THE ROLE OF ABSCISIC ACID METABOLISM IN WESTERN WHITE PINE (*Pinus monticola* Dougl. Ex D. Don) SEED DORMANCY

By

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ABSTRACT

Western white pine seeds exhibit deep dormancy at maturity and require several months of moist chilling to reach maximal germination capacities. Toward increasing the efficiency of dormancy-breakage and improving the rate and synchronicity of germination, the effectiveness of various modified pre-chilling and chilling treatments were determined. A key parameter that defined an efficient dormancy-breaking protocol included a lengthy higher temperature soak prior to moist chilling; high moisture content and air exchange during moist chilling were also important. Dormancy of this species is primarily "coat-enhanced", imposed by the seed coat, nucellar membrane, and megagametophyte. However, other physiological aspects of the dormancy mechanism have not been explored – in particular the contribution of abscisic acid (ABA) and its metabolism. To achieve this, ABA and the metabolites, phaseic acid, dihydrophaseic acid, 7'-hydroxy ABA, and ABA-glucose ester, were quantified in western white pine seeds during three treatments that terminated dormancy. These treatments were: (1) a 98-d moist chilling period, (2) exposure to the ABA-biosynthesis inhibitor fluridone and gibberellic acid, and (3) the removal of the hard seed coat. Overall, any treatment that terminated dormancy and increased germination capacity also resulted in dramatic decreases in ABA content in both embryo and megagametophyte tissues. The catabolism of ABA occurred via several routes, depending on the stage and the seed tissue; the pathways included 8'- and 7'-hydroxylation of ABA, and ABA conjugation. In seed populations that remained dormant, ABA was maintained or returned to high levels after a transient decrease. Thus, it has become evident that ABA biosynthesis (i.e. a higher or equal capacity for ABA biosynthesis versus catabolism) is important for dormancy maintenance. As seeds transition to a germinable state, changes in ABA flux – i.e. shifts in the ratio between biosynthesis and catabolism – occur to support a catabolic state. Further evidence to support this contention comes from expression studies of zeaxthanin epoxidase (*ZEP*), 9-*cis* epoxycarotenoid dioxygenase (*NCED*), and abscisic acid 8'-hydroxylase (*CYP707A*) genes during moist-chilling-induced dormancy termination. As seeds transitioned from a dormant to germinable state, expression of ABA biosynthetic genes (*ZEP* and *NCED*) decreased while an ABA catabolic gene (*CYP707A*) increased.

Keywords: Abscisic acid metabolism, seed dormancy, germination, *Pinus*, *de novo* synthesis, coat-enhanced dormancy, cytochrome P450

To my loving and supportive parents and a friend that knows me the best……

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ABBREVIATIONS

7'OH ABA	7'-hydroxy abscisic acid
8'OH ABA	8'-hydroxy abscisic acid
ABA	abscisic acid
ABA-GE	abscisic acid glucose ester
aba / *ABA* / ABA	abscisic acid auxotroph (*mutant* / *GENE* / PROTEIN)
abi / *ABI* / ABI	abscisic acid insensitive (*mutant* / *GENE* / PROTEIN)
BLAST	Basic Local Alignment Search Tool
bp	base pair
C_x	$Carbon_{(number\ of\ carbons)}$ (e.g. five-carbon = C_5)
cDNA	complimentary or copy DNA
d	d(s)
ddH_2O	de-ionized distilled water
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPA	dihydrophaseic acid
DTT	dithiothreitol
ES	electrospray (ionization)
Fluridone	1-methyl-3-phenyl-5-(3'-[trifluoromethyl]phenyl)-4(1*H*)pyridinone
GA	gibberellic acid
GA_{4+7}	gibberellic acid A4 / A7 mixture (70:30)
h	hour(s)
HPLC	high performance liquid chromatography
min	minute(s)
MRM	multiple reaction monitoring
mRNA	messenger RNA
MS/MS	tandem mass spectrometer
N	nucleotides Adenine, Cytosine, Guanine, or Thymine
NCBI	National Center for Biotechnology Information
neo-PA	neo-phaseic acid
PA	phaseic acid
PCR	polymerase chain reaction
R	purine nucleotides Adenine or Guanine
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
rRNA	ribosomal RNA
RP-LC	reversed phase-liquid chromatograph
RT-PCR	reverse transcriptase PCR
SD	standard deviation of a series or mean
SE	standard error of a series or mean
Tris	tris(hydroxymethyl)-aminomethane
vp / *VP* / VP	viviparous (*mutant* / *GENE* / PROTEIN)
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
Y	pyrimidine nucleotides Cytosine or Thymine

CHAPTER 1

Seed dormancy and the connection to abscisic acid and its metabolism

1.1 Introduction

Reproduction is undoubtedly the most important stage in the lifecycle of an organism. Two of the most important plant phyla, the angiosperms and gymnosperms, rely upon the production of seeds to accomplish this task. The quiescent seed, as the reproductive propagule, allows for dispersal in both time and space and is a principal reason why these phyla have been so successful. Once a seed meets favourable environmental conditions (e.g. of light, water, temperature, O_2), germination can commence. *Germination* includes those events from the start of imbibition or water uptake by the quiescent dry seed and culminates with the extension of the radicle until it protrudes through surrounding structures such as the endosperm, megagametophyte, and testa (Bewley and Black, 1994). Yet some seeds fail to complete germination in seemingly favourable conditions even though they are still viable. These *dormant* seeds often need environmental cues such as periods of warm-dry conditions (after-ripening), moist chilling (stratification) or even smoke for *dormancy* to be overcome (e.g. Adkins et al., 1986; Egerton-Warburton, 1998; Flematti et al., 2004).

It may seem unusual that a seed exhibits dormancy, a built-in block to germination, but it may not be favourable for a seed to germinate in all ideal conditions. For once a seed has initiated growth and becomes a seedling, it passes from its most stress-resistant state to its most stress-susceptible state, during which it is highly vulnerable to environmental conditions, and there is no reversal to a resistant state. If a seed germinates in the fall it may not have time for successful establishment before winter and may ultimately expire. However, a seed that passes through winter and germinates in the spring has more time for vegetative growth and a better chance of survival. Thus, in nature, dormancy is an adaptive trait that improves survival by optimizing the distribution of germination over time (Bewley, 1997).

