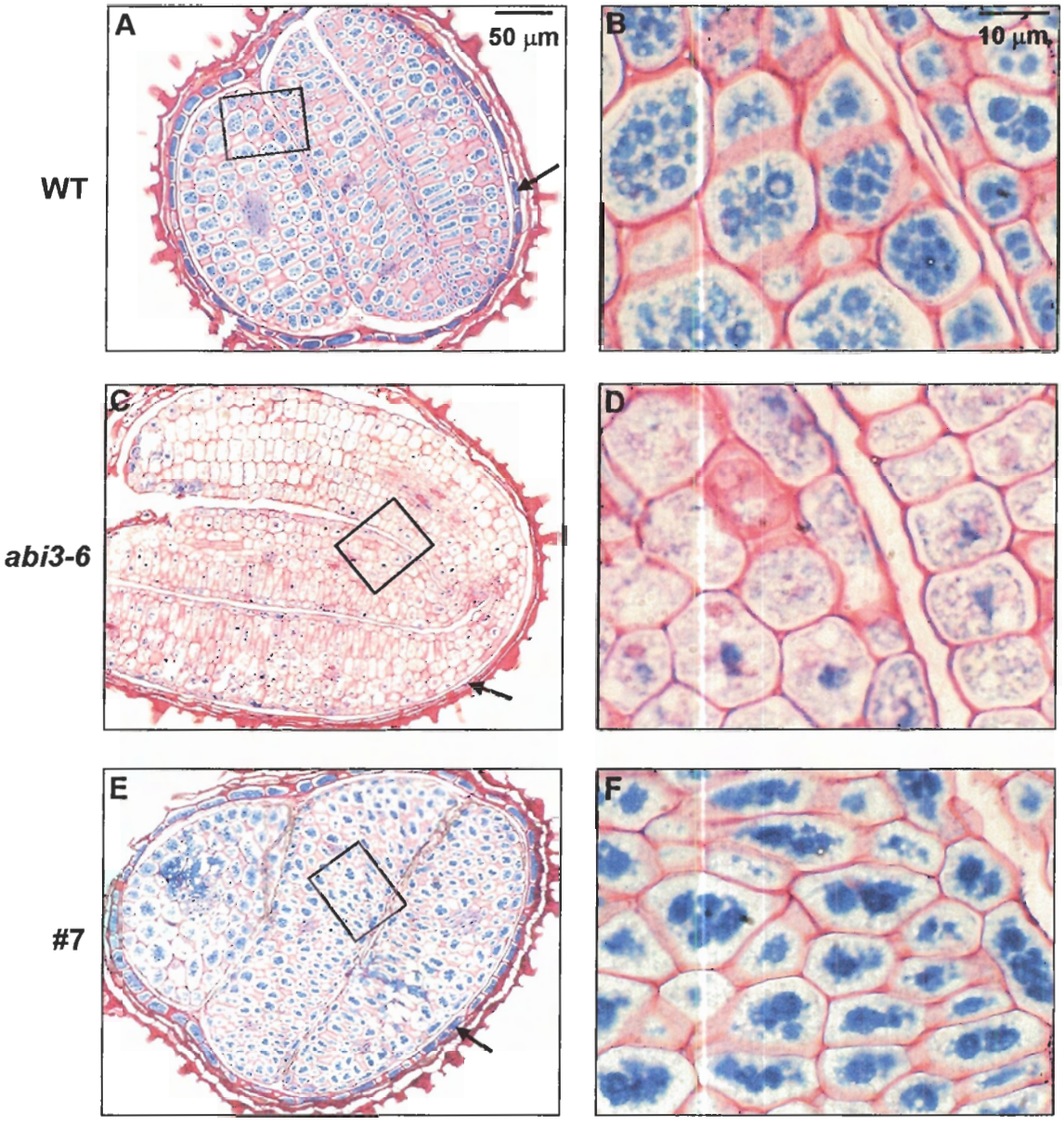


Figure 4.4. Subcellular characteristics of seeds of the wild-type (WT), *abi3-6*, and *35S-CnABI3* transgenic plants (line #7). Images of the right panel represent cells (embryo storage parenchyma cells) within the boxed areas of the left panel images. Arrows on the left panel images indicate the aleurone layer.

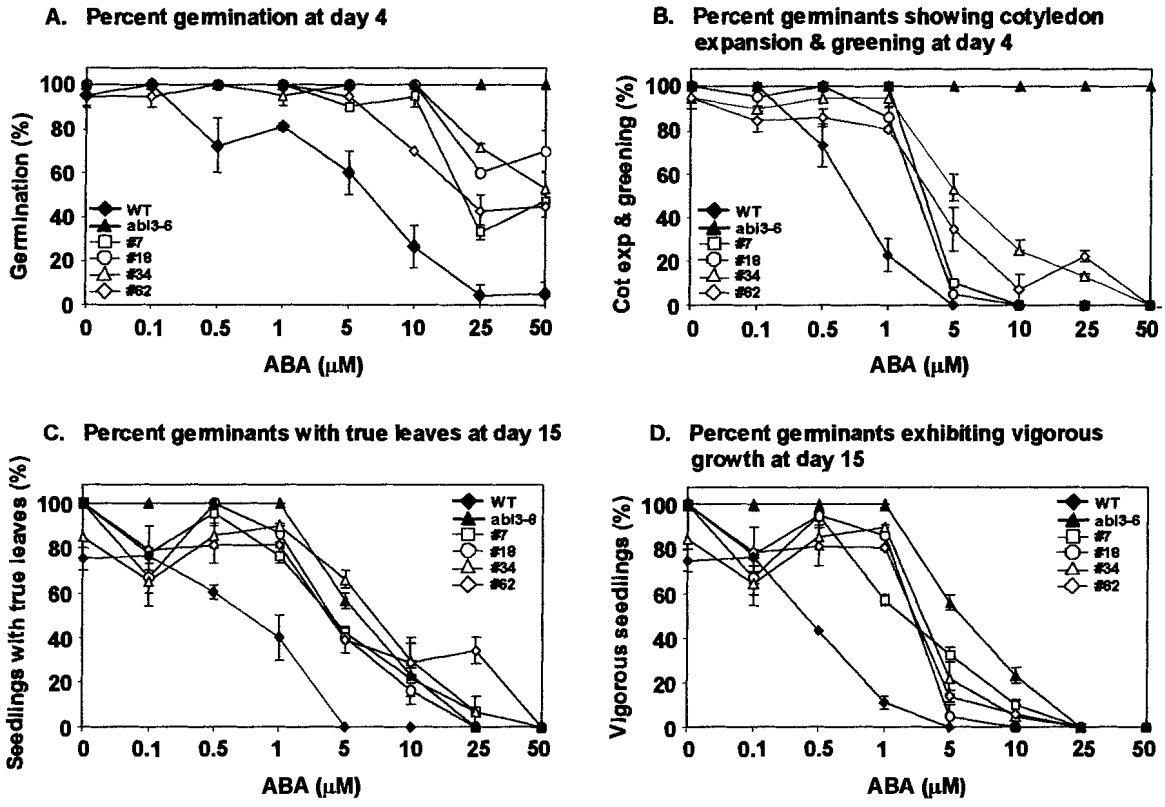


Figure 4.5. Effect of exogenous ABA on germination and post-germinative growth of seeds of the wild-type (WT), *abi3-6*, and *35S-CnABI3* transgenic plants (four lines). Dry seeds of wild type and *35S-CnABI3* transgenic plants, and immature (20-22 DAP) seeds of *abi3-6* plants, were subjected to moist chilling for 4 days. Various concentrations of *S*-(+) ABA were applied to the minimal medium plates at the beginning of moist chilling period and plates were transferred to germination conditions to monitor percent germination as well as the growth characteristics of the germinants. Germination percentages are based on radicle emergence. Approximately 50 seeds were used in each treatment, and triplicate treatments were carried out for each ABA concentration.

differences in sensitivities (as far as germination is concerned) persisted during post-germinative growth (Fig. 4.5B-D), which may indicate an inability of the conifer ABI3 protein to fully restore ABA sensitivity.

4.3.4 Synergistic effect between CnABI3 and sugar in the inhibition of post-germinative growth

Sugar and ABA signaling pathways interact to control different processes during the life cycle of plants. The ectopic expression of the *CnABI3* gene greatly enhanced the ability of glucose to inhibit the post-germinative growth of *Arabidopsis* seedlings (Fig. 4.6). Treated seeds of all four transgenic lines exhibited similar phenotypes in response to glucose and/or ABA; thus, for simplicity, only seeds from line #7 are shown as representatives. At relatively low concentrations of glucose (0.5-1% glucose), the transgenic plants exhibited stress characteristics (Fig. 4.6 and data not shown). Presence of 0.2 μM ABA in addition to 1% glucose caused the transgenic seedlings to appear even more stressed and further induced anthocyanin accumulation in the cotyledons. Even by day 12, the transgenic seedlings exhibited only a partial recovery. In contrast, the growth of the *abi3-6* mutant seedlings (and wild-type seedlings) was not inhibited by 1% glucose and/or 0.2 μM of ABA but the morphologies of the seedlings were different (Fig. 4.6). Application of 2% glucose generally led to similar outcomes as the 1% glucose treatment. Presence of 1 μM ABA in addition to 2% glucose caused both the wild-type and transgenic seedlings to lose their ability to develop green cotyledons and true leaves. Again, the transgenic seedlings showed a more severe stress response than that exhibited by wild-type seedlings. For example, the latter retained the capacity to develop some roots. Unlike the *abi3-6* seedlings, both the wild-type and transgenic seedlings failed to

recover over time. When the glucose concentration was raised to 6%, which is normally inhibitory to growth, all lines (including the *abi3-6* mutant) showed severe stress responses and major accumulation of anthocyanins in the cotyledons. Only the *abi3-6* mutant showed full recovery at day 17 and developed true leaves and shoots. Interestingly, despite an inability of wild-type seedlings to fully recover by day 17 (or at any later stage), they nonetheless exhibited enhanced growth of the primary root which was greater than that of the *abi3-6* seedlings, although the differences in average length were not significant (Fig. 4.7). However, the *abi3-6* seedlings had very well developed lateral root systems, while the wild-type seedlings characteristically showed only growth of the primary root. Root lengths of seedlings from all four transgenic lines were diminished considerably (to 1-2 mm on average), which clearly indicates that the CnABI3 protein and glucose act synergistically in the inhibition of post-germinative growth. Addition of ABA (1 μM) in combination with 6% glucose, led to similar responses as those elicited by 6% glucose alone, with the exception of the stunted root growth exhibited by wild-type seedlings at day 17. Once again, the *abi3-6* mutant was able to recover in contrast to the complete inhibition of post-germinative growth of both the wild-type and the transgenic seedlings (Fig. 4.6).

4.4 Discussion

4.4.1 Seed phenotypes, desiccation tolerance and accumulation of proteins implicated in desiccation tolerance

In contrast to the *abi3-6* mutant seeds that are green and wrinkled at maturity, the visible phenotypes of seeds expressing the *CnABI3* gene were completely restored to

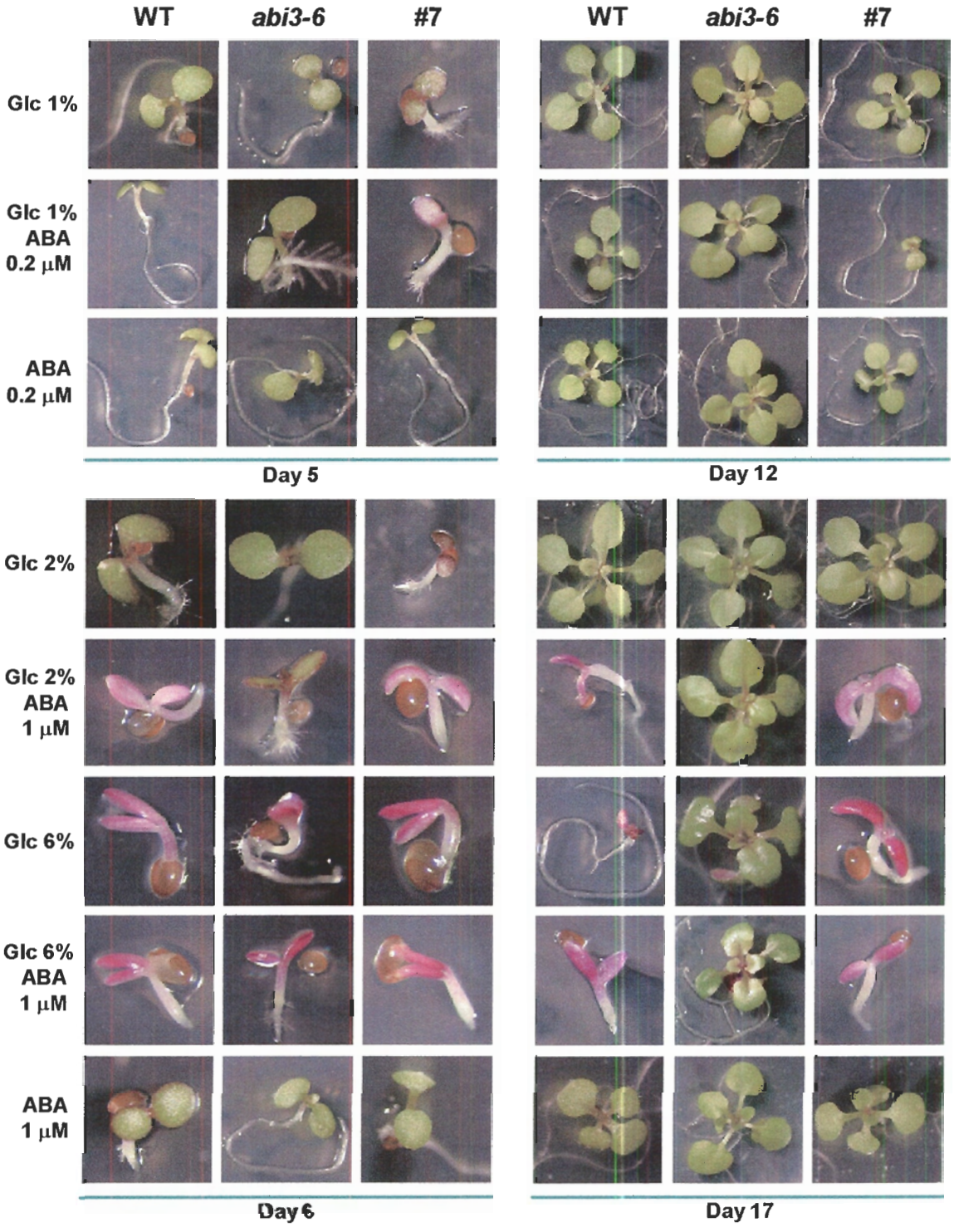


Figure 4.6. Synergistic effect of CnABI3, glucose and ABA in the inhibition of post-germinative growth. Images are cropped at various magnifications in order to show more details for the smaller seedlings that did not grow normally.

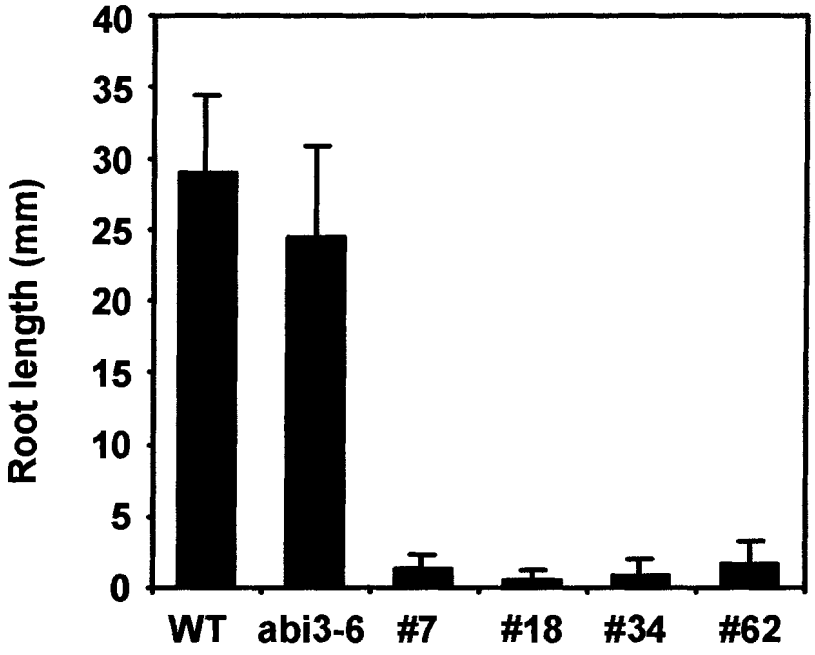


Figure 4.7. Primary root lengths of seedlings at day-17 on 6% glucose medium. Glucose was added to the minimal medium at the beginning of incubation. Data are based on three replicates of approximately 50 seedlings each.

those of the wild type. The mature transgenic seeds were yellow-brown (presumably due to chlorophyll breakdown during late development and desiccation) and exhibited more normal seed fill. In addition, unlike the *abi3-6* background, the transgenic seeds that had undergone maturation drying were able to germinate and develop into healthy seedlings to the same extent as those of wild-type plants. These phenotypes provided convincing evidence that the conifer *CnABI3* gene was functioning in *Arabidopsis*. They further added to the ease of selection of positive transformants, while additionally eliminating lines in which gene silencing (or non-functional integration) occurred. Indeed, of the 77 transgenic lines isolated on the basis of hygromycin-resistant T1 seeds and PCR confirmation of the presence of the *CnABI3* gene, over 20 lines produced non-germinable green wrinkled T2 seeds similar to the *abi3-6* background (data not shown).

Seeds of the transgenic lines expressing the *CnABI3* gene were fully filled and exhibited virtually 100% germination in the mature desiccated state, indicating a complete restoration of desiccation tolerance. While the underlying basis of desiccation-tolerance is multifaceted, several proteins have been implicated in this process including specific small heat-shock proteins (HSPs) of the cytosolic classes (I and II) that accumulate in seeds of several plant species (reviewed in Kermode and Finch-Savage, 2002; see references therein). In *Arabidopsis*, class I small HSPs are first detected during mid-maturation and become most abundant in dry seeds; the proteins decline rapidly during germination (Wehmeyer *et al.*, 1996). The *ABI3* gene product may activate expression of genes encoding specific small HSPs during seed development; *abi3-6* seeds have undetectable amounts of HSP17.4 (Wehmeyer and Vierling, 2000). Interestingly, a chimeric gene consisting of the small HSP gene promoter linked to GUS shows strong

expression in mutant seeds that are heat-stressed, indicating that the genes are under distinct developmental and stress regulation (Wehmeyer and Vierling, 2000). The water channel protein α-tonoplast intrinsic protein that accumulates during seed maturation in the parenchyma cells of seed storage organs may contribute to cytoplasmic osmoregulation and/or to the survival of seed tissues during desiccation and/or subsequent rehydration (Johnson *et al.*, 1989). Likewise, oleosin is suggested to preserve the integrity of oil bodies during seed maturation drying (Frandsen *et al.,* 2001). The accumulation of both of these proteins as well as dehydrin-related polypeptides was very much reduced in seeds of the *abi3-6* mutant plants; the restoration of desiccation tolerance of seeds of the transgenic lines expressing the *CnABI3* gene was accompanied by a restoration of the accumulation levels of these putative desiccation protectants.

4.4.2 Rescued reserve accumulation in transgenic seeds expressing the CnABI3 gene

Wild-type seeds of *Arabidopsis* accumulate several seed storage proteins during their development including the 12S globulins (cruciferins A1, B and C) and 2S albumins (At2S1, At2S2, At2S3, At2S4 and At2S5) (reviewed in Fujiwara *et al.,* 2002). The accumulation of these seed storage proteins (e.g. subunit polypeptides of cruciferins in the ~28-34 and ~18-23 kDa range and 3- and 9-kDa 2S albumins) was markedly reduced in *abi3-6* mutant seeds; mature seeds of all four transgenic lines exhibited restored accumulation levels of these storage proteins to those characteristic of wild-type seeds. Lipids were significantly reduced in seeds of the *abi3-6* mutant; transgenic expression of CnABI3 fully restored the levels to those characteristic of the wild-type seeds. Thus functioning of the conifer CnABI3 protein in respect of this key developmental process was equal to that of the *Arabidopsis ABI3* gene. In contrast to the ability of CnABI3 to

fully restore the accumulation levels of the 12S globulins, the maize VP1 protein is unable to promote the expression of the cruciferin C gene in *abi3-6* seeds (Suzuki *et al.*, 2001).

ABI3 is thought to regulate seed storage-protein gene expression by acting synergistically with other transcription factors (e.g. FUS3 and LEC1, LEC2 and others) that participate in combinatorial control (Parcy *et al.*, 1997; Kroj *et al.*, 2003). ABI3/VP1 may recruit additional DNA-binding proteins to the promoters of storage-protein genes via its ability to alter chromatin structure (e.g. nucleosome positioning) (Li *et al.*, 2001). Regulation of *At2S3* gene expression appears to involve *FUS3* and *LEC2* that bind directly to promoter elements (RY repeats 1 and 2), while ABI3 acts in an indirect manner (likely via its interaction with bZIP proteins that bind to the G-box) (Kroj *et al.*, 2003). The low accumulation levels of storage proteins in mature *abi3-6* seeds intimate that FUS3, LEC1 and LEC2 are unable to compensate for the loss of ABI3 function in terms of the regulation of storage protein synthesis. The restored storage-protein accumulation in transgenic lines suggests that CnABI3 effectively interacts with these other regulatory factors.

Seeds of the transgenic lines and wild-type seeds had total protein contents that corresponded well with seed sizes. For example, the average sizes of seeds of the transgenic lines were similar to (line #7), slightly smaller (lines #18 and #34), or larger (line #62) than that of the wild-type plants (data not shown) and this was reflected in their total protein contents. Such was not the case for seeds of the *abi3-6* mutant. While the *abi3-6* seeds had drastically lower accumulation levels of storage proteins at maturity, their total protein amount per seed was surprisingly close to that of the wild-type and the

transgenic lines. We suspect that the *abi3-6* mutant seeds accumulate more free amino acids during their development, than do wild-type seeds. Free amino acid accumulation occurs in seeds of a storage-protein-deficient soybean line which lacks the ability to synthesize two major globulin storage proteins (11S glycinin and 7S β-conglycinin), but nonetheless contains a total nitrogen content comparable to that of mature wild-type seeds (Takahashi *et al.,* 2003). Alternatively the *abi3-6* seeds may accumulate large amounts of proteins (other than storage proteins) as a result of a premature induction of syntheses associated with post-germinative growth. For example, the chlorophyll a/b binding protein 3 (cab3), whose synthesis is repressed in wild-type seeds until the post-germinative stage, is induced in *abi3-6* seeds during seed development (Suzuki *et al.,* 2001). Because of their inability to perceive maturation signals, the mutant seeds may continue to synthesize proteins related to both development and germinative/post-germinative programmes, although the former do not accumulate. Seeds of double mutants of *Arabidopsis* (*aba/abi3*) do not complete the seed developmental programme and there is a premature (yet incomplete) switching to a germination programme. During development (14 to 20 DAP) the low amounts of various maturation-specific proteins are degraded and proteins characteristic of germination are induced, in the absence of germination (Koornneef *et al.*, 1989; Meurs *et al.,* 1992).

In addition to defective *ABI3* gene function directly affecting storage-protein gene expression, plants carrying the *abi3* mutation (the weakest allele, *abi3-1*) also partition more resources into seed development than do wild-type plants. These extra resources are available as a result of delayed senescence of the cauline leaves in the mutant; also apparent are changes in the distribution of photosynthate (Robinson and Hill, 1999).

An interesting finding was the increased accumulation of ER molecular chaperones BiP and PDI in mature *abi3-6* seeds as compared to the mature seeds of the wild-type and transgenic lines. Since both BiP and PDI act as molecular chaperones and play roles during and after protein synthesis (i.e. aiding in protein folding, protein translocation and in the disposal of misfolded polypeptides), the high levels of these proteins in the mutant seeds may be suggestive of higher protein synthetic activities prior to seed desiccation (assuming there is a cessation of protein synthesis in the dry seed). Generally these ER resident molecular chaperones are elevated under circumstances where newly synthesized proteins become misfolded, for example, as a consequence of exposure to environmental stresses (e.g. heat stress or oxidative stress). It may be relevant that the proteins of maturation-defective mutants of *Arabidopsis* (e.g. *abi3* and *lec* mutants) appear to be more susceptible to denaturation during heating (Wolkers *et al.*, 1998). Proteins in dry wild-type seeds do not denature at temperatures up to 150 °C; those of dry desiccation-sensitive seeds (*lec1-1*, *lec1-3* and *abi3-5*) denature at 68, 89 and 87 °C, respectively. In contrast, in desiccation-tolerant seeds (*abi3-7* and *abi3-1*), denaturation commences above 120 and 135 °C, respectively.

Our examination of the subcellular characteristics of storage parenchyma and aleurone layer cells of *abi3-6*, wild-type and transgenic seeds revealed that the former exhibited altered protein storage vacuole number (and possibly morphology) and various structural differences (a virtual loss of integrity of aleurone layer cells and altered cell walls surrounding storage parenchyma cells). Defects in cell wall biosynthesis appear to be secondary effects of *ABI* (e.g. *ABI8*) gene mutations (Brocard-Gifford *et al.*, 2004). While the reduced storage protein content of the mutant cells likely contributed to the

reduction in protein storage vacuole number, nonetheless the storage parenchyma cells appear highly vacuolated. Sequestration of ABA within developing transgenic tobacco seeds (effected by expression of an anti-ABA single chain variable fragment antibody) leads to a marked disruption of storage reserve deposition, to the extent that the storage parenchyma cells of the seed more closely resemble plant vegetative cells (Phillips *et al.*, 1997). The transgenic seeds exhibit other features of seedlings, including green-chloroplast-containing cotyledons, desiccation intolerance and premature activation of the shoot apical meristem. The highly reduced accumulation of small HSPs in *abi3-6* seeds (e.g. HSP 17.4) (Wehmeyer and Vierling, 2000) may contribute to an altered morphology of protein storage vacuoles; a putative role for HSP17 in assisting in the packaging of storage proteins in protein bodies (thus determining organelle shape) has been suggested (Miroshnichenko *et al.*, 2005).

4.4.3 ABA sensitivity is only partially rescued in transgenic seeds expressing the CnABI3 gene

Seeds expressing the *CnABI3* gene exhibited much higher sensitivities to exogenous *S*-(+)-ABA as far as their germination is concerned, as compared to seeds of the *abi3-6* mutant, which can germinate even in 300 μM racemic ABA (Nambara *et al.*, 1994). However, ABA sensitivity was considerably lower than that of the wild-type seeds. These responses may be attributed to functional differences between the gymnosperm ABI3 protein and its angiosperm counterpart or may be a consequence of using the modified CaMV 35S promoter to drive *CnABI3* gene expression in *Arabidopsis* seeds. Since the *CnABI3* gene was constitutively expressed in the transgenic lines, we might expect them to exhibit a higher (or at least equivalent) level of sensitivity to ABA

compared to the wild-type. In *abi3-6* seeds constitutively expressing the maize *VP1* gene, ABA sensitivity is fully restored to that characteristic of wild-type seeds (Suzuki *et al.,* 2001). Thus, the incomplete restoration of ABA sensitivity in the present study may indeed be indicative of differences between the CnABI3 protein and the angiosperm ABI3 protein in the domains related to ABA responses.

4.4.4 ABA sensitivity and synergistic effect between CnABI3 and sugar in the inhibition of post-germinative growth

Sugars play important roles in metabolism as well as in the regulation of many developmental processes, including germination and early post-germinative growth (reviewed in Gazzarrini and McCourt, 2001; Finkelstein and Gibson, 2002). When present at low concentrations, sugars generally promote germination and early post-germinative growth; at higher concentrations, these processes are inhibited. During early post-germinative seedling development/growth, sugars can repress nutrient mobilization, hypocotyl elongation, cotyledon greening and expansion, and shoot development. High sugar accumulation during early seedling development may elicit a protective mechanism (i.e. developmental arrest), as it likely reflects poor growth conditions at a stage when the young seedling is highly vulnerable. There is evidence that the inhibition of seedling growth by high concentrations of exogenous sugars may occur by increasing ABA biosynthesis (reviewed in Gazzarini and McCourt, 2001).

Expression of the *CnABI3* gene conferred sugar hypersensitivity and enhanced the inhibition of post-germinative growth of seedlings of the transgenic lines incubated in various concentrations of glucose. Whether the high concentrations of glucose promoted endogenous ABA synthesis that in turn inhibited early seedling growth is not clear.

However, this hypothesis is generally inconsistent with the findings of the present study. Because the lines expressing the *CnABI3* gene exhibited lower ABA sensitivities than that of the wild-type, inhibition of growth should be weaker in the former. However, the enhanced growth inhibition of the transgenic seedlings intimates that there are complex interactions between sugar signalling pathways and CnABI3. Further, the synergistic effect between CnABI3 and sugar in the inhibition of growth is obviously not limited to the seedlings' responsiveness to ABA. Of course I cannot directly compare the results of exogenous ABA application and a treatment that may increase the endogenous level of ABA.

Although poorly understood, transduction of sugar signals involves interactions with hormone signal transduction networks involved in ABA, GA, cytokinin, ethylene and auxin action (Gazzarrini and McCourt, 2001; Finkelstein and Gibson, 2002; Arroyo *et al.,* 2003; Price *et al.,* 2003). *ABI4, ABI5* and *CTR1* are all involved in sugar responses, although their expression is differentially regulated by glucose (Arroyo *et al.,* 2003); over-expression any of *ABI3, ABI4* or *ABI5* genes in *Arabidopsis* confers glucose hypersensitivity (Brocard *et al.,* 2002; Finkelstein *et al.,* 2002). Furthermore, sugar insensitive mutants (exhibiting reduced sensitivity to the presence of sugars during germination and early seedling development) were later discovered to be allelic to previously known mutations in ABA biosynthesis or sensitivity (Finkelstein *et al.,* 2002 and references therein). Thus hormonal and sugar regulation of gene expression and plant function are intimately linked, particularly in the case of the transition from seed maturation to germination (Bradford *et al.,* 2003).