In the agricultural and silvicultural industry, dormancy is generally an undesirable trait since the grower can usually ensure conditions, in the field or greenhouse, that are optimal for their crop. Here, rapid germination and growth are paramount and production success is measured in terms of biomass. In agriculture, extensive breeding programs have reduced the degree of dormancy; this has been a traditional way to improve crop traits in general for many decades. In the forest industry, breeding programs in seed

orchards are complicated by the fact that the generation time for trees are untimely long and many species are characterized by a deeply manifested dormancy. Ultimately, dormancy (or lack there of in some cases, e.g. preharvest sprouting) still remains a problem in agriculture and forestry. From a scientific standpoint, research into dormancy, its induction, maintenance, and termination, is still in its adolescence and until we understand the mechanisms underlying dormancy, we can only contribute in a limited way to the improvement of agricultural and forestry operations. However, in recent years, substantial progress has been made towards understanding some of the molecular mechanisms underlying dormancy, which have been accelerated through the analysis of mutants (e.g. those of maize and *Arabidopsis*) that are deficient in abscisic acid (ABA) biosynthesis or response. Cloning of the genes from these mutants has opened up avenues to reduce dormancy by altering specific traits (genes) through the technology of genetic transfer. Further, we can apply knowledge from these species to other less-studied more-challenging species such as conifers. This review will center on two aspects: (i) the involvement of ABA in seed dormancy induction, maintenance, and termination and (ii) our current knowledge of ABA metabolism, its biosynthesis and catabolism.

1.2 Types of seed dormancy

Seed dormancy can be the result of an innate capacity of the mature seed to germinate, termed primary dormancy, or may occur as a result of unfavourable germination conditions encountered upon seed dispersal, termed secondary dormancy (Fig. 1.1). Primary dormancy is established during seed development and will be focused on in this review. Primary dormancy or the causes there of, can be further classified into two major types: coat-imposed or coat-enhanced dormancy and embryo dormancy. In coat-enhanced dormancy the seed remains dormant because the surrounding structures prevent germination through one or a combination of the following: interference with water uptake, mechanical restraint, interference with gas exchange, supplying inhibitors to the embryo, and/or preventing the exit of inhibitors from the embryo (Bewley and Black, 1994; see chapter 4 for further discussion). In embryo dormancy, it is the embryo that is dormant and the embryonic axis will not elongate even if the embryo is isolated and placed naked to germinate.

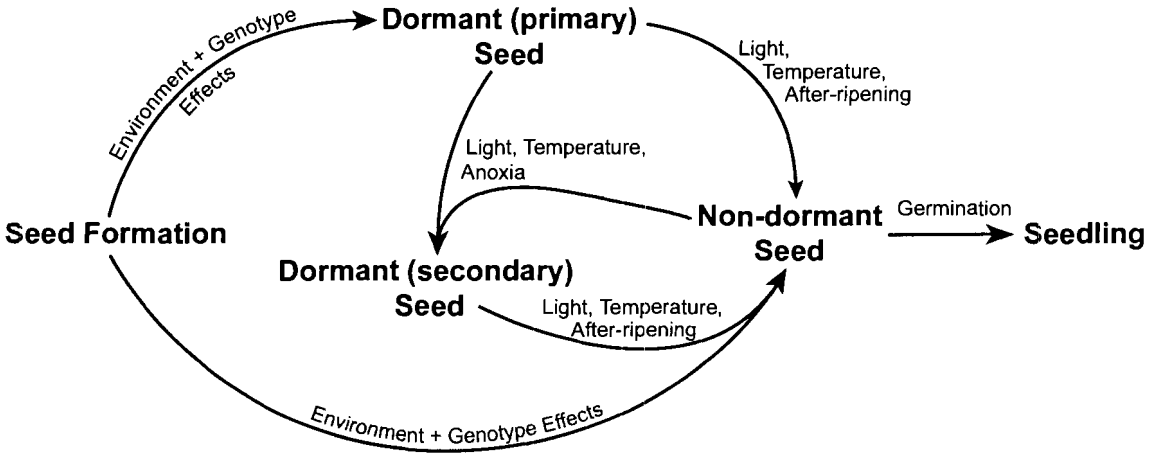


Fig. 1.1. The transition from dormancy to germination in seeds. From Bewley and Black (1994), with kind permission of Springer Science and Business Media (Plenum Publishing).

In many cases the inhibitor inside the seed, either within the embryo itself or extra-embryonic tissues such as the endosperm or megagametophyte, is thought to be ABA (Table 1.1). For instance, when *Nicotiana plumbaginifolia* seeds are imbibed there is an accumulation of ABA in dormant seeds but not in seeds that have been allowed to afterripen (Grappin et al., 2000). The ABA-biosynthesis inhibitor fluridone (section 1.5) and GA_3 (GA = gibberellic acid, see section 1.3) are efficient in breaking dormancy; both of which also inhibited accumulation of ABA during imbibition (Grappin et al., 2000). That *de novo* ABA synthesis may be important for dormancy imposition during imbibition has also been reported in *Arabidopsis thaliana* (Cape Verde Islands, Cvi, ecotype), barley (*Hordeum vulgare*), beechnut (*Fagus sylvatica*), Douglas-fir (*Pseudotsuga menziesii*), lettuce (*Lactuca sativa*), sunflower (*Helianthus annus*), and tobacco (*Nicotiana plumbaginifolia*) (Le Page-Degivry and Garello 1992; Wang et al. 1995; Bianco et al. 1997; Le Page-Degivry et al. 1997; Yoshioka et al. 1998; Grappin et al. 2000; Ali-Rachedi et al. 2004) (see chapter 5 for further discussion).