The expression of the *CnABI3* gene strongly inhibited root growth of the transgenic seedlings on the 6% glucose medium, which contrasted dramatically with the effects of sugar on root growth of the wild-type seedlings. The roots of the wild-type seedlings kept growing for a prolonged time (i.e. more than a month, but the seedlings were never able to develop shoots); the transgenic seedlings exhibited no root elongation, even after 30 days (data not shown). Similar results were reported for transgenic expression of the maize *VP1* gene in *abi3-6* plants; in these transgenic seedlings, the maize VP1 protein enhanced ABA inhibition of root growth (Suzuki *et al.,* 2001). Moreover, the overexpression of an *ABI3* gene in a wild-type *Arabidopsis* background also inhibits root growth in the presence of ABA (Parcy *et al.,* 1994). A mechanism for the role of ABI3 in the regulation of lateral root development has been reported recently (Brady *et al.*, 2003).

4.4.5 Novel features of the gymnosperm ABI3 orthologue

The gymnosperm ABI3 orthologue has similar but not identical functions to its angiosperm counterparts. The complementation work of the present study revealed unique features of the CnABI3 protein and uncovered novel functions of ABI3/VP1 proteins in general. Two physiological processes that were not fully complemented in the transgenic plants by the conifer protein included an incomplete restoration of ABA sensitivity as far as the inhibition of seed germination is concerned and an inability to fully suppress early flowering. In these respects, CnABI3 was also clearly different from VP1. Some of these results may be indicative of differences between the CnABI3 protein and angiosperm ABI3/VP1 proteins in the domain(s) that confer ABA sensitivity. Differences between the wild-type and transgenic seeds with respect to their ability to

metabolize exogenous ABA could underlie differences in *apparent* ABA sensitivity. However, our analyses of the ability of a metabolism-resistant ABA analogue (*S*-(+)-8'-acetylene ABA) to inhibit seed germination as compared to the natural *S*-(+)-ABA, argue against this (data not shown). The effects of the *abi3-6* mutation on ABA accumulation or on flux in other hormone pathways have not been determined and so we cannot comment on whether the differences in ABA sensitivity are modulated by hormonal cross-talk, e.g., a differential sensitivity to, or accumulation of, other hormones. The early flowering phenotype associated with mutant *abi3-6* plants may be due to the termination of quiescence-associated processes (normally maintained by ABI3) as a result of a lack of functional ABI3 protein (Kurup *et al.*, 2000). Yeast two-hybrid screens using B2 and B3 domains of the *Arabidopsis* ABI3 protein as bait identified a CONSTANS-related factor as one of four putative interacting proteins (Kurup *et al.*, 2000) and there is a genetic interaction between *ABI3* and *DET1* (*DEETIOLATED1*) in the control of flowering (Rohde *et al.*, 2000a). There may be differences in the domains of the conifer protein that interact with CONSTANS (or other regulators) such that flowering time under long-day conditions is less effectively modulated in the transgenic plants. It would be of interest to examine the diurnal/circadian changes in the expression, abundance and localization of CONSTANS (or downstream genes/proteins controlled by CONSTANS, such as FT [FLOWERING LOCUS T] and SOC1 [*SUPPRESSOR OF OVEREXPRESSION OF CO1*) under short and long-day conditions in the mutant, transgenic and wild-type *Arabidopsis* plants.

The *Arabidopsis ABI3* and maize *VP1* gene orthologues are both from angiosperms and show greater sequence similarly; thus, it is perhaps surprising that the

CnABI3 protein fully restored 12S and 2S storage protein accumulation, in contrast to the VP1 protein, which was unable to promote 12S cruciferin gene expression in *abi3-6* seeds (Suzuki *et al.*, 2001). The domains responsible for activating seed-storage-protein genes and for mediating ABA sensitivity appear to be distinct in the CnABI3 protein. The *abi3* mutation reduces storage lipid accumulation during seed development; as was the case for storage proteins, the CnABI3 protein was able to restore lipid accumulation and also promoted the synthesis of the oil body membrane protein oleosin in the transgenic lines. The structural abnormalities of the storage parencyhmya and aleurone layer cells (including thin cell walls and altered protein body morphology) were fully rescued by the conifer ABI3 protein, likely as a result of rescued biosyntheses – of cell wall components (e.g. hydroxyproline-rich glycoproteins), storage proteins and small HSPs (and/or TIPs) involved in the maintenance of protein body shape.

Perhaps the most striking novel function not previously uncovered is the ability of CnABI3 protein to modulate ER molecular chaperone abundance either directly or indirectly. ER resident molecular chaperones are elevated under circumstances where newly synthesized proteins become misfolded, for example, as a consequence of exposure to environmental stresses (e.g. heat stress or oxidative stress). While secondary factors may be operative here (related to ABI3 effecting changes in protein biosynthesis), there may be a potential role for ABI3-related proteins in the sensing of ER stress, or they may possess an ability to interact with other proteins to reduce ER stress. Recently, ABI3 has been shown to modulate the expression of a peroxiredoxin, an antioxidant implicated in the sensing of stress (Haslekas *et al.*, 2003a,b) and I have evidence from yeast two-

hybrid analyses of a specific interaction between CnABI3 and a protein of the heat stress signal transduction cascade (Chapter 5).

4.4.6 Gymnosperms and angiosperms share a high degree of conservation of ABI3 functions

Despite having novel features, it is striking that an *ABI3* gene orthologue of a gymnosperm (derived from the conifer species, yellow-cedar, *Chamaecyparis nootkatensis*) functions nearly perfectly in the angiosperm *Arabidopsis thaliana*. In Chapter 3, I demonstrated that the CnABI3 protein is able to activate seed storage-protein gene promoters (e.g. those of the napin and vicilin genes from *Brassica napus* and *Pisum sativum*, respectively) in transgenic tobacco leaves. Here I demonstrate that the functional complementation effected by the conifer protein extends to most key developmental processes, indicating a high degree of conservation of ABI3 functions between gymnosperms and angiosperms. These findings are useful for related research on seeds of conifers in which the definitive experiments may be less feasible to conduct in part because of their very long life cycles and deep dormancy. ABI3 of angiosperms participates in the regulation of processes central to seed development. The conifer system has been instrumental in showing a potential role for ABI3 in dormancy maintenance (Chapter 3; Zeng *et al.*, 2003). Defining some of the unique properties of CnABI3 (and some of the proteins that act in concert with CnABI3) may well contribute to a greater understanding of a direct role for ABI3 in the regulation of the dormancy-to-germination transition.

Chapter 5:
Identification of Proteins That Interact with CnABI3 Using a Yeast Two-Hybrid Approach

5.1 Introduction

As ABA responsive transcription factors, ABI3/VP1 proteins have a broad range of functions. When activating target gene expression, they do not work alone. For example, they act through ABREs (ABA-Responsive Elements) on target DNA to regulate ABA-responsive gene expression. However, they do not bind to ABREs directly by themselves, but rather interact with other proteins (e.g. DNA binding proteins and transcription factors), forming a complex in order to function. It is likely that ABI3 and VP1 interact with different proteins to effect their different functions. The yeast two-hybrid assay is an efficient method to detect protein-protein interactions in vivo. In angiosperms, several proteins that interact with ABI3 and VP1 have been identified by this method (Hobo *et al.*, 1999; Jones *et al.*, 2000; Kurup *et al.*, 2000; Nakamura *et al.*, 2001). A basic region leucine zipper (bZIP) factor, TRAB1, was isolated and showed true interactions with both VP1 and ABA-responsive elements (Hobo *et al.*, 1999). This work provided evidence that VP1 regulates ABA-induced transcription by interacting with another factor that binds to ABRE directly. Later, it was found that *Arabidopsis* ABI3 protein physically interacts with ABI5, also a bZIP factor and a homologue of TRAB1 (55% similar) (Nakamura *et al.*, 2001). Four other transcription factors have been

identified and showed specific interactions in vivo and in vitro with ABI3 consisting of its B2 and B3 domains (Kurup *et al.*, 2000). The same group also isolated three proteins that interact with wild oat (*Avena fatua*) VP1 (Jones *et al.*, 2000). Expression analysis of these interacting proteins in dormant and non-dormant embryos of *abi3, lec1, fus3* and wild type led the authors to suggest that these proteins participate in the regulatory network that controls embryo development, dormancy and germination (Jones *et al.*, 2000; Kurup *et al.*, 2000). Another interesting finding is that GF14, a 14-3-3 protein, interacts with VP1 and EmBP1 and is a part of the VP1 regulatory complex in the Em promoter. GF14 might act as a structural linkage between these transcriptional factors (Schultz *et al.*, 1998). Recently, two *Arabidopsis* bZIP proteins, AtbZIP10 and AtbZIP25, were found to physically interact with ABI3 and regulate seed storage-protein gene expression (Lara *et al.*, 2003).

The identification of the above proteins contributes to the understanding of ABI3/VP1 functions. However, some of the suggestions are still speculative, not conclusive. Since ABI3/VP1 play roles in many processes and functions not only in seeds, but also in vegetative tissues, it is very likely that other proteins interacting with them have not yet been identified. Screening of ABI3/VP1 interacting proteins is still an exciting research subject to pursue. In addition, conifer species may have some unique characteristics in this aspect; therefore, identification of yellow-cedar proteins that interact with CnABI3 has been carried out by yeast two-hybrid analyses.

5.2 Materials and methods

5.2.1 Construction of bait vector and target cDNA library

Stratagene's CytoTrap XR library construction kit (Stratagene, La Jolla, CA, USA) was used for the yeast two-hybrid assay to detect protein-protein interactions between CnABI3 and target proteins. The system contains a bait vector pSos, a target vector pMyr and a temperature-sensitive yeast mutant strain cdc25H that grows at 25 °C but not at 37 °C. The gene of interest (encoding the bait protein) is cloned in pSos to form a fusion protein with hSOS when expressed in vivo. A cDNA library is inserted into the pMyr vector and expressed as a fusion protein with a myristylation peptide which anchors the fusion protein to the plasma membrane. When both fusion proteins are coexpressed in the cdc25H cells, a physical interaction between the bait protein and a protein encoded by a cDNA in the target vector brings the hSOS protein and the myristylation signal together. It localizes the hSOS protein to the plasma membrane and activates the RAS-signalling pathway to enable cdc25H cells to grow at 37 °C (an otherwise restrictive growth temperature for this strain). The coding region of *CnABI3* cDNA was cloned into pSos, engineered in-frame and downstream of the hSOS protein between restriction sites *Nco*I and *Sal*I. Total RNA of yellow-cedar seeds (year 1 developing seeds and mature seeds at different stages of a 3-month stratification treatment) was extracted using a method optimized for conifers (Wang *et al.* 2000) and mRNA was purified with the PolyATtract®mRNA isolation system II (Promega, Madison, WI, USA). A cDNA library was constructed using 7 μg of mRNA and the resultant cDNA library was inserted into the pMyr vector between restriction sites *Eco*RI and *Xho*I following the instruction manual of the kit.

5.2.2 *Yeast two-hybrid analyses and identification of target cDNAs encoding proteins that interact with CnABI3*

Yeast cdc25H competent cells were first transformed with pSos CnABI3. Positive transformants were verified to be suitable in the yeast two-hybrid assay. A yeast cell culture from a single colony containing pSos CnABI3 was used in the preparation of competent cells. The pMyr cDNA library was transformed to the competent cells according to methods in the instruction manual of the CytoTrap kit. Possible protein-protein interactions were identified by the growth ability of co-transformed yeast cells on SD/Galactose (–UL) medium at 37 °C (a restrictive temperature for the mutant cdc25H strain). Putative positive colonies were picked up and verified at least twice by growing them under the restrictive conditions. Colonies that passed these verifications were used for plasmid extraction and subsequent transformation into *E. coli DH5α* competent cells. The corresponding pMyr cDNA plasmids were purified from *E. coli* and then co-transformed with pSos CnABI3 into cdc25H yeast cells to verify protein-protein interactions again. Positive interacting constructs were then sequenced with primers on pMyr vector at both ends of the inserted cDNAs for further analyses.

5.2.3 *Amplification of 5'-end of cDNA using a 5'-RACE (Rapid Amplification of cDNA Ends) method*

The isolated cDNAs encoding CnABI3-interacting proteins were not full length and lacked the 5'-end of the coding regions. A 5'-RACE method was employed to amplify the missing fragments using Invitrogen's "5' RACE system for rapid amplification of cDNA ends, Version 2.0" (Invitrogen life technologies, Carlsbad, CA, USA). Purified mRNA (140 ng) from yellow-cedar seeds was used for the first-strand cDNA

amplification. Gene specific primers designed near the 5'-end of each of the known sequences were used for reverse transcription. Poly-dCs were added to the first strand cDNA by tailing dCTP to the 3'-end of the cDNA with TdT (Terminal deoxynucleotidyl Transferase). For subsequent PCR amplification, the forward primers were universal anchor primers from the kit and reverse primers were a second gene specific primer from each of the genes. The second gene specific primer was upstream of the first one so that a nested PCR was carried out to ensure that the correct gene was amplified. Resultant PCR fragments were cloned in a pBluescript vector and sequenced for analyses.

5.2.4 Deletion constructs of CnABI3 in pSos to analyze domains of CnABI3 that interact with target proteins

In order to analyze which domains of the CnABI3 protein interact with each of the isolated target proteins, deletion constructs of CnABI3 were engineered to include a number of combinations of the four functional domains (A1, B1, B2 and B3). Resultant pSos CnABI3(Δ) constructs are: pSos A1, pSos A1B1, pSos A1B1B2, pSos B1B2B3, pSos B2B3 and pSos B3. They were all made inframe and downstream of hSOS in pSos. To achieve this, the pSos CnABI3 was digested with *Kpn*I and *Sal*I; a 2500 bp fragment, which contains the whole *CnABI3* gene coding-region and ~ 100 bp vector DNA, was recovered. This 2500-bp fragment was then digested with *Bam*HI (for constructing pSos A1), *Bst*BI (for constructing pSos A1B1) and *Eae*I (for constructing pSos A1B1B2), respectively. The corresponding fragments containing the correct domains were recovered and blunt ended, followed by *Sal*I linker ligation and digestion with *Eco*RI and *Sal*I. The three resultant fragments were then ligated with *Eco*RI - *Sal*I prepared pSos vector to obtain the final constructs. To construct pSos B1B2B3, the pSos CnABI3 was

digested with *Bam*HI to get rid of the sequence encoding the A1 domain. The pSos vector DNA (containing CnABI3 ΔA1) was then blunt ended and self ligated. To construct pSos B2B3 and pSos B3, the pSos CnABI3 vector was digested with *Bam*HI and *Mlu*I to yield a 1670 bp fragment. This fragment was then digested with *Bst*BI (for pSos B2B3) and *Eae*I (for pSos B3) and blunt ended. The two fragments were separately ligated with *Bam*HI linker with either one extra nucleotide (CGGATCCG, for pSos B2B3) or three extra nucleotides (CGCGGATCCGCG, for pSos B3). They were subsequently digested with *Bam*HI and *Sal*I and ligated with pSos CnABI3 DNA prepared with these two enzymes.