Perhaps more importantly though, there is considerable evidence that ABA plays a role in regulating the onset of dormancy during seed development and can ultimately contribute to the degree of dormancy present in a mature seed (section 1.3). For example, the ABA-deficient *sitiens* mutant of tomato possesses a very thin testa, only 1 cell layer

thick, whilst wild-type testas are 4-5 cell layers thick (Hilhorst and Downie, 1996). The ability of the *sitiens* mutant to complete germination at much lower osmotic potentials relative to the wild-type was dependent on the mutant testa, lessening the resistance to penetration by the radicle.

Table 1.1. Some seeds containing germination inhibitors. From Bewley and Black (1994), with kind permission of Springer Science and Business Media (Plenum Publishing).

Species	Seed Tissue	Inhibitor
Acer negundo	Pericarp	ABA
Avena fatua		ABA
		Short-chain fatty acids
Beta vulgaris	Pericarp	Phenolic acids
		cis-cyclohexene-1-2-diccarboximide
		Inorganic ions
Corylus avellana	Testa, embryo	ABA
Eleagnus angustifolia	Pericarp, testa	Coumarin
	Embryo	Coumarin
Fraxinus americana	Pericarp	ABA
	Embryo	ABA
Medicago sativa	Endosperm	ABA
Nicotiana plumbaginifolia	Not determined	ABA
Prunus domestica	Embryo	ABA
Rosa canina	Pericarp, testa	ABA
Taxus baccata	Embryo	ABA
Triticum spp	Pericarp/testa	Catechin, tannins

From a historical perspective, applied ABA has long been implicated in preventing germination of many mature, nondormant embryos and can inhibit the rise in activities of several key enzymes involved in reserve mobilization as these are usually post-germinative events (Bewley, 1997; Kermode, 1995, and references therein). However, recently it was found that applied ABA does not inhibit reserve mobilization that occurs prior to germination (at least in some species). In *Arabidopsis*, lipid mobilization in the 1-cell layer think endosperm was not inhibited by 10-20 μM ABA; however, lipid mobilization in the embryo, which occurs post-germinatively, was (Pritchard et al., 2002; Penfield et al., 2004). In addition, rupture of the seed coat and endosperm and mobilization (degradation) of the cell walls surrounding the radicle also proceeded in the presence of 20 μM ABA. Interestingly, these processes (lipid mobilization and cell wall hydrolysis) still required the action of GA (Penfield et al., 2004). In tobacco (*N. tabacum*)

seeds, oil mobilization as assessed by ^{13}C-NMR was not inhibited by 10 μM ABA (Manz et al., 2005). The authors suggest that, similar to *Arabidopsis*, it is reserves in the 5-cell layer thick endosperm of tobacco that are not inhibited by exogenous ABA. In addition, applied ABA did not inhibit testa rupture or the water-holding capacity of the micropylar endosperm or radicle in tobacco seeds (Manz et al., 2005).

Applied ABA may prevent germination by inhibiting specific processes within the embryo itself, as is the case in embryos of *Brassica napus*. Schopfer and Plachy (1985) found that ABA inhibited cell wall elasticity and loosening (both late germination events) in embryos, preventing radicle extension and thus preventing the completion of germination. It must be noted however, that *B. napus* embryos are non-dormant and require application of ABA to prevent radicle extension. Thus, the mode or site of action of exogenous ABA and endogenous ABA in preventing germination and maintaining a dormant state could be quite different (Bewley, 1997). Recently, an arabinogalactan-protein (AGP) in the cell walls of *Arabidopsis* roots has been implicated in the ABA inhibition of seed germination (van Hengel et al., 2003). The *agp30* mutant germinates faster in the presence of 10 μM ABA and the expression of *ABI5* and *AtEM6* were reduced in *agp30* mutants compared to wild-type. Further, the effect of this mutation was shown to be the result of an enhanced force exerted by the radicle rather than changes to the testa. The authors suggest that AGP30 plays a role in ABA perception perhaps by interacting or modifying properties of cell surface receptors or other partner molecules associated with ABA perception (although this mutation did not effect ABA sensitivity in germination assays). However, since AGPs have also been implicated in cell elongation they may affect this process rather than any direct association with ABA response (van Hengel et al., 2003). This example further demonstrates that there are active processes within the radicle that can prevent its extension. In fact there is a well-developed model showing how ABA and GA act to promote and repress germination respectively much of which is beyond the scope of this review (Fig. 1.2). Here ABA can inhibit, while GA promotes, radicle turgor and extension and the rise in enzymes responsible for degrading cell wall regions around the radicle (although evidence for ABA's involvement in the latter is somewhat tenuous) (see chapter 4, Bewley, 1997, Bentsink and Koornneef, 2002, Kermode, 2004, for further review).

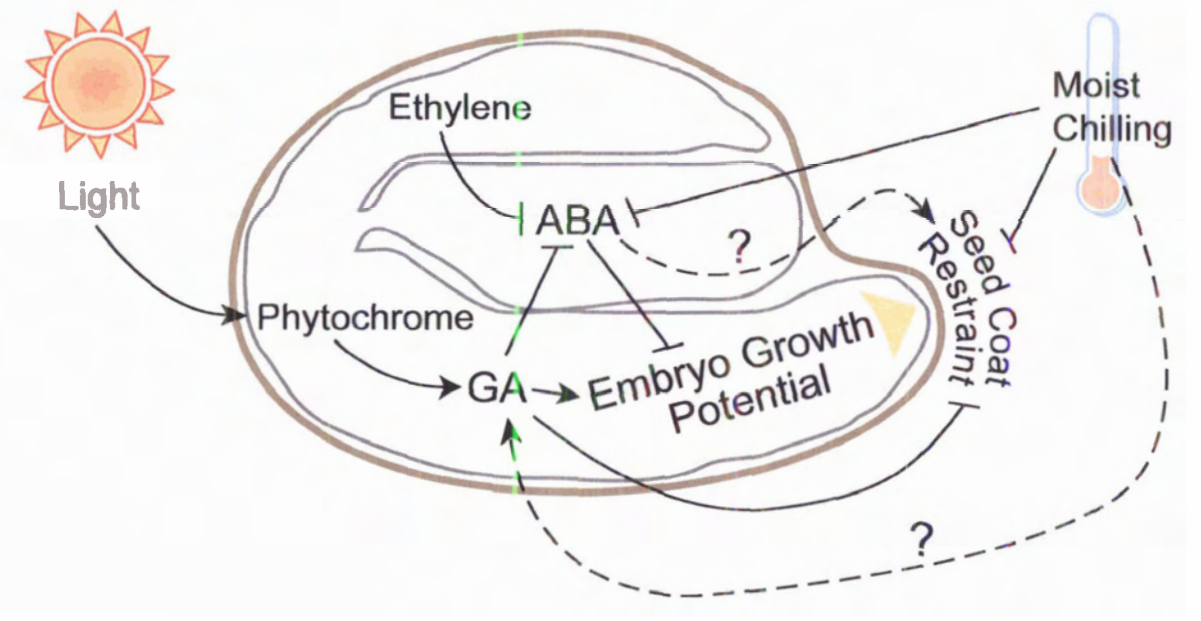


Fig. 1.2. Factors, both hormonal and environmental, affecting germination through embryo growth potential and seed coat restraint.

1.3 ABA's involvement in seed development

ABA is involved in several specific processes during seed development and, in general, is thought to keep the seed in a developmental/anabolic program (versus a germinative/catabolic one) while on the mother plant (for reviews see Kermode, 1995; Holdsworth et al., 1999; Finkelstein and Rock, 2002). Typically, ABA content in developing seeds is low in early development during histodifferentiation and embryo pattern formation, rises to peak during mid-development when cells are expanding and reserves accumulate, and falls as the seed undergoes maturation drying when the embryo (seed) becomes metabolically quiescent, dormant, and desiccation tolerant (Bewley and Black, 1994; Meinke, 1995; Bewley, 1997). In *Arabidopsis*, crosses between wild-type and ABA-deficient mutants have shown that a first peak in ABA, immediately preceding the maturation phase, is derived from maternal tissues while a second lesser peak in the embryo occurs later in preparation for desiccation. These reciprocal crosses show that only ABA produced by the embryo itself, and not maternal ABA, was necessary for dormancy imposition (Karssen et al. 1983). However, the first maternal peak in ABA is

also important and Raz et al. (2001) show that maternal ABA, along with *FUS3* and *LEC* genes (see section 1.4), is needed to prevent precocious germination.

In addition to preventing vivipary and imposing dormancy, ABA has also been implicated in a number of other mid to late embryogenic events including: the deposition of storage reserves and acquisition of desiccation tolerance. For example, ABA is required for accumulation of the β subunit of β-conglycinin in developing soybean cotyledons in culture. Fluridone, an inhibitor of carotenoid biosynthesis and ABA biosynthesis (see section 1.5), negates this increase (Bray and Beachy, 1985). In *Brassica napus* embryos in culture, normal embryogeny (i.e. prevention of precocious germination and reserve accumulation) proceeds if ABA is present in the culture medium (Crouch and Sussex, 1981). Application of fluridone during maize seed development *in planta* can also cause precocious germination but only if applied during 5-15 DAP (versus 9-14 DAP) (Fong et al., 1983).

However, ABA's precise role in development and maturation has been questioned over the years. Using cotton seed as a model, Galau et al. (1991) point out that the temporal presence of ABA and the expression of the various developmental events (i.e. maturation and post-abscission) do not correlate. It was suggested that abscission of the ovule from the parent plant plays a key role in later development, preparing the embryo for desiccation and the early events of germination and that ABA acts as an environmental regulator rather than a developmental one. Barrett et al. (1989) failed to show any difference in *in vitro* translated proteins when pea embryos were cultured in ABA, fluridone, or both. In the *Arabidopsis aba1* mutant, with only 5% the ABA of wild-type, abundance of some developmentally-associated mRNAs was not significantly reduced (Parcy et al., 1994).

However, these studies are complicated by the fact that the ABA threshold needed to initiate certain processes, such as expression of seed storage protein (SSP) genes, may be far less than the actual ABA content (i.e. ABA concentrations are saturating). Further, the ABA threshold (sensitivity) needed to induce processes such as dormancy or desiccation tolerance varies (as demonstrated by the range of phenotypes in ABA mutants). Accordingly, even different genes differ in their ABA responsiveness. For example, expression of several late embryogenesis abundant (LEA) genes in white spruce

(*Picea glauca*) somatic embryos respond differently to different concentrations of ABA and even vary in their response to ABA stereostructure (the natural S-(+)-ABA versus unnatural R-(-)-ABA enantiomers) (Dong and Dunstan, 1997).

Complicating variable ABA thresholds and sensitivities is the finding that sensitivity often changes during development. In alfalfa (*Medicago sativa*), sensitivity to ABA decreases linearly during the course of seed development and mature dry embryos require a high concentration of ABA to prevent their germination (Xu and Bewley, 1991). Embryos of sprouting-susceptible wheat (*Triticum aestivum*) lost sensitivity to ABA as the grain matured, while the sprouting-resistant cultivar continued to show sensitivity to ABA through desiccation (Walker-Simmons, 1987). In transgenic tobacco, vicilin and napin SSP gene promoters fused to GUS are responsive to exogenous ABA during development; however, after premature drying ABA-induction is virtually abolished (Jiang et al., 1996). In general, a seed's sensitivity to ABA declines during development, especially during desiccation (Kermode, 1995).