The six deletion constructs and the full-length *CnABI3* construct were individually cotransformed with each of the interacting plasmids (pMyr CnAIPs) into yeast cdc25H competent cells. Growth phenotypes were analyzed to determine their detailed protein-protein interactions.

5.3 Results

5.3.1 Three proteins were identified by yeast two-hybrid assay that interact with CnABI3

The pSos bait vector containing *CnABI3* was first transformed both alone and co-transformed with either of the two control pMyr plasmids (pMyr MAFB and pMry Lamin C from the kit) into yeast cdc25H competent cells. Results showed that CnABI3 itself and with control proteins did not cause any non-specific false positive interaction phenotypes (data not shown), indicating it is suitable to use the CytoTrap system for the identification of CnABI3 interacting proteins. For two-hybrid analyses, yeast competent

cells were prepared using pSos CnABI3 transformed cells. This improved co-transformation efficiency significantly because each cell already had the bait vector in it at the time of the library (pMyr cDNAs) transformation. From about 3 million transformants screened, initially more than a hundred candidate colonies showed various degrees of growth on selective medium at 37 °C. These candidates were subjected to at least two rounds of verification in yeast and finally 14 colonies grew very well under the restrictive conditions, indicating positive interactions between the CnABI3 protein and those proteins encoded by cDNAs in the pMyr vector. Plasmids were isolated from all the 14 yeast colonies and subsequently transformed into *E. coli DH5α* competent cells. Transformants were incubated on chloramphenicol medium to select cells containing pMyr plasmids. Purified pMyr plasmids from the *E. coli* cells were transformed back to yeast cells again together with pSos CnABI3, and all the 14 pMyr plasmids were verified to have positive protein-protein interactions with CnABI3 in vivo. Sequencing results of the 14 cDNAs showed 12 of them encode the same protein and the other two encode different proteins. They were named as *CnAIP1*, *CnAIP2* and *CnAIP3* (for *CnA*BI3 *I*nteracting *P*roteins).

For all the three proteins, the cDNAs derived from pMyr constructs were missing the 5'-end of the coding sequences. The missing coding regions were successfully amplified by a 5'-RACE method. The cDNA and amino acid sequences of the three CnAIPs have been submitted to the GenBank and their accession numbers are shown in Table 5-1.

Table 5-1. Some characteristics of the three proteins that interact with CnABI3. Protein size and pI are analyzed at http://www.expasy.org/. Cellular localization is predicted at http://psort.nibb.ac.jp ; typical domains are analyzed with http://smart.embl-heidelberg.de and http://www.ebi.ac.uk/InterProScan ; protein similarities are searched and compared using BLAST (http://www.ncbi.nlm.nih.gov/) and WU-BLAST2 (http://blast.wustl.edu/).

	CnAIP1	**CnAIP2**	**CnAIP3**
GenBank Accession Numbers	AY268950 (N) AAP31311 (P)	AY268951 (N) AAP31312 (P)	AY268952 (N) AAP31313 (P)
cDNA	1220 bp	1474 bp	1916 bp
Protein Size	298 AA, 32 kDa	438 AA, 47.2 kDa	541 AA, 59 kDa
Predicted pI	9.3	6.48	9.54
Cellular Localization	Nucleus (88%)	Nucleus (94%)	Nucleus (60%)
Similar to	*HSP-associated factor *TPR protein	**FRIGIDA* family genes of *Arabidopsis* *hydroxyproline-rich glycoprotein	*Gymnosperm putative proteins
Typical Domains Present in protein*	-TPR (Tetratricopeptide repeat) domain -STI1-like domain	Frigida family domain	Zinc finger, C2H2-type

5.3.2 Some characteristics of the three CnABI3-interacting proteins

CnAIP1 encodes a 298-amino acid protein with a predicted PI of 9.3 and an 88% possibility of nuclear localization (Table 5-1). Similarity analysis by BLAST and WU-BLAST2 programs indicated that CnAIP1 encodes a putative HSP (heat shock protein)-associated factor. CnAIP1 shows significant homology to an HSP-associated protein in *Arabidopsis* (gene *At4g22670*), with 69% identities and 77% positives between amino acids 6 – 297 of CnAIP1 (Table 5-1 and Fig. 5.1 for alignment).

Protein domain analysis showed that CnAIP1 contains three TPR (Tetratricopeptide Repeat) domains (Fig. 5.1). This makes CnAIP1 a good candidate to interact with CnABI3 because proteins containing TPRs are generally involved in a variety of functions including protein-protein interactions, the assembly of multi-protein complexes, transcription control, cell cycle regulation and protein transportation. CnAIP1 also contains MPGG repeats stretching ca. 50 amino acids and a STI1-like domain (STI1 encodes an HSP-binding protein) at the C-terminal region (Fig. 5.1).

CnAIP2 gene encodes a 438-amino acid protein with a molecular weight of 47.2 kDa and a theoretical pI of 6.48 (Table 5-1). It has a 94% possibility of localization in the nucleus. The amino acid sequence of CnAIP2 shows high degree of similarities to the *FRIGIDA* family genes of *Arabidopsis*. For example, it has 52% identities, 70% positives to the C-terminus of *At5g48385* of *Arabidopsis* between 1 to 283 amino acid residues of CnAIP2 (Table 5-1; Fig. 5.2). *At5g48385* is one of the 7-member *FRIGIDA* family genes of *Arabidopsis* (Michaels et al., 2004). The other six members also have considerable

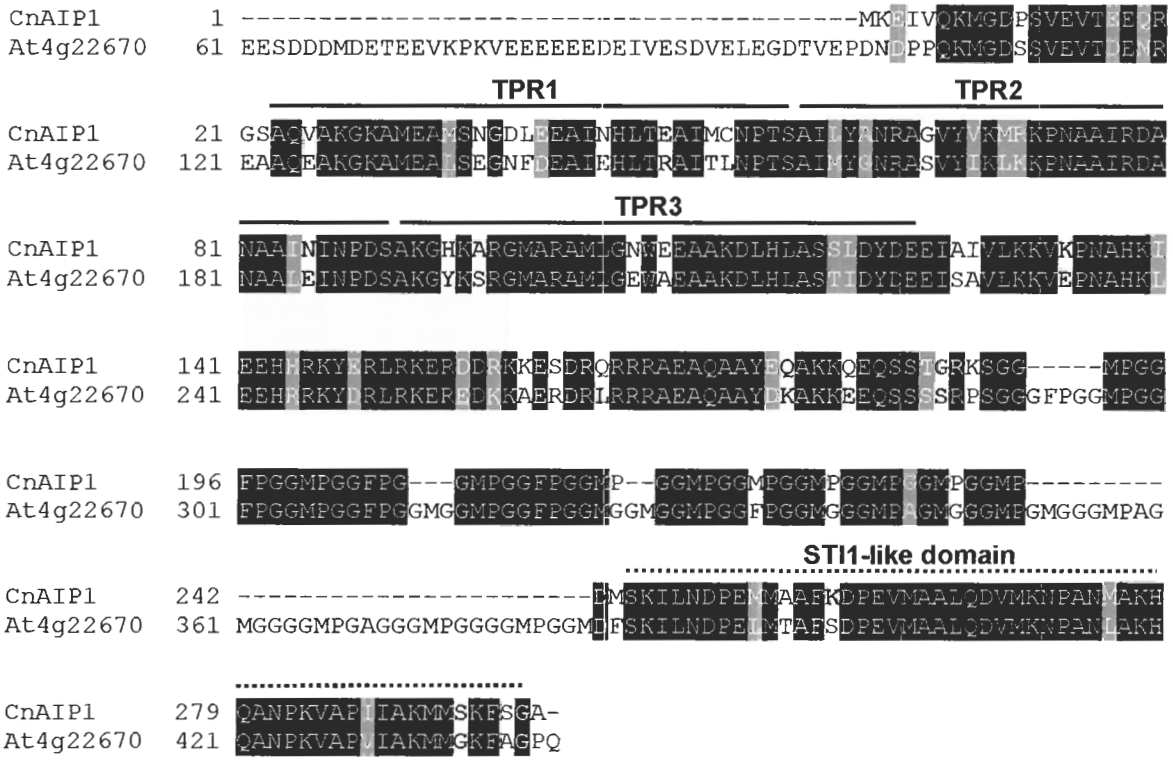


Figure 5.1. Alignment between CnAIP1 of yellow-cedar and At4g22670 of Arabidopsis. GenBank accession numbers are AAP31311 (CnAIP1) and CAB79222 (At4g22670). Identical amino acids are shown in black shade and positive amino acids are shown in grey shade. Three TPR domains are indicated with solid lines above; the C-terminal STI1-like domain is indicated with a dotted line. Overall similarities of the two proteins are 69% identities and 77% positives between amino acids 6 – 297 of CnAIP1. Alignment was generated with programs ClustalW and Boxshade.

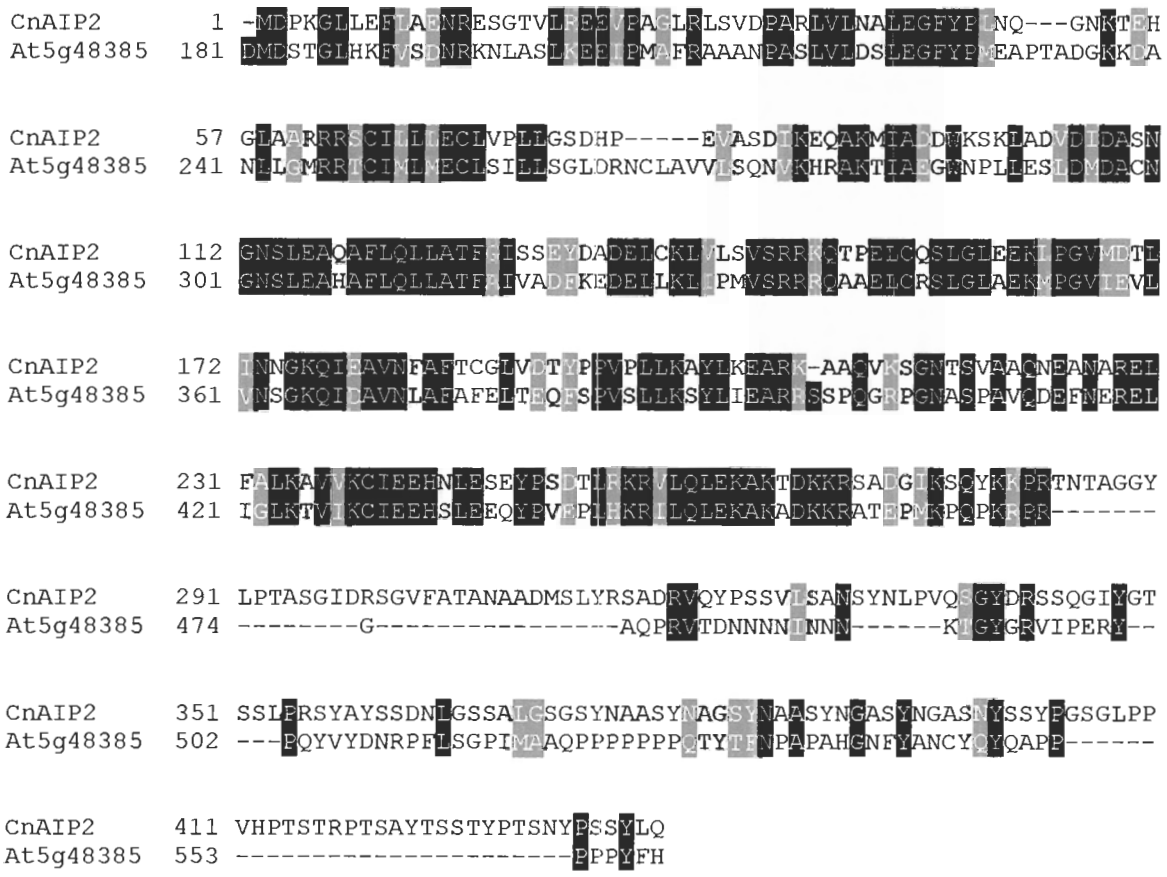


Figure 5.2. Alignment between CnAIP2 of yellow-cedar and At5g48385 of *Arabidopsis*. GenBank accession numbers are AAP31312 (CnAIP2) and NM_180592 (At5g48385). Identical amino acids are shown in black shade and positive amino acids are shown in grey shade. Overall similarities of the two proteins are 52% identities and 70% positives (between amino acids 1 – 283 of CnAIP2). Alignment was generated with programs ClustalW and Boxshade.

similarities to CnAIP2 with more than 50% positives for each of them. *FRIGIDA* genes are major players in the blocking of flowering, which may imply that ABI3 plays roles in the transition from vegetative growth to reproductive stage (see Chapter 6). CnAIP2 also has high homology to some putative proteins from rice (GenBank Accession No.: AK099919) and maize (GenBank Accession No.: AY109393).

As the largest of the three, the *CnAIP3* gene encodes a 541-amino acid protein with a predicted pI of 9.5 and a 60% possibility of nuclear localization (Table 5-1). BLAST analysis showed that CnAIP3 had high homology with two putative proteins of loblolly pine (*Pinus taeda*) (Fig. 5.3). The two partial proteins of loblolly pine were derived from an EST database (GenBank Accession Nos.: DR690502 and DR388332) and they possibly belong to the same protein. Parts of CnAIP3 also showed high similarities with other gymnosperm proteins (partial proteins from EST databases), such as proteins from Douglas-fir (*Pseudotsuga menziesii*) (CN635644), maritime pine (*Pinus pinaster*) (CR393801) and white spruce (*Picea glauca*) (DR594181) (data not shown). However, high homology was not found between CnAIP3 and any angiosperm protein, although some domains of CnAIP3 showed similarities to the domains of proteins such as the sec24 transport family protein (*Arabidopsis At3g44340*, $E=1.4e^{-07}$, 56.8 bits), snRNP-B (small nuclear ribonucleoprotein associated protein-B; *Arabidopsis At4g20440*, $E=7.9e^{-05}$, 44.8 bits) and putative transcription factors (GenBank Accession No.: EAL89768, $E=2e^{-05}$, 53.9 bits).