ABA contents and sensitivities are also regulated by environmental perturbations (e.g. of light, temperature, or water availability). In wheat, temperature influenced dormancy during development and in the mature grain and is ascribed to changes in ABA content or sensitivity (Walker-Simmons, 1990; Garello and Le Page-Degivry, 1999). Water stress during development of *Sorghum bicolor* increased the seed's capacity to germinate during development – correlated with a sharp decrease in ABA content and sensitivity (Benech Arnold et al., 1991). Seeds of the phytochrome-deficient (*pew1*) mutant of *Nicotiana plumbaginifolia* accumulate higher levels of ABA in the mature seeds – suggesting that ABA metabolism is controlled, at least in part, by a phytochrome-mediated light signal (Kraepiel et al., 1994; see section 1.7). Thus, during seed development, developmental processes (manifest in gene expression) respond to changing amounts of ABA and to the different sensitivities of individual organs, cells, and even genes. Accordingly, ABA amounts and sensitivities change as part of both the genetic program and as a result of environmental interaction.

Undoubtedly ABA does play a major role in seed development (deposition of storage reserves, acquisition of desiccation tolerance, prevention of vivipary, and induction of primary dormancy) and more compelling *in planta* support for ABA's involvement

comes from work with ABA mutants. The ABA-deficient tomato *sitiens* mutant, with ABA content approx 10% of wild-type, has reduced dormancy and seeds germinate viviparously in overripe fruits (Groot et al., 1991). The ABA biosynthetic mutants of maize, *Arabidopsis*, and *N. plumbaginifolia* (e.g. *viviparous (vp)*-5 or *vp14*, *aba1*, and *aba*2 respectively), show decreased levels of endogenous ABA, exhibit reduced dormancy, and in the case of the maize mutant fail to develop desiccation tolerance and germinate precociously on the ear (Karssen et al., 1983; Tan et al., 1997; Frey et al., 1999; White et al., 2000). Similarly, over- or antisense-expression of the ABA biosynthetic gene *ABA2* of *N. plumbaginifolia*, encoding zeaxanthin epoxidase, produced transgenic seeds with either delayed germination (increased dormancy) and increased levels of ABA or rapid germination (decreased dormancy) and a reduction in ABA content, respectively, when compared to the wild-type plants (Frey et al., 1999). However, these biosynthetic mutants or transgenic plants fail to demonstrate the full extent of ABA's role in seed development, owing to the fact that ABA synthesis is decreased but not abolished due to leaky or redundant pathways (see section 1.5). ABA contents in maize *vp14* embryos are approx 30% of the wild-type (Tan et al., 1997), suggesting that these lower levels are adequate to induce correct seed development (e.g. deposition of most SSPs) and only later maturation stages are affected (e.g. degree of dormancy, capacity for precocious germination).

Additional mutants have also been isolated that are diminished in their ABA-responsiveness, or ABA-sensitivity, and are able to germinate in ABA concentrations otherwise inhibitory to wild-type seeds (see Finkelstein et al., 2002, for review). Like the ABA-biosynthetic mutants, the resultant phenotypes of ABA-insensitive mutants vary depending on the gene product and the severity of the allele in question. For example, the *Arabidopsis abscisic-acid-insensitive-5-1* (*abi5-1*) mutant has a relatively weak phenotype – the seeds are desiccation tolerant, weakly dormant, slightly resistant to ABA inhibition of germination, and alter only a subset of embryonic or ABA-inducible transcripts (Finkelstein, 1994; Finkelstein and Lynch, 2000). *ABI5* has been identified as a basic region leucine zipper (bZIP) transcription factor and is among a sub-family of 13 bZIPs in *Arabidopsis* (Finkelstein and Lynch, 2000; Bensmihen et al., 2005). In reference to its weaker phenotype, it has recently been suggested that loss of a family member may

be partially compensated by increased expression of other family members (Finkelstein et al., 2005). Similarly, seeds of the *abi3-1* mutant display a reduced dormancy but are desiccation tolerant and non-viviparous (Koornneef et al., 1984; Koornneef et al., 1989). ABI3 is a transcription factor from the B3 domain family that regulates many events during seed maturation (Giraudat et al., 1992; see Finkelstein et al., 2002, for further information). However, null alleles of *abi3* (e.g. *abi3-3, abi3-4, abi3-6*) are severe and fail to complete seed maturation producing green, desiccation-intolerant seeds that are very insensitive to ABA and have reduced SSP accumulation (see section 1.4; Nambara et al., 1992; Ooms et al., 1993). The embryos, however, are morphologically normal and produce plants indistinguishable from that of the wild-type (Nambara et al., 1992). Interestingly, a similar phenotype to this *abi3* mutant was achieved in transgenic tobacco seed by expressing an ABA-specific single-chain-Fv (scFv) antibody that binds and sequesters free ABA (Phillips et al., 1997).

1.4 Examples of other regulators of seed development

ABA (and the signal it transduces) is not the only regulator of mid and late stages of seed development. Several other *Arabidopsis* loci have been identified which affect later embryogenesis (and subsequent germination). Some do not appear to be strictly related to hormone synthesis or response but may subsequently affect these processes (see section 1.7 for further discussion). These are the leafy colyledon (*lec*) class mutants: *lec1*, *lec2*, and *fusca3* (*fus3*) (Keith et al., 1994; Meinke et al., 1994). Similar to *ABI3*, these three loci encode transcription factors regulating genes during seed development. *LEC1* encodes a homolog of the CCAAT-binding factor HAP3 subunit (Lotan et al. 1998) while *FUS3* and *LEC2* belong to the B3-domain protein family that includes *ABI3* (Luerßen et al. 1998, Stone et al. 2001).

Phenotypically the cotyledons of these mutants develop trichomes and produce anthocyanins in their tips (which together give a leaf-like appearance). Like some of the maize *vp* and more severe *Arabidopsis abi3* mutants, *lec1* and *fus3* embryos can show vivipary, desiccation intolerance, and reduced protein storage accumulation, yet show a normal response to ABA (Keith et al., 1994; Meinke et al., 1994; West et al., 1994). Interestingly, embryos of null *abi3*, *fus3*, and *lec1* alleles all show characteristics of

seedlings during development (i.e. characteristics that develop after germination!) such as premature activation of the shoot apical meristem (SAM) and vascular tissue differentiation (Keith et al., 1994; West et al., 1994; Nambara et al., 1995). The transgenic tobacco expressing an anti-ABA scFv gene also shows characteristics of a seedling: green-chloroplast-containing cotyledons, reduced protein and lipid reserves, desiccation intolerance, and premature activation of the SAM (Phillips et al., 1997). These mutant and transgenic plants demonstrate that there are active pathways that repress the germinative/post-germinative program during embryo development.

Studies, especially with double mutants of *abi3*, *fus3*, and/or *lec1*, have revealed interactions between these three loci. These loci control both separate and overlapping developmental and biochemical pathways (references above and Parcy et al., 1997; Holdsworth et al., 1999; Nambara et al., 2000; Vicient et al., 2000). For instance, *ABI3* seems to interact with both *FUS3* and *LEC1* to promote chlorophyll breakdown and suppress anthocyanin accumulation during late embryogenesis (Parcy et al., 1997). *ABI3* and *FUS3* also interact, both synergistically and additively, to promote or repress expression of certain SSP, LEA, or 'germination-related' genes (Nambara et al., 2000). However, the MYB gene, *AtMYB13*, is de-repressed in *lec1* and *fus3* mutants but in a severe *abi3* mutant *AtMYB13* exists at low levels similar to the wild-type (Kirik et al., 1998). The LEA gene, *AtEm1*, is also increased (de-repressed) in the *lec1* mutant but in *abi3* and *aba1* mutants it is reduced – needing both ABA and the *ABI3* protein for its expression (Parcy et al., 1994; Vicient et al., 2000).

In deference to their suggested functions, each of these genes has different expression patterns during development, allowing for their concerted action to promote later stages in seed development such as development of desiccation tolerance and induction of dormancy while repressing germinative and post-germinative functions (Holdsworth et al., 1999, references therein). *ABI3*, *FUS3*, and *LEC1* are currently only known to have functions specific to the seed and its maturation program (although *ABI3* has also been suggested to play a role in vegetative quiescence, see below). When *LEC1* is ectopically expressed in vegetative tissues of transgenic *Arabidopsis* embryo-like growth structures are produced (Lotan et al., 1998). Similarly, ectopic expression of *ABI3* in leaves confers

the ability to accumulate several seed-specific mRNAs (e.g. SSP genes) in the presence of endogenously applied ABA (Parcy et al., 1994; Zeng et al., 2003).

In addition to their differential, synergistic, and additive functions on development, it is also noteworthy that: (i) Parcy et al. (1997) found that *lec1* mutant seeds are not sensitive to ABA contradicting Meinke et al. (1994), (ii) *FUS3* and *LEC1* positively regulate the abundance of the ABI3 protein (Parcy et al., 1997), (iii) immature *fus3* mutant siliques accumulate one third of the wild-type level of ABA, but by maturity *fus3* siliques attain wild-type levels (Nambara et al., 2000), and (iv) all three genes have been found to encode transcription factors which are thought to exert their effects by protein-DNA and/or protein-protein interactions (Giraudat et al., 1992; Lotan et al., 1998; Luerβen et al., 1998). A model has been presented where FUS3 and LEC1 act in a similar pathway, upstream of ABI3, notably because *fus3* and *lec1* themselves cause similar phenotypic/developmental effects, more widespread than that of *abi3* and can regulate the amount of ABI3 protein (Bonetta and McCourt, 1998, references therein and above). Supporting this contention, but not proving it, is the finding that *FUS3* seems to regulate ABA content during development (see section 1.7; Nambara et al., 2000). Further, Raz et al. (2001) suggest FUS3 and LEC-type genes regulate embryo growth arrest while ABI3 primarily affects subsequent embryo dormancy. Recently, it has been suggested that *LEC1* acts upstream of both *FUS3* and *ABI3* as inducible ectopic expression of *LEC1* induced both *FUS3* and *ABI3* expression, *FUS3* and *ABI3* were down-regulated in a *lec1* mutant background, and lastly ecotopic expression of SSP genes (driven by *LEC1*) were reduced in an *abi3* or *fus3* background (Kagaya et al., 2005).

Additional mutants with seemingly increased dormancy (lack of germination) are related to GA biosynthesis and response. For example, the *Arabidopsis* mutant *sleepy-1* (*sly1*) was recovered as a suppressor of the *abi1* mutation (Steber et al., 1998). *Sly1* is characteristic of a GA-response mutant, severely dwarfed plants, dark green foliage, and underdeveloped petals and stamen, yet cannot be rescued by exogenous GA application (see Karssen, 1995; Steber et al., 1998 and references therein for a further description of these mutants). GA has long been known as a promoter of germination and post-germinative events as demonstrated by the classic experiments with the barley half grain and the identification of GA as a promoter of α-amylase induction in the cereal aleurone

layer (Paleg, 1960; Chrispeels and Varner, 1967). A hormone balance theory has been invoked where ABA and GA act antagonistically to repress and promote dormancy breakage/germination, respectively (Karssen and Lacka, 1986; Karssen, 1995, references therein). However, it is generally thought that GAs are important for the promotion and maintenance of germination while ABA exerts its effects mainly during seed development, regulating dormancy induction (Bewley, 1997; Jacobsen et al., 2002; see chapter 5 for further discussion). More recently, it has been suggested that GA may play an antagonistic role during seed development – a premise that has been widely dismissed because GA mutants progress through seed development much like their wild-type counterparts (Karssen, 1995, references therein). However, it has been proposed that the balance of GA and ABA helps govern germination and maturation pathways, respectively, during maize seed development (White et al., 2000). Using either GA-deficient mutants or application of exogenous GA-biosynthesis inhibitors, White et al. (2000) found that a GA deficiency early in seed development suppresses vivipary in ABA-deficient kernels and restores desiccation tolerance. Further, GA deficiency (GA synthesis inhibition) in immature maize embryos in culture can enhance anthocyanin accumulation and accumulation of several maturation-phase mRNAs such as SSPs or LEAs (all deemed ABA responsive processes) (White and Riven, 2000). Thus, it seems that the increase in ABA during seed development not only promotes the maturation program (e.g. deposition of storage reserves, dormancy induction, and desiccation tolerance) but also represses the germinative one (e.g. precocious germination). This premise is supported by these studies in maize and also by the work on transgenic tobacco expressing an anti-ABA antibody (Phillips et al., 1997). Other mutants either associated with ABA, such as the null *abi3* mutants, or with a separate developmental pathway, such as the *fus3*, *lec1,* and *lec2* mutants, also demonstrate that there are factors present that actively repress germinative processes during seed development/maturation (Keith et al., 1994; West et al., 1994; Nambara et al., 1995; Raz et al., 2001). The mutants *fus3-2* and *lec2-1* but not *lec1-3* display a GA-dependent premature germination phenotype as demonstrated by crosses with the *ga1-3* biosynthetic mutant (Raz et al., 2001).