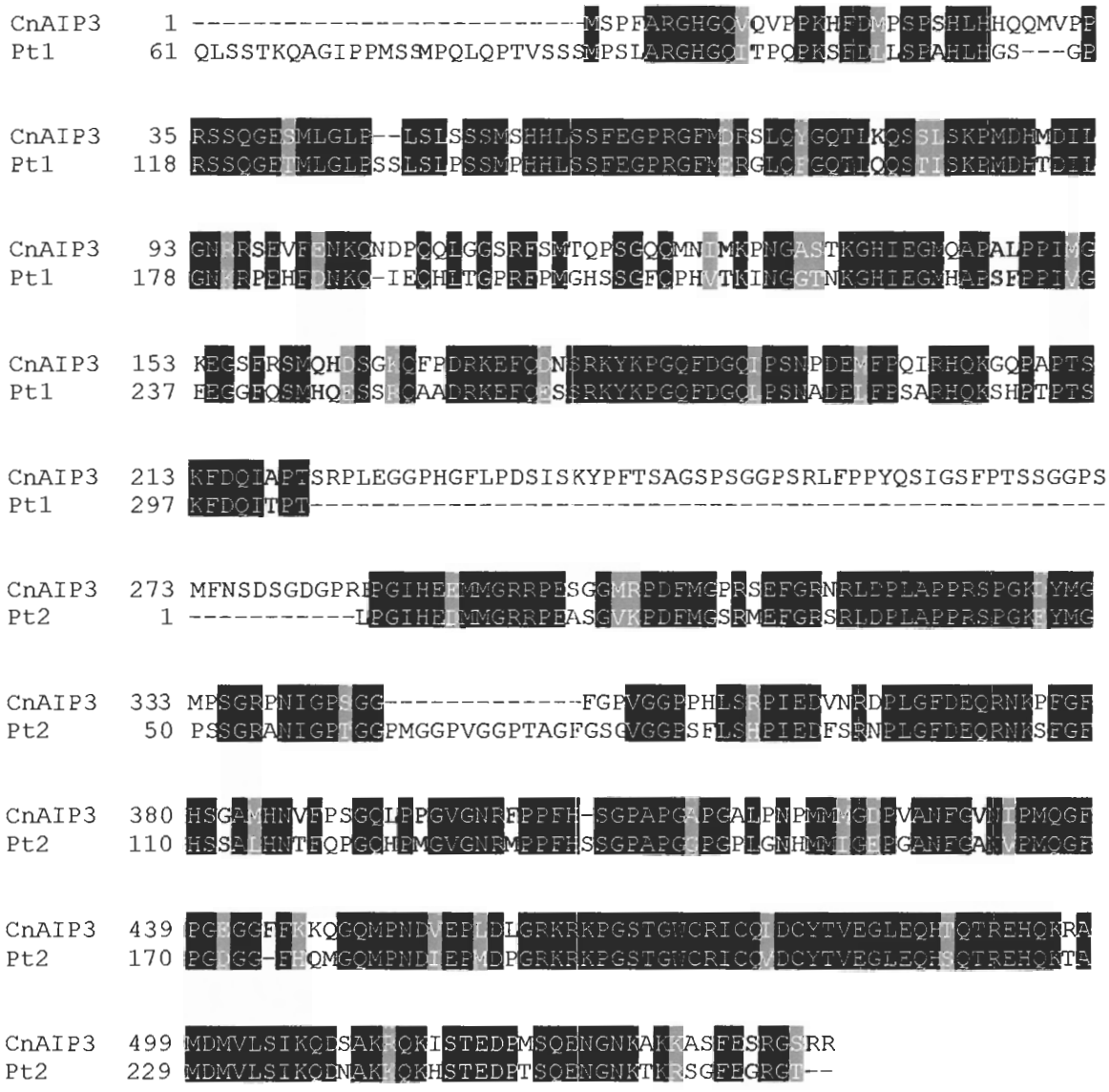


Figure 5.3. Alignment between CnAIP3 of yellow-cedar and two putative proteins of loblolly pine (*Pinus taeda*). Pt1 and Pt2 of loblolly pine are partial proteins derived from an EST database. GenBank accession numbers are AAP31313 (CnAIP3), DR690502 (Pt1) and DR388332 (Pt2). Identical amino acids are shown in black shade and positive amino acids are shown in grey shade. Overall similarities for CnAIP3 and Pt1: 65% identities and 76% positives (between amino acid 1 – 220 of CnAIP3); for CnAIP3 and Pt2: 72% identities and 80% positives (between amino acid 285 – 539 of CnAIP3). Alignment was generated with programs ClustalW and Boxshade.

5.3.3 Analyses of interacting domains of CnABI3

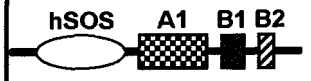
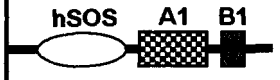
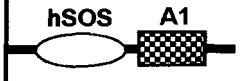
As in other ABI3/VP1 proteins, CnABI3 has four conserved domains: an acidic activation domain A1 and three basic domains B1, B2 and B3. In order to test which domains participate in the interactions with each of the three proteins, deletion constructs of CnABI3 in the bait vector were made to include a number of combinations of the four domains. As shown in Table 5.2, seven different constructs of *CnABI3* were used to cotransform yeast cells with the three interacting protein genes in the pMyr vector to examine protein-protein interactions. Each of the cotransformations was done twice and the same results were obtained (Table 5-2). Results revealed that the B1 and B2 domains of CnABI3 were required for interactions with CnAIP1 and CnAIP2, while all three B domains were required for interaction with CnAIP3. Domain A1 is not needed for any of the interactions.

5.4 Discussion

5.4.1 The selection of CytoTrap yeast two-hybrid system

Stratagene's CytoTrap XR library construction kit was used in this study because this system is based on generating fusion proteins whose interactions act in the yeast cytoplasm to induce cell growth under restrictive conditions. This has a major advantage over conventional transcription-activation based two-hybrid systems because CnABI3 itself is a transcriptional factor and may result in false positives. This was true for the screen of interacting proteins with *Arabidopsis* ABI3 and wild oat VP1 (Jones *et al.*, 2000; Kurup *et al.*, 2000). A GAL4 transcription factor based system was used in these

Table 5-2. Interactions between CnAIPs and different domains of CnABI3. Protein-protein interactions were detected in vivo by yeast two-hybrid approach. Deleted domains are labeled with "Δ". Positive interactions are shown as "+" and no interactions are indicated as "—".

CONSTRUCTS		CnAIP1		CnAIP2		CnAIP3	
		1st	2nd	1st	2nd	1st	2nd
	CnABI3 (Full)	+	+	+	+	+	+
	CnABI3 (ΔB3)	+	+	+	+	–	–
	CnABI3 (ΔB2B3)	–	–	–	–	–	–
	CnABI3 (ΔB1B2B3)	–	–	–	–	–	–
	CnABI3 (ΔA1)	+	+	+	+	+	+
	CnABI3 (ΔA1B1)	–	–	–	–	–	–
	CnABI3 (ΔA1B1B2)	–	–	–	–	–	–

assays and constructs containing B1B2B3 domains of ABI3 and VP1 were found to have non-specific activation of the reporter gene. Therefore, the researchers had to use only B2B3 domains of ABI3 and VP1 for their screening (Jones *et al.*, 2000; Kurup *et al.*, 2000). However, partial sequences of the bait proteins may not fold correctly and not result in their real conformation. The missing part (i.e. the B1 domain) is actually the interaction site in some instances; for example, the B1 domain mediates the interaction between ABI3 and ABI5 (Nakamura *et al.*, 2001). In this study, the B1 domain of CnABI3 is needed in the interactions with all the three proteins identified. Therefore, a whole bait protein is more appropriate to identify the unknown interactions. The use of full-length coding sequence of CnABI3 rendered the subsequent assays more reliable.

5.4.2 Identification and verification of interactions

In total, about three million colonies were examined to detect protein-protein interactions with CnABI3. This should be an adequate number to cover the whole cDNA library. In addition, a modification I made to the kit significantly increased the transformation efficiency. The yeast competent cells for target library transformation were prepared from cells already carrying the bait vector pSos CnABI3, rather than cotransforming the bait vector and the target vector together at the same time as recommended by the kit. Putative interacting protein candidates were subjected to a few rounds of verifications including purification of the interacting plasmids from *E. coli* transformation and subsequent new two-hybrid tests in yeast cells. Furthermore, six deletion constructs of CnABI3 were generated to perform two-hybrid assays with the three putative interacting proteins. These assays not only identified the individual domains of CnABI3 that participate in each interaction, but also verified again that the

interactions between CnABI3 and the three proteins were real, at least in vivo in yeast cells. The ultimate verification of true interactions in plant cells needs to be carried out using functional analyses in plants (see Chapter 6).

5.4.3 Some features of the interacting proteins

The three identified CnAIPs are not homologues to any of the known ABI3/VP1 interacting proteins. This is not surprising because of the far evolutionary distance between gymnosperm and angiosperm species. Within angiosperms, proteins identified to interact with ABI3 of *Arabidopsis* (Kurup *et al.*, 2000) are different from those that interact with wild oat VP1 (Jones *et al.*, 2000), even though the screens were done by the same group at the same time. Up to now, about 15 proteins (including the three CnAIPs) have been found to have physical interactions with ABI3/VP1. This number is expected to increase as more studies of the functions of ABI3/VP1 proteins in different tissues at different stages are undertaken.

All the three proteins were predicted to be localized in the nucleus, which is a positive feature expected of possible partners of the transcriptional factor CnABI3. Their similarities to other known proteins from BLAST results intimate their possible functions. CnAIP1 is highly similar to a heat-shock-protein related factor. Initially this was worrisome because heat shock proteins are common false positives in yeast two-hybrid analyses. However, it is very likely to be a true interaction with CnABI3 in this screen. The reasons for this are as follows: first, commonly HSP false positives are proteins such as HSP90 and HSP70. Based on BLAST searches, CnAIP1 is not an HSP per se, but an HSP-associated factor or HSP-interacting protein (its homologue in

Arabidopsis is *At4g22670*, or *AtHip1*, for *Arabidopsis thaliana HSP-interacting protein 1*). In my yeast two-hybrid assays, 14 constructs in total were verified to have positive interactions with CnABI3; 12 of them encoded the same protein, CnAIP1 (the other two encoded CnAIP2 and CnAIP3, respectively). This indicates that the interaction with CnAIP1 was very specific. If CnABI3 could easily bind to HSPs non-specifically, one would expect that homologues of the usual HSPs, such as HSP90 and HSP70, would be among the isolated clones. Secondly, ABI3 regulates HSP gene expression, especially that of sHSPs (small heat shock proteins) during late embryogenesis (Wehmeyer and Vierling, 2000; Vehmeyer *et al.*, 1996). The mechanism of the regulation is not yet understood. ABI3 may interact with this heat-shock related factor and play roles in activating heat-shock protein syntheses during seed development and in stress responses. A very encouraging report is that the homologue of CnAIP1 in *Arabidopsis*, AtHip1, is an orthologue of Hip (HSP70-Interacting Protein) of mammalian cells (Webb *et al.*, 2001). In mammalian cells, Hip is a component of the cytoplasmic chaperone heterocomplex that regulates signal transduction via interaction with hormone receptors and protein kinases (Webb *et al.*, 2001). Hip-Hsp70 association stabilizes the progesterone receptor at an intermediate stage in the assembly of the functional receptor complex, and results in a receptor conformation that prevents premature interaction with hormone in the cytosol (Prapapanich *et al.*, 1996, 1998). Similarly, CnAIP1 may interact with CnABI3 to play regulatory roles in the signal transduction pathways of the plant hormone abscisic acid.

CnAIP2 is closely similar to *FRIGIDA* family genes, which makes this interaction very interesting. FRIGIDA is a crucial regulator in the control of delaying flowering time

and FRL1 (FRIGIDA LIKE 1) is specifically required in this regulation (Michaels *et al.*, 2004; Johanson *et al.*, 2000). If ABI3 interacts with FRIGIDA family proteins to participate in flowering regulation, this expands ABI3 functions beyond seed development to include the important transition from vegetative to reproductive growth (see Chapter 6).

CnAIP3 has high homology to some gymnosperm proteins but lacks significant similarities to angiosperm proteins. This indicates that CnAIP3 is a relatively young gene and is more specific to conifers. It is unknown what kind of protein CnAIP3 encodes; some local sequences inside CnAIP3 have a certain degree of similarity to the domains of proteins such as snRNP-B (small nuclear ribonucleoprotein associated protein-B), sec24 transport family protein and putative transcription factors. This makes CnAIP3 an interesting candidate to interact with CnABI3 and regulate target gene expression.

Chapter 6:
Functional Analyses of Proteins That Interact with CnABI3

6.1 Introduction

There are a dozen proteins in angiosperms that have been shown to interact with ABI3/VP1 transcription factors. At least four of them are bZIP proteins. They are TRAB1 (Hobo *et al.*, 1999a), ABI5 (Lopez-Molina *et al.*, 2002), AtbZIP10 and AtbZIP25 (Lara *et al.*, 2003). ABI5 is a component of ABA signalling both in seeds and vegetative tissues and its functions have been investigated extensively (Nakamura *et al.*, 2001; Brocard *et al.*, 2002; Lopez-Molina *et al.*, 2002; Arroyo *et al.*, 2003). Functions of TRAB1 and AtbZIP10/25 are also understood well (Hobo *et al.*, 1999a, b; Lara *et al.*, 2003). AIP2 was recently found to be an E3 ligase of ubiquitin-26S proteasome pathway and has a role in ABI3 degradation (Zhang *et al.*, 2005). However, other proteins such as ABI3 interacting proteins AIP1, 3, RPB5 (Kurup *et al.*, 2000) and wild oat VP1 interacting proteins AfVIP1-3 (Jones *et al.*, 2000) are not clearly understood yet.

The three CnABI3 interacting proteins identified in Chapter 5 are the first ones from gymnosperm species. To find out their biological roles particularly in relation to interacting with ABI3 in plants is crucial for the verification of true interactions and will aid in further understanding the multiple functions of ABI3.

Of the three *CnAIPs*, the one most interesting to me was *CnAIP2* because of its significant homology to *Arabidopsis FRIGIDA* family genes. *FRI* and *FRL1 (FRIGIDA LIKE 1)* genes are major regulators in the blocking of flowering, imposing a vernalization requirement in *Arabidopsis* plants with a functional *FRI* allele (Stinchcombe *et al.*, 2004). *FRI* positively regulates *FLC* (*FLOWER LOCUS C*) expression and *FRL1* is specifically required for this up-regulation (Michaels *et al.*, 2004). *FLC* in turn negatively regulates *SOC1*, a gene that promotes flowering. Because of the role of *FRIGIDA* in flowering regulation, this intimates a potential role for *ABI3* in the control of the phase transition from vegetative to reproductive growth if there is a bonafide interaction between CnABI3 and CnAIP2. The available means to investigate the nature of a CnABI3-CnAIP2 interaction are limited using conifer species; therefore, *Arabidopsis thaliana* was selected as the testing system, taking advantage of the respective homologous genes. Several key aspects need to be addressed for possible interactions between *ABI3* and *FRIGIDA* genes of *Arabidopsis*: (i), To examine whether ABI3 physically interacts with FRIGIDA proteins in vivo. This can be tested using a yeast two-hybrid approach as described in Chapter 5. Since it is unknown if *ABI3* directly interacts with *FRI* or with other *FRIGIDA* family genes which participate in *FRI's* function, three genes were used for the test – *FRI, FRL1* and *At5g48385* (which has the highest similarity to *CnAIP2* among *FRIGIDA* genes). (ii), To investigate if *ABI3* is needed for delaying flowering regulated by *FRI.* This can be studied by crossing *abi3* mutant plants with *FRI* plants (containing a functional *FRI* gene) and by over-expressing *ABI3* constitutively in *FRI* plants. If the interaction is necessary for *FRI's* function, F2 homozygous *abi3 FRI* plants are expected to flower much earlier (because of the lack of an ABI3 protein), as compared to the *FRI*

line which exhibits a very late flowering phenotype. *FRI* plants overexpressing *ABI3* constitutively may have a "never flowering" or a significantly delayed flowering phenotype as compared to the *FRI* line. (iii), To test if *FRIGIDA* genes interact with *ABI3* in the regulation of seed dormancy. The cDNA library for the yeast two-hybrid analysis was constructed with mRNA isolated from yellow-cedar seeds; thus, *CnAIP2 (FRI)* is expressed in seeds and the ABI3-FRI interaction may contribute to dormancy inception and other maturation processes. Dormancy and germination/growth characteristics of *abi3 FRI* and overexpressed-*ABI3 FRI* lines are being examined.

CnAIP1 encodes a heat-shock protein-associated factor which may interact with CnABI3 to regulate HSPs and sHSPs during late embryogenesis or during stress responses. Small heat shock proteins have been suggested to play a general protective role during seed development of *Arabidopsis* because of the greatly reduced sHSPs amount in desiccation intolerant mutants (Wehmeyer and Vierling, 2000; Vehmeyer *et al.*, 1996). Very interestingly, the authors propose that ABI3 activates HSP17.4 transcription during seed development. The mechanism for this regulation is not known yet. Reverse genetics approaches are used to analyze functions in relation to the interaction between *ABI3* and *At4g22670* (*CnAIP1* homologue of *Arabidopsis*). Analysis of *Arabidopsis* mutants with defects in this gene (such as T-DNA inserted mutants) will uncover the significance of the interaction.

Expression analyses using northern and western blots were conducted to examine mRNA levels and protein abundance of the three *CnAIPs* and *CnABI3* in yellow-cedar seeds at different stages. These analyses will reveal the approximate stages during which

the specific protein-protein interactions occur, and thus contribute to an elucidation of their possible roles in the control of dormancy and germination/growth.

6.2 Materials and methods

6.2.1 Expression of CnAIPs and CnABI3 in yellow-cedar seeds (northern blots, antibody production and western blots)

Yellow-cedar seeds of seed lot 30156 were subjected to a dormancy-breaking treatment and then placed in germination conditions (30 °C days, 20 °C nights with an 8-h photoperiod) (Chapter 3; Ren and Kermode, 1999). Megagametophytes and embryos were excised from seeds at different stages and stored at – 80 °C before RNA and protein extraction. Northern and western blot analyses were carried out to examine mRNA and protein levels of *CnAIPs* and *CnABI3*. Methods for RNA and protein extraction, northern and western blot analyses were the same as those in Chapter 2 "Materials and Methods". An exception is that native protein gels were used for western blot analyses.

For northern blots, cDNAs of *CnAIP1, CnAIP2* and *CnAIP3* derived from the pMyr vector were used to prepare ^{32}P-labelled probes. The cDNA for the *CnABI3* probe was the same fragment as that noted in Chapter 2.

For western blots, antibodies of CnAIPs and CnABI3 were produced by genetic immunization at GENEART GmbH (Germany). The cDNAs of the four genes were supplied to GENEART and the company subcloned each cDNA into an expression vector. Vector DNA was directly injected to mice to express native proteins and to elicit antibody production. Since the antibodies were produced from proteins directly synthesized in vivo, they are suitable to detect the native forms of the proteins. Therefore,

native proteins of yellow-cedar seeds were extracted and native gels were performed for western blot analysis; SDS and beta-mercaptoethanol were omitted in all the steps of the western blot procedure. A 1:100 dilution of all four antibodies was used.

6.2.2 Analysis of SALK line T-DNA inserted mutants of At4g22670

The gene *At4g22670* has high similarity to *CnAIP1* and is likely the homologue of *CnAIP1* in *Arabidopsis*. T2 seeds of six SALK lines of T-DNA inserted *At4g22670* mutants were obtained from the Arabidopsis Biological Resource Centre (Ohio State University, USA) (Alonso *et al.*, 2003). Only four of the lines were able to germinate on Kanamycin plates and were used for further analysis. Seeds of mutants and wild type (Col.) were subjected to a 4-day moist chilling treatment (at 4 °C) before transferring to germination conditions (21 °C, 16-h light period). After germination, seedlings were transferred to soil and allowed to grow the full life cycle in a growth chamber to monitor any phenotypic differences between the mutant and the wild-type plants. Mature seeds from these plants were harvested and total seed protein extracts were analyzed by SDS-PAGE (see Chapter 4, Materials and Methods 4.2.2 and 4.2.4).

In parallel experiments, seeds of the mutants and WT were subjected to heat shock treatments during and after germination; and were grown at the above-mentioned normal conditions after the heat treatment (recovery phase) . The heat shock treatment was started from 21 °C, rising 4 degrees per h to 41 °C; thereafter 41 °C was maintained for four hours followed by a return to 21 °C at the same rate of 4 °C/h.

To identify mutant plants with homozygous T-DNA insertions, three PCR primers were designed based on the T-DNA insertion position in SALK038084 line. The forward

primer (5'ACCCTTCCCTTCTCACTACTCC3') was in the first exon of *At4g22670*; the reverse primer (5' TGAATCCCCCATCTGTATCGAA 3') spanned the third intron and the fourth exon. A second reverse primer (5'TGGTTCACGTAGTGGGCCATCG3', http://signal.salk.edu/tdna_FAQs.html) was on the left border of the T-DNA. With the T-DNA insertion, PCR is predicted to amplify a 435-bp fragment; without the T-DNA insertion, an 826-bp genomic DNA fragment should be produced. Produce of a single 435-bp band on the gel indicates a homozygous T-DNA insertion, while the presence of both bands indicates heterozygous status.

6.2.3 Cloning of three FRIGIDA family genes and ABI3 gene of Arabidopsis

The cDNAs (full-length coding sequences) of the *Arabidopsis FRI* gene and the putative CnAIP2 homologue *At5g48385* were amplified by RT-PCR. Total RNA was extracted from a functional *FRIGIDA* line CS6209 (obtained from the Arabidopsis Biological Resource Center, Ohio State University, USA). The first strand cDNA was reverse transcribed with 4 μg of total RNA and oligo$(dT)_{18}$ primer using the "RevertAid H Minus First Strand cDNA Synthesis Kit" (Fermentas Inc., Burlington, ON, Canada). Subsequent PCR amplification was carried out with PfuUltra™ High-Fidelity DNA Polymerase (Stratagene, La Jolla, CA, USA). The cDNA of *FRIGIDA LIKE 1 (FRL1)* gene was directly amplified by PCR using genomic DNA because this gene is intronless. For primers of all the three genes, an *Eco*RI recognition sequence was introduced in the forward primer and a *SalI* site was included in the reverse primer (no *Eco*RI or *SalI* sites are present in any of the three genes). The cDNA of the *Arabidopsis ABI3* gene was

Table 6-1. Primers used for amplifying *ABI3* and three *FRIGIDA* family genes of *Arabidopsis thaliana.*

Gene	Forward Primer (5' → 3')	Reverse Primer (5' → 3')
FRI	CACCGAATTCATGTCCAATTATCCACCGAC	AGTCGACCTATTTGGGGTCTAATGATG
FRL1	CACCGAATTCATGACGGCGAGTGAGACTAT	AGTCGACCTACTGAGAATAATAAGGCGG
At5g48385	CACCGAATTCATGGAGGATACTCGGTCAGT	AGTCGACCTAGTGAAAGTAAGGAGGAG
ABI3	CACCGTCGACGATGAAAAGCTTGCATGTGG	CGACGCGTTTTCATTTAACAGTTTGAGA

amplified by PCR using a plasmid template (supplied by Dr. Giraudat, Centre National de la Recherche Scientifique, France). A *Sal*I site was introduced in the forward primer and a *Mlu*I site was introduced in the reverse primer. The sequence CACC was added at the 5'-end of the forward primers for all the four genes so that all the PCR products were cloned into a TOPO vector (pENTR™/D-TOPO® Cloning Kit, Invitrogen Life Technologies, Carlsbad, CA, USA) for sequencing and subsequent cloning. Primer sequences for the four genes are shown in Table 6-1.

6.2.4 Yeast two-hybrid assays on FRIGIDA family proteins and ABI3 (CnABI3)

The three *FRIGIDA* family gene cDNAs were cloned into the pMyr vector MCS sites between *Eco*RI and *Sal*I, in frame and downstream of the myristylation sequence. The *Arabidopsis ABI3* cDNA was cloned in the pSos vector, in frame and downstream of hSOS. Yeast two-hybrid analysis was carried out to examine protein-protein interactions (see Chapter 5 Methods). The pSos CnABI3 and pMyr CnAIP2 were also used for cross-examinations (between CnABI3 and three FRIGIDA proteins, and between ABI3 and CnAIP2).

6.2.5 Generating crossed lines of FRI x abi3 and FRI x 35S-CnABI3

In order to investigate if ABI3 interacts with FRI to regulate flowering time, crosses were made between line CS6209 (carrying functional *FRI* alleles) and the *abi3-6* mutant to generate homozygous *FRI abi3-6* lines in F2 plants. To isolate homozygous *abi3-6* lines, mature F2 green seeds (before desiccation) were selected and germinated immediately without moist chilling treatment (mature seeds containing a functional *ABI3* allele are brown and unable to germinate without moist chilling). An additional

characteristic of homozygous *abi3-6* mutant plants was that there were no trichomes on the leaves of F2 plants. From F2 homozygous *abi3-6* plants, homozygous *FRI* lines were selected by PCR method. To distinguish *FRI* and *fri* alleles, rapid DNA extraction from F2 leaves and PCR amplification was carried out using the "REDExtract-N-Amp^{TM} Plant PCR kit" (Sigma, Saint Louis, MO, USA). Primer design was based on the 16-bp deletion in the Columbia ecotype (Johanson *et al.*, 2000). PCR amplification was carried out with a forward primer (5'-AGATTTGCTGGATTTGATAAGG-3') near the end of exon1 and a reverse primer (5'-ATATTTGATGTGCTCTCC-3') in the intron after exon1. A 224-bp fragment for the *FRI* allele and a 208-bp fragment for the *fri* allele are the expected amplification products. PCR products were separated on a 3% high-resolution agarose gel (Ambion Inc. Austin, TX, USA).

The CS6209 plants were also crossed with #7 plants (*abi3-6* plants previously transformed with *35S-CnABI3*, Chapter 4; Zeng and Kermode, 2004) to generate over-expressed *CnABI3* in *FRI* lines. The #7 plants were used as the male parents and the CS6209 plants as the female parents. The F1 seeds (containing a FRI allele) were germinated on hygromycin plates to select *35S-CnABI3* lines.

6.2.6 Flowering time measurement

Seeds of different lines were placed on half strength MS plates and subjected to a 4-day moist chilling treatment (at 4 °C) before transfer to a growth chamber for germination. Seedlings at similar stages after germination (~4-5 days) were transferred to soil and grown in a growth chamber (Conviron Model E15, Winnipeg, Manitoba, Canada) at 21 °C with 16-h light period and average light intensity (photosynthetic photo

flux) of 96 μmol/m^2/s (Quantum Meter, APOGEE Instruments Inc. Logan, UT, USA). Plants of all the tested lines were grown side by side in the same growth chamber. Growth performance was monitored daily. Flowering time was scored as the number of days from the time that seedlings were transferred to soil to the day when the first flower bud emerged. Numbers of rosette leaves were also recorded at the time of flowering.

6.2.7 Seed dormancy analysis of different FRI and ABI3 lines

To test if *FRIGIDA* genes interact with *ABI3* to regulate seed dormancy and germination, freshly harvested mature seeds of different lines were sown on water-saturated filter papers in Petri dishes and immediately incubated under germination conditions (21 °C, 16-h light period) without moist chilling treatments (because cold treatment releases dormancy). For *abi3-6* background, mature green seeds before desiccation were used since these seeds lose their viability after desiccation. For *ABI3* background, mature desiccated seeds were used. Germination percentage was scored after 7 days of incubation (*abi3-6* seeds germinated after 1 day of incubation since they were not dormant). The criterion for germination was radicle protrusion. Additional seeds of *ABI3* background were stored in dry conditions at room temperature and germination tests will be repeated using seeds at different times of storage (fresh ABI3 seeds are dormant and their germination rates are very low). The degree of seed dormancy for each line can be compared with the number of days of seed dry storage (afterripening) required to reach 50% of germination (Clerkx *et al.*, 2004). All tests were carried out in duplicates, each with ~50 seeds.

6.3 Results

6.3.1 Expression of CnABI3 interacting proteins in yellow-cedar seeds

Embryo and megagametophyte samples of yellow-cedar seeds at different stages of a full dormancy-breaking treatment, and during germination/post-germinative growth were used to examine mRNA and protein levels of *CnABI3* and the three interacting proteins. As shown in Figure 6.1A, mRNA levels of *CnABI3* were high during the stages of stratification and declined after germination. Interestingly, the expression of *CnAIP1* and *CnAIP3* remained relatively constant during all stages examined. For *CnAIP2*, the mRNA level in embryos was initially low in seeds subjected to only a 3-day water soak, but increased markedly during the dormancy-breaking warm/cold treatment. A decline in transcripts occurred at the later stages. The same general pattern of changes was evident in megagametophytes.