In summary, ABA's presence during seed development is widespread. ABA is involved in many aspects from reserve deposition to desiccation tolerance to dormancy imposition during development and in the mature seed. The examples alluded to above demonstrate how ABA can impact seed development and dormancy but also show how other factors are involved in a complex web of interactions. This web includes the interaction of various hormonal pathways (e.g. ABA with GA but also ethylene and perhaps auxin) as well as factors not currently associated with hormonal signaling (e.g. *lec1*) (see section 1.7 for further discussion). A model was presented recently where ABA was ascribed to function as a 'status quo' hormone and would function, for example, by maintaining or preventing developmental phase changes such the one during seed development to dormancy to germination. In contrast, GA could be described as a 'transitionary' hormone, promoting growth and changes in developmental states (McCourt et al., 2005). For example, GA is needed for the initiation of germination and can influence the timing of leaf emergence and decrease the time to flowering (Evans and Poethig, 1995, Bentsink and Koornneef, 2002). Further supporting this contention, the ABA-related factor ABI3, whose transcripts were detected in apices of *Arabidopsis* seedlings during growth in the dark, seems to help induce vegetative quiescence processes such as the prevention of leaf and plastid formation (Rohde et al., 1999; Rohde et al., 2000b). Thus, ABI3 not only plays a role in seed development but also helps maintain quiescence processes in other tissues (e.g. in meristem activity) – such as apices of dark-grown etiolated seedlings (see Rohde et al., 2000a). More recently, it has been proposed that light negatively modulates *ABI3* expression, mainly through changes in *phytochrome B* (*phyB*) (Mazzella et al., 2005). Although phyB is not the direct signal, light perceived by phyB in the seed regulates the later developmental decision that leads to either seedling vegetative quiescence or photomorphogenesis (Mazzella et al., 2005).

It should be noted that in addition to being thought of as an inhibitory or 'status quo' hormone, ABA is also involved in initiating and up-regulating several processes. As mentioned above ABA is considered necessary for the deposition of storage reserves and factors important in desiccation tolerance. However, ABA is also important during early seed development as it helps prevent seed abortion and promote embryo growth (Cheng et al., 2002; Frey et al. 2004). Further, ABA, at least at some basal level, is needed for

proper vegetative development. For example, leaves of *aba1* mutants are abnormally shaped and fail to develop clearly distinct spongy and palisade mesophyll layers (Barrero et al., 2005).

1.5 ABA Biosynthesis

Early research on the origins of ABA within higher plants suggested, that as a C_{15} sesquiterpenoid, ABA was synthesized *directly* from the C_5 building units isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) via farnesyl diphosphate (FPP) (Zeevaart and Creelman, 1988). Like many sesquiterpenes, it was thought that the IPP and DMAPP needed for ABA synthesis in higher plants were products of the mevalonic acid (MVA) pathway in the cytosol. These statements have since been discounted and it is now known that ABA in higher plants is synthesized *indirectly* via the tetraterpene C_{40} carotenoids and that the IPP and DMAPP needed to form carotenoids, and thus ABA itself, are formed from the methylerythritol 4-phosphate (MEP) pathway in the plastids (see Appendix, Figs. A1-A4; Rodríguez-Concepcíon and Boronat, 2002; Taylor et al., 2005).

1.5.1 Zeaxathanin to ABA

During the last decade the steps in the ABA biosynthetic pathway from zeaxanthin to ABA have been further elucidated and clarified so that the framework is now clearly established (for reviews see Cutler and Krochko, 1999; Zeevaart, 1999; Nambara and Marion-Poll, 2005; Taylor et al., 2005). Zeaxanthin is generally regarded as 'the starting point' for ABA synthesis since mutants blocked downstream of zeaxanthin show symptoms typical of ABA-deficiency (e.g. wilty phenotype) rather than carotenoid-deficiency (Taylor et al., 2000). However, zeaxanthin is not committed solely to ABA biosynthesis and the molecules zeaxanthin, antheraxanthin, and violaxanthin participate in the xanthophyll cycle involved in protection against photoinhibition and photooxidative stress (Havaux et al., 2005, references therein).

Biochemical studies along with work with mutants have provided the evidence that ABA is synthesized via carotenoids and xanthophylls. For instance, (i) the carotenoid biosynthetic inhibitors, fluridone and nonflurazon, which block the phytoene desaturase

(PDS) enzyme, also inhibit ABA buildup (Bartels and Watson 1978; Zeevaart and Creelman, 1988), (ii) *in vivo*-labeling studies using $^{18}O_2$ are consistent with ABA formation from a large precursor such as an oxygenated carotenoid (Zeevaart et al., 1989; Rock and Zeevaart, 1991), (iii) a cell-free system from oranges revealed that all-*trans*-violaxanthin, all-*trans*-neoxanthin, and 9'-*cis*-neoxanthin can all act as precursors for xanthoxin and ABA (Cowan and Richardson, 1997), and (iv) many mutants, e.g. the corn *viviparous* (*vp*) mutants, that are defective in carotenoid and xanthophyll biosynthesis are also deficient in ABA (McCarty, 1995, references therein).