Antibodies against CnABI3 and the three CnAIPs were produced using a genetic immunization approach by directly expressing the proteins in vivo in mice to stimulate antibody production (GENEART GmbH, Germany). Therefore, native proteins are suitable for western blot assays. The quality of these antibodies needs to be tested carefully to determine if they can specifically recognize their respective antigens. Western blot results indicated that antibodies for CnABI3 and CnAIP3 were not ideal and resulted in several cross-reactive bands (data not shown). For CnAIP1, levels of the protein were largely unchanged during the different stages from seed stratification to early post-germinative growth, similar to the mRNAs (Fig. 6.1A, B). CnAIP2 blot showed a more interesting pattern of changes and the lower band (indicated in Fig. 6.1B) may be the authentic protein. As was the case with the transcript levels, the protein level

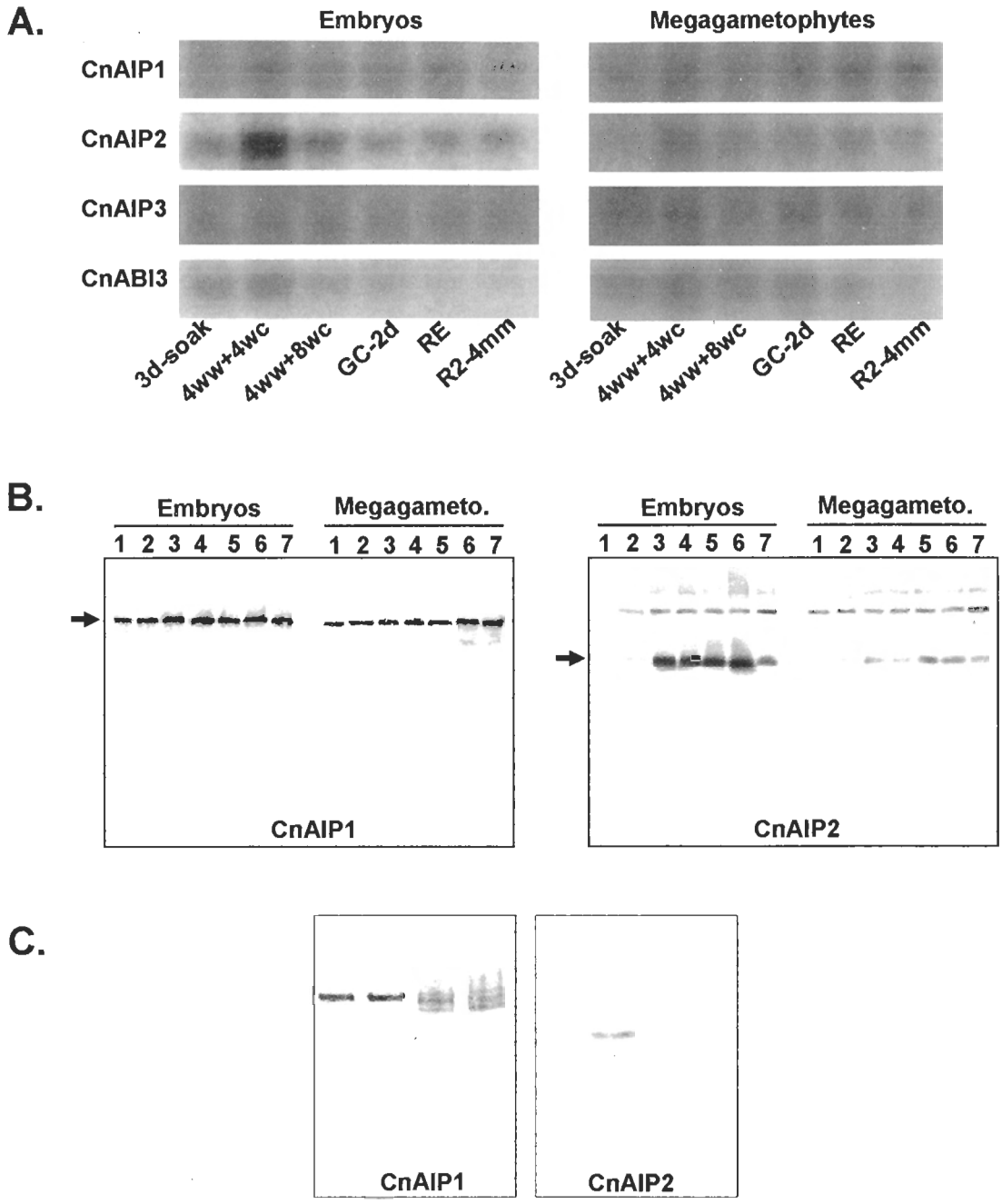


Figure 6.1. Expression of CnAIPs and CnABI3 in yellow-cedar seeds. **A,** Northern blot examining mRNA levels of CnAIPs and CnABI3 during different stages of dormancy-breaking treatments and germination/growth. **B,** Western blots of CnAIP1 and CnAIP2 using native gels. Stages of samples are: **1,** 3-day water soak; **2,** 4-week warm (following the 3-day water soak. Same for the following samples); **3,** 4-week warm + 8-week cold; **4,** 2-day at germination conditions; **5,** Radicle Emergence; **6,** Radicle 2-mm; **7,** Radicle 4-mm. Arrows indicate the possible correct proteins. **C,** Western blots comparing yellow-cedar and western white pine samples with anti-CnAIP1 and anti-CnAIP2 antibodies. In each blot, the first two samples are yellow-cedar seed extracts and the third and forth samples are western white pine seed-proteins at similar stages (3-day water soak and 2-mm growth).

was initially low but increased substantially after warm/cold dormancy-breaking treatments, especially after some exposure to cold. The level of CnAIP2 protein stayed high for a longer period than that of mRNA, indicating little degradation of CnAIP2 protein after syntheses. The levels of CnAIP2 protein at different stages also intimated that it is required for germination and very early seedling growth, and potentially for dormancy-breakage. Another interesting observation is that protein levels of CnAIP2 in megagametophyte samples were much lower than in embryos (Fig. 6.1B), which again may indicate that CnAIP2 is specifically needed for elongation of the radicle and not for reserve mobilization in megagametophytes.

Antibodies were also tested with western white pine seed samples (along with yellow-cedar samples) and more diffuse bands appeared on the blots (Fig. 6.1 C). The results indicate that these antibodies are highly specific for yellow-cedar proteins. In addition, these antibodies did not recognize any *Arabidopsis* proteins (data not shown), again indicating their specificity to yellow-cedar proteins.

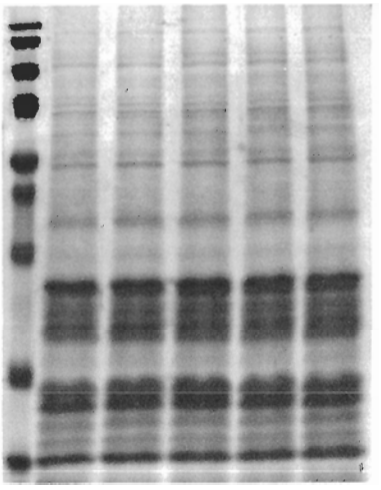
6.3.2 Analysis of CnAIP1 homologue in Arabidopsis

As indicated in Chapter 5, *CnAIP1* is highly similar to an *Arabidopsis* gene *At4g22670*; the two are very likely homologous. In order to investigate CnAIP1's possible functions in yellow-cedar, it is more feasible to look at the functions of *At4g22670* since the approaches available in yellow-cedar are limited. *At4g22670* is a heat-shock protein-associated factor and may participate in the regulation of HSPs or small heat-shock proteins (sHSPs). Six SALK-lines of T-DNA inserted *At4g22670* mutants were obtained from the Arabidopsis Biological Resource Center (Ohio State

A

LINES	T-DNA positions
1. SALK_016817	300-UTR5
2. SALK_038084	Exon
3. SALK_059751	Exon
4. SALK_118916	300-UTR5

B



C

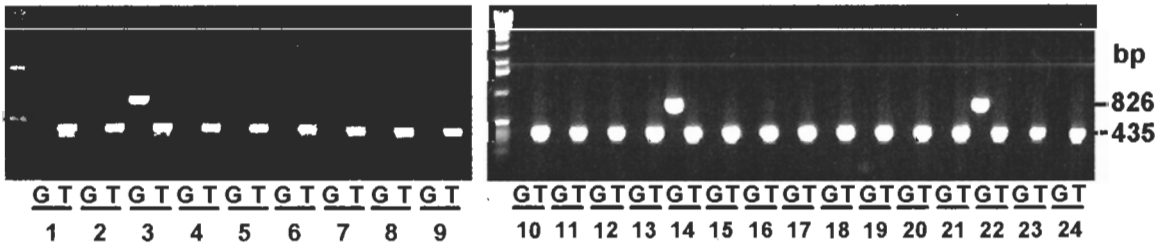


Figure 6.2. Analysis of *Arabidopsis* mutants with *T-DNA* insertions in the gene *At4g22670* (*SALK* lines). **A.** Positions of *T-DNA* insertions in the four lines. **B.** Seed total protein gel stained with Coomassie blue (50µg protein/lane). **WT**, wild-type (*Col*). **C.** PCR to select homozygous T-DNA insertion lines from SALK_038084 T3 plants. *G* – amplifying genomic DNA; *T* – amplifying T-DNA insert.

University, USA). The T-DNA was inserted at different sites of this gene (Fig. 6.2 A). Germination tests showed that only four of the lines were kanamycin-resistant and these were used for further analysis (Fig. 6.2 A). Any new phenotypes of these mutants would shed light on what functions this gene might play. However, from seed germination to the growth phase of the lifecycle, plants of the four lines did not show any apparent phenotypes that were different from those of the wild-type plants. Heat shock treatments during and after germination were performed on the mutant and WT lines but again failed to reveal any noticeable differences (data not shown). Seed total-protein gels stained with Coomassie blue showed virtually identical bands from the mutant and WT lines (Fig. 6.2 B).

T3 Plants of SALK-038084 line were screened by PCR and 21 out of 24 plants contained homozygous T-DNA inserts (only #3, #14 and #22 were heterozygous) (Fig. 6.2C). However, none of homozygous plants showed any abnormal characteristics when grown side by side with WT plants. More detailed analyses are being carried out to explore the functions of this gene.

6.3.3 Functional analysis of interactions between ABI3 and FRIGIDA family genes

The amino acid sequence of CnAIP2 has high similarities with the FRIGIDA (FRI) family proteins of *Arabidopsis thaliana* (Chapter 5). *FRI* and *FRL1 (FRIGIDA LIKE 1)* genes are major players in the blocking of flowering in *Arabidopsis*. It is interesting to investigate if *ABI3* interacts with *FRIGIDA* genes to regulate flowering time, or if the interactions in seeds regulate seed dormancy and germination. A physical interaction occurs between yellow-cedar CnABI3 and CnAIP2 proteins. Thus, it is first

Table 6-2. Interactions between ABI3 and FRIGIDA family proteins of *Arabidopsis*. CnABI3 and CnAIP2 of yellow-cedar were used as controls for cross examination. *ABI3* and *CnABI3* were cloned in the pSos vector; the *FRIGIDA* genes were cloned in the pMyr vector. Interaction analyses were carried out using a yeast two-hybrid approach. Positive interactions are shown as "+" and no interactions shown as "– ".

	ABI3	CnABI3
FRI	–	–
FRL I	–	–
At5g48385	–	–
CnAIP2	+	+

necessary to verify if physical interactions also occurs between the two counterparts of *Arabidopsis*.

Three of *FRIGIDA* family genes, *FRI, FRL1* and *At5g48385*, were cloned from *Arabidopsis* by RT-PCR using mRNA or PCR with genomic DNA (for the intronless *FRL1* gene). PCR products were inserted into a TOPO vector and sequenced to ensure the sequences were 100% correct (FRI and FRL1 were amplified again because of errors introduced by PCR the first time). The full coding sequences for the three genes are 1830 bp, 1413 bp and 1677 bp, respectively. They were subcloned into pMyr vector of the CytoTrap kit; engineered in-frame and downstream of the myristylation signal peptide sequence (see Chapter 5). The cDNA of *Arabidopsis ABI3* was also amplified by PCR, and confirmed by sequencing before it was subcloned into the pSos vector. pMyr constructs of the three *FRIGIDA* genes, as well as the *CnAIP2* gene were cotransformed into yeast cdc25H competent cells together with either pSos ABI3 or pSos CnABI3 to analyze protein-protein interactions. Results did not show positive interactions between FRIGIDA proteins and ABI3 (Table 6-2). Interestingly, there was a strong physical interaction between ABI3 of *Arabidopsis* and CnAIP2 of yellow-cedar (Table 6-2).

In order to investigate if *ABI3* interacts with *FRIGIDA* genes to regulate flowering time, crosses were made to generate ABI3 null and over-expressing mutants in plants carrying functional *FRIGIDA* alleles. To achieve this, *abi3-6* mutants were crossed with CS6209 to generate *FRI abi3-6* lines. Plants containing homozygous alleles for both genes have been obtained by screening ~200 F2 plants. If *ABI3* interacts with *FRIGIDA* to delay flowering, these *FRI abi3-6* plants are expected to have an early flowering phenotype (because of the lack of an ABI3 protein). At the same time, plants expressing

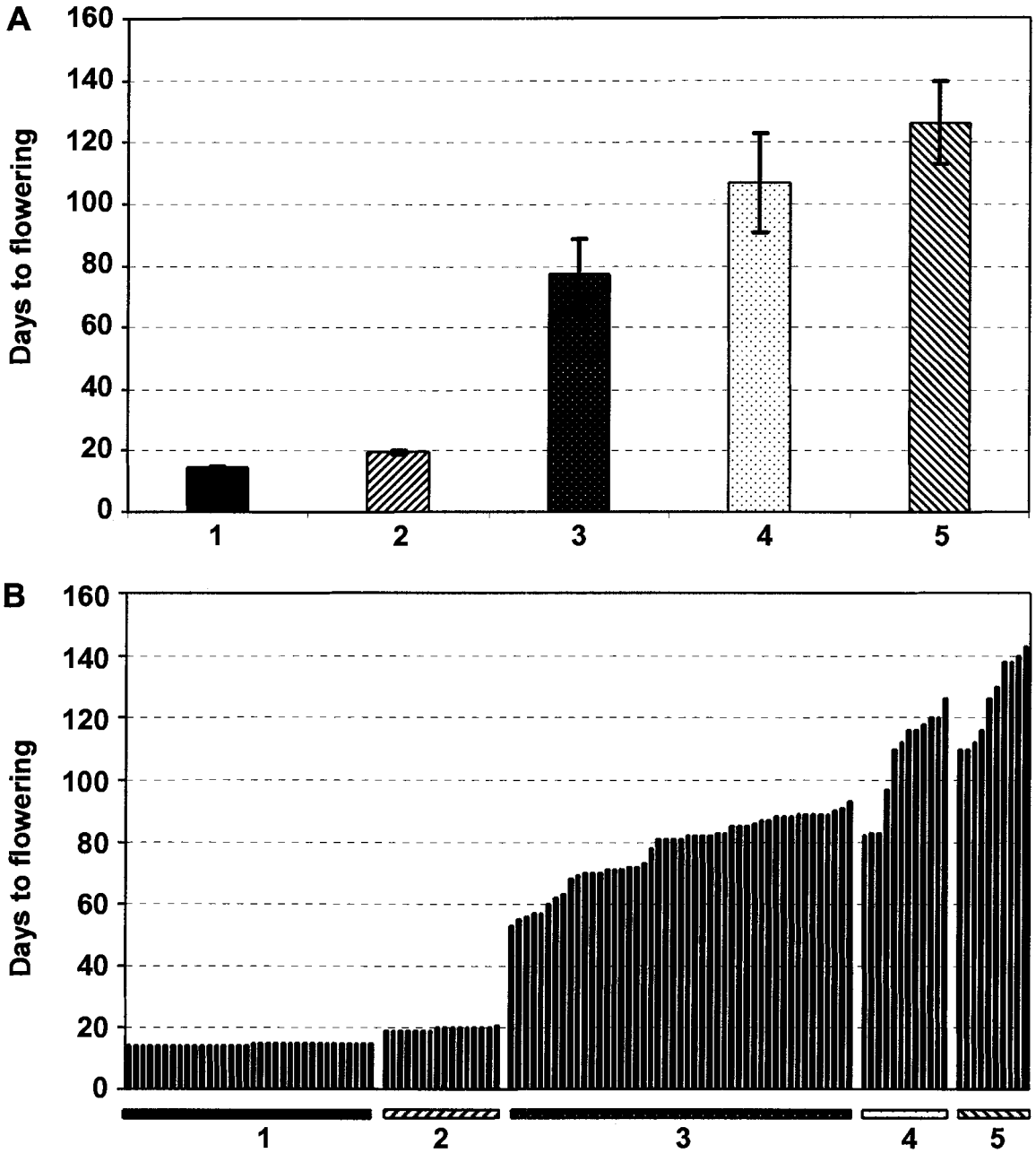


Figure 6.3. Flowering times of plants from different lines of *Arabidopsis*. **A.** Average flowering time of each genotype. **B.** Flowering time of individual plants showing variations in each genotype. Each bar represents an individual plant. Genotypes compared are: **1,** *fri abi3-6* (*abi3-6* mutants); **2,** *fri ABI3* (WT-*Col.*); **3,** *FRI abi3-6* (F2 plants carrying homozygous *FRI* and *abi3-6*); **4,** *FRI ABI3* (CS6209); **5,** *FRI 35S-CnABI3* (F1 crossed plants).

CnABI3 constitutively (#7 plants, carrying *35S-CnABI3*, see Chapter 4) were also crossed to CS6209 plants. Progenies containing both *FRIGIDA* and constitutively overexpressed *CnABI3* might flower significantly later than CS6209 plants. Indeed, as shown in Figure 6.3, the *FRI abi3-6* plants flowered from 53 to 93 days, substantially earlier than CS6209 plants (carrying *FRI* with a functional *ABI3* gene), which flowered from 82 to 126 days. The overexpressing *FRI 35S-CnABI3* lines (F1 plants) flowered even later than the CS6209 plants, ranging from 110 to over 143 days (some of them had still not flowered at the time of writing my thesis, more than 160 days). As a sharp contrast, the *abi3-6* and the WT (Col) plants (carrying a non-functional *fri* allele), flowered significantly earlier than all the *FRI* lines (Fig. 6.3). Also note that the flowering times for *FRI* plants of the same genotype showed a considerable long range (i.e. more than 40 days between the first and the last *FRI* plant flowered). In addition, the flowering times of different *FRI* genotypes were overlapping.

CnAIP2 was isolated in a yeast two-hybrid assay using a cDNA library constructed with mRNAs from seeds. This intimates that the CnAIP2 protein functions in seeds and possibly interacts with CnABI3 to control seed dormancy and germination. Therefore, it was worthwhile examining if any interactions between *ABI3* and *FRIGIDA* genes affected the degree of seed dormancy. Germination analyses on different lines showed that all the *abi3-6* plants, regardless of whether they carry *fri* or *FRI*, were totally non-dormant and fully germinated overnight (Table 6-3). Germination of freshly harvested seeds of CS6209, *FRI 35S-CnABI3* and Columbia WT plants were similar at 7 days, all showed a low % germination (Table 6-3). More germination tests are underway

Table 6-3. Germination of freshly harvested mature seeds from different lines of *Arabidopsis*. Seeds were placed on water saturated filter papers in Petri dishes and directly incubated under germination conditions without moist chilling treatment.

Genotype		Germination Percentage (%)	
		Day1	Day7
1	*fri abi3-6*	100 ± 0	
2	*fri ABI3*		4 ± 1
3	*FRI abi3-6*	99 ± 1	
4	*FRI ABI3*		4.5 ± 0.5
5	*FRI 35S-CnABI3*		8 ± 2

to compare dormancy of the lines accurately using seeds after different times of dry storage (afterripening).

6.4 Discussion

6.4.1 Expression of CnAIPs in yellow-cedar seeds during dormancy-breakage and germination/growth

CnABI3 was found to play a role in dormancy maintenance in yellow-cedar seeds (Chapter 3; Zeng *et al.*, 2003). The expression levels of the three *CnAIPs* at different stages of dormancy termination and during/after germination were examined to determine if they were also involved in seed dormancy control. Antibodies for the three CnAIPs and CnABI3 were produced by genetic immunization by directly expressing proteins in vivo in mice. Preliminary results showed that the antibodies for CnAIP1 and CnAIP2 were likely highly specific because they reacted CnAIP1 blots to a single polypeptide and to a dominant polypeptide on CnAIP2 blots. These antibodies were very specific to yellow-cedar proteins; recognition of western-white pine proteins was diminished and they did not bind to *Arabidopsis* proteins at all. However, antibodies against CnAIP3 and CnABI3 were not ideal.

The level of CnAIP1 mRNA and protein was constant throughout all the stages, indicating a constitutive function. In contrast, protein and mRNA levels of CnAIP2 demonstrated a clear temporal change. Initially the amount of CnAIP2 was low in mature seeds (and after a 3-day water soak) but was induced after a period of moist chilling. Protein and mRNA levels were correlated very well in this change. The elevated level of protein in the embryos indicates that CnAIP2 is needed during germination and very

early post-germinative growth. At the same time, western blots also revealed that CnAIP2 stayed somewhat constant and is not specific for post-germinative events such as programmed cell death and reserve mobilization in the megagametophytes. Since CnAIP2 is induced during a dormancy-breaking treatment, it may be required for the transition to germination.

6.4.2 Undetermined functions of CnAIP1 homologue in Arabidopsis

Because of the limitations of methods that can be used to analyze gene functions in conifer species, *Arabidopsis thaliana* was chosen for a comparative analysis using homozygous genes to the yellow-cedar ones, hoping that they play similar functions in both species. This was true for *CnABI3* and *ABI3* as demonstrated in Chapter 4. A reverse genetics approach was employed to study the *CnAIP1* homologue in *Arabidopsis*. CnAIP1 has almost 70% identities in amino-acid sequence to that of the *Arabidopsis* gene *At4g22670*. Mutants produced by T-DNA insertion into this gene were used to examine any abnormal phenotypes as compared to the WT plants. However, no visible differences were observed during germination, nor during the entire growth cycle. Seedlings subjected to heat shock treatments responded similarly and there were no differences on gels of total seed proteins. Possible reasons for this may be due to the existence of functionally redundant genes in *Arabidopsis*. *At4g22670* (or *AtHip1*) encodes a heat shock protein associated factor and there is a great possibility that multiple genes share the same function; for example, *AtHip2* may be able to compensate the loss of *AtHip1* functions (Webb *et al.*, 2001). It is also possible that subtle differences in phenotypes exist but require other analyses to reveal them. Therefore, more experiments are being carried out for a more detailed examination. Small heat-shock proteins (sHSPs)

are abundantly expressed during seed maturation and play a general protective role in desiccation tolerance. Interestingly, ABI3 might activate the synthesis of sHSPs such as HSP17.4 (Vehmeyer *et al.*, 1996; Vehmeyer and Vierling, 2000). In sunflower embryos, ABI3 activates the expression of a chimaeric gene driven by a small heat-shock protein hsp17.7 promoter. In addition, ABI3 and heat shock factors have synergistic effects in this activation (Rojas *et al.*, 1999). These results lead to similar speculations about the significance of the interaction between CnABI3 and CnAIP1 during development of yellow-cedar seeds.

6.4.3 Does ABI3 interact with FRIGIDA genes to regulate flowering?

CnAIP2 is highly similar to *Arabidopsis FRIGIDA* family genes (especially *At5g48385*). *FRI* is a major regulator in the delay of flowering and variations in *FRI* gene quantitatively determine flowering time (Michaels *et al.*, 2004; Johanson *et al.*, 2000; Stinchcombe *et al.*, 2004). If ABI3 truly interacts with FRI, then ABI3 very likely participates in the control of flowering. Previous work suggests that ABI3 may play a role in the flowering process. For example, one of the *Arabidopsis* ABI3-interacting proteins is similar to CONSTANS, a gene that acts in flowering control pathways (Kurup *et al.*, 2000). In addition, *abi3* mutants (such as *abi3-6*) show early flowering phenotypes (see Chapter 4). For these mutants, one explanation could be that the overall growth of the plants is faster and results in earlier flowering. However, ABI3 may play a more direct role, controlling vegetative growth and maintaining the current developmental stage. Thus it may act at several key transitions and participate in delaying flowering.

Since the *Arabidopsis ABI3* and the yellow-cedar *CnABI3* genes are orthologues, and *FRI* genes are highly similar to *CnAIP2*, the first question was whether *ABI3* and *FRI* proteins also physically interact with each other in vivo. Because it was unknown which of the *FRI* family genes might interact with *ABI3*, three of them were selected for yeast two-hybrid analysis. The reasons that these three genes were chosen were that *FRI* is clearly a major regulator in blocking flowering and *FRL1* is required for *FRI's* function; the third gene selected was *At5g48385* because among *FRIGIDA* family genes, it has the highest homology to *CnAIP2*. All three genes were separately cloned into the pMyr vector and *ABI3* was cloned into pSos for protein-protein interaction analysis. However, none of the three *Arabidopsis* proteins interacted with ABI3 in yeast cells. It could be that the FRIGIDA proteins do not physically interact with ABI3; or if there is any interactions, another member of the FRIGIDA family may be required in the oligomer along with ABI3 (presumably this member participates in FRI's functions). Alternatively, the interacting proteins are incorrectly folded when expressed as fusion proteins in the yeast two-hybrid system. The CnABI3 of yellow-cedar did not interact with the FRIGIDA proteins either. Interestingly, there was a strong interaction between ABI3 of *Arabidopsis* and CnAIP2 of yellow-cedar. These results indicate that although CnAIP2 has great sequence similarities with FRIGIDA proteins, the domains for protein-protein interactions may differ from each other. This raises the question of whether the *CnAIP2* and *FRIGIDA* genes play similar roles in the two distant species. It would be safe to say that even if they share some functions, they also likely have unique characteristics and different roles as well.

Flowering time data from the crossed lines clearly demonstrated the roles of *ABI3*, as well as *FRI*, in the regulation of flowering. Evidence was not enough to conclude that physical interaction between ABI3 and FRI was required for flowering regulation; however, this possibility cannot be ruled out either. All the *FRI* plants flowered significantly later than the *fri* plants, confirming the dominant role of a functional FRI protein in this process. The *FRI* plants carrying the *abi3-6* allele flowered earlier than those carrying the *ABI3* allele, which was promising. However, the earliest still took 53 days to flower and the later ones took as long to flower as some of the *FRI* plants with an *ABI3* gene. This is in comparison to about 20 days required for the WT (Col) plants that had a non-functional *fri* gene. If *FRI* must interact with *ABI3* in order to function, the *FRI abi3-6* plants were expected to flower much earlier on average. There is a possibility though, that without the presence of a functional ABI3 protein, FRI might interact with another factor with partial function similar to ABI3; for example, a B3 domain protein such as FUS3 or LEC2. It is interesting that when *CnABI3* was over expressed in *FRI* plants, the average flowering time was indeed later compared to Cs6209 *FRI* plants; again, there was a considerable overlap between the two genotypes. Regardless of whether there was a *FRI* or *fri* background, *abi3-6* plants exhibited early flowering compared to *ABI3* plants, which indicates that *ABI3* might directly interact with some factors other than *FRI*. Most importantly, *ABI3* is somehow involved in the regulation of flowering.

Preliminary data from germination tests do not show that *FRI* is involved in seed dormancy. It is in agreement with the expression of *CnAIP2* in yellow-cedar seeds that *CnAIP2* is induced after a period of cold treatment during stratification.

Functional analyses for *CnAIP1* and *CnAIP2* were carried out in *Arabidopsis* and there is always the question that if the *Arabidopsis* homologous genes (based purely on sequence similarities) have the same functions as those in conifer species. *Arabidopsis* is suitable for preliminary investigations. Ultimately, the functions of yellow-cedar proteins still need to be tested in yellow-cedar itself.

Chapter 7:
Conclusions and Future Work

7.1 Conclusions

This study has focused on the characterization and functional analyses of the first cloned gymnosperm *ABI3* gene, *CnABI3*. Research has been carried out with numerous approaches in multiple biological systems including conifer species, tobacco, *Arabidopsis* and yeast cells.

The yellow-cedar *CnABI3* was verified to be a true orthologue of *ABI3/VP1* genes of angiosperms; it shared great similarities with its angiosperm counterparts, while exhibiting unique characteristics as well. A role of CnABI3 in dormancy maintenance and in the inhibition of germination/growth of conifer species was established. Results also revealed a potential role of western-white pine ABI3 in oxidative stress responses during early seedling growth. The ability of CnABI3 to function as a transcriptional activator was demonstrated by ectopically expressing the *CnABI3* gene in tobacco leaves to activate genes driven by seed storage-protein gene promoters. Functional conservation of ABI3 between gymnosperms and angiosperms was elucidated by a near perfect performance of the *CnABI3* gene in *Arabidopsis abi3-6* plants. Three yellow-cedar proteins that physically interact with CnABI3 were identified using a yeast two-hybrid approach; their possible biological functions in relation to CnABI3 were investigated in

yellow-cedar and in *Arabidopsis*. Results suggest a global role for ABI3 in the control of many key transitions in seeds and plants, and a potential role in the control of flowering.

7.2 Future work

The immediate future work will be continuing studies on the functional analyses of CnABI3-interacting proteins. More experiments need to be carried out in detail. For all the three *CnAIPs*, their expression in yellow-cedar will be examined with seeds at more stages including developing seeds, and seeds after stress treatments (e.g. oxidative stress and heat stress treatment). For *CnAIP1*, reverse genetics approaches will be continued to find out what roles the *CnAIP1* homologue in *Arabidopsis* may play. Whether ABI3 physically interacts with the At4g22670 protein will also be analyzed using in vivo and/or in vitro methods. Expression analysis of related genes (e.g. genes encoding sHSPs) will be conducted in different lines (T-DNA insertion, *abi3*, *WT*, and crossed lines) of *Arabidopsis* to explore the functions of the interaction between *ABI3* and *At4g22670*. For *CnAIP2*, more analysis will be carried out on different *FRI* and *fri* plants. F2 and F3 plants with homozygous *35S-CnABI3* and *FRI* genes will be monitored. Expression of genes downstream of *FRI* (such as *FLC* and *SOC1*) will be analyzed in different lines. Plants constitutively overexpressing *Arabidopsis ABI3* in the *FRI* background will be generated and analyzed.

Longer-term future work will try to answer the question of whether ABI3 is a global regulator throughout the plant lifecycle to control key transitions between different developmental stages. In vegetative growth, ABI3 is usually undetectable; it may be expressed in only a few shoot apical meristem cells, and interact with other factors to

determine cell fate. Once cell fate is determined, e.g. in a leaf primordium, *ABI3* gene may cease to express. This can be examined by in situ hybridization (mRNA, protein) or in situ RT-PCR using shoot tips from plants with various genetic backgrounds (mutants, overexpressing lines, crossed lines and WT) of different species. Almost all the known functions of *ABI3* are involved in safeguarding and maintaining plants in the present status (e.g. seed dormancy inception and maintenance, monitoring early post-germinative growth together with ABI5, elevated expression in the shoot apex during dark growth, and a possible role in the blocking of flowering). Therefore, by interacting with other regulators, ABI3 may serve as a global "gatekeeper" to maintain the current developmental stage and prevent seeds, seedlings, and plants from entering the next stage prematurely.

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