In brief, ABA is formed from zeaxanthin as follows. Within the plastid (e.g. chloroplast), zeaxanthin proceeds to antheraxanthin, violaxanthin, neoxanthin, and finally xanthoxin. Xanthoxin is then converted to ABA in the cytosol by two enzymatic steps via abscisic aldehyde (AB aldehyde) (Fig. 1.3; Cutler and Krochko, 1999). ABA-biosynthetic mutants have been invaluable tools for dissection of the biosynthetic pathway, just as they helped in defining a role for ABA in seed development and dormancy (Table 1.2).

1.5.1.1 Epoxy-carotenoid synthesis, formation of the 9 *cis* isomers

Zeaxanthin is converted to violaxanthin, via the intermediate antheraxanthin, by the enzyme zeaxanthin epoxidase (ZEP) (Fig. 1.3). ZEP was first cloned from the *aba2* mutant of *N. plumbaginifolia* (*NpZEP*) by insertional mutagenesis and encodes a chloroplast-imported protein with sequence similarities to FAD-binding monooxygenases that requires ferredoxin as a reducing agent (Bouvier et al., 1996; Marin et al., 1996). *NpZEP* was able to complement the *aba1* mutation of *Arabidopsis* (Marin et al., 1996); confirming that the *aba1* locus was a *ZEP* from previous biochemical work (Rock and Zeevaart, 1991). Mutants deficient in ZEP have been isolated from *Arabidopsis* in numerous mutant screens (subsequently termed *AtZEP*) (Koornneef et al., 1982; Merlot et al., 2002; Xiong et al., 2002) and also in rice (*Oryza sativa*) (Agrawal et al., 2001).

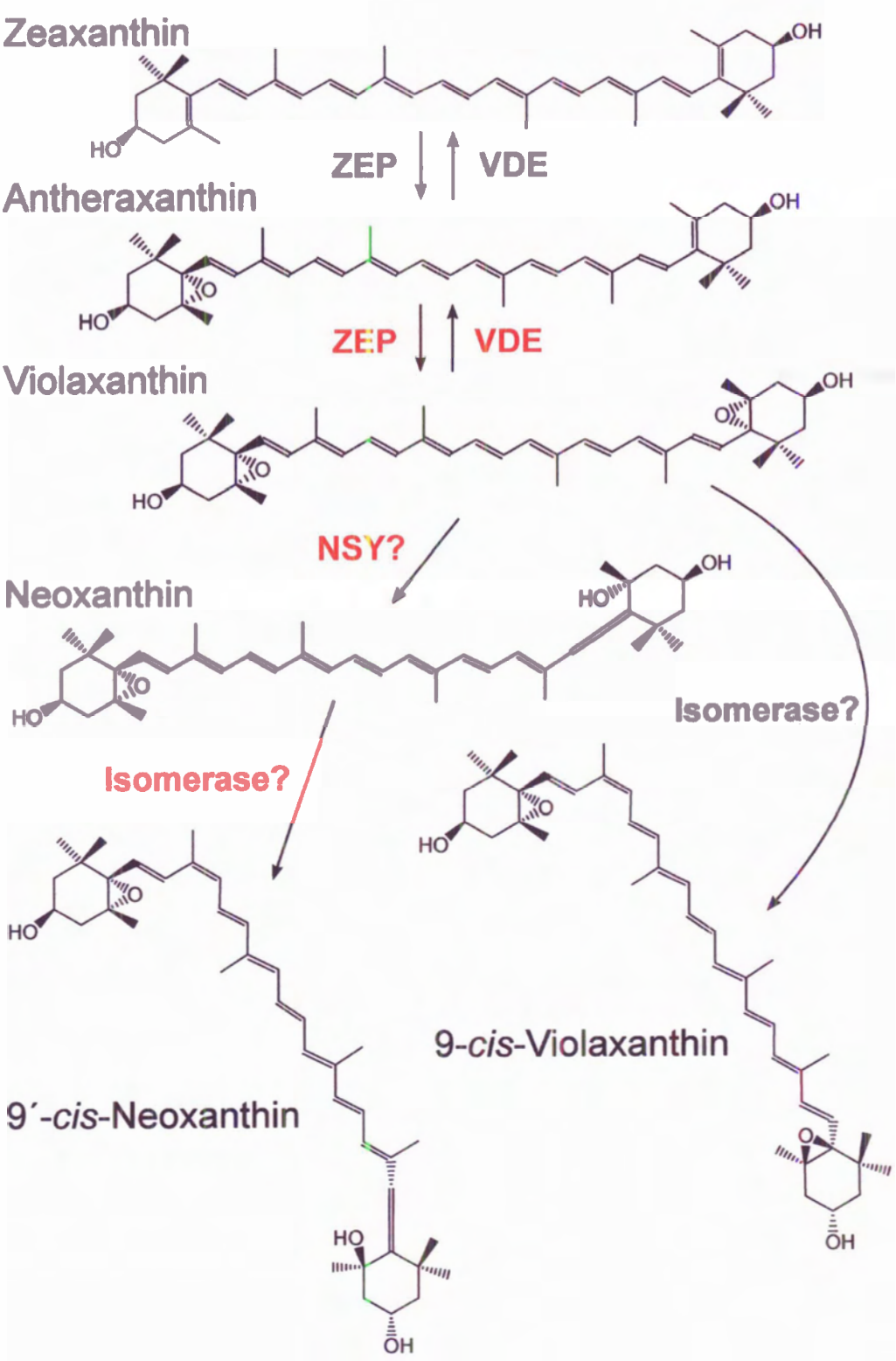


Fig. 1.3. Formation of the *cis* isomers of violaxanthin and neoxanthin from zeaxanthin. ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NSY, neoxanthin synthase.

Table 1.2. ABA auxotroph mutants impaired in the downstream steps of the ABA biosynthesis pathway (zeaxanthin to ABA).

Species	Mutant	Gene function (enzyme)	References
Arabidopsis thaliana	*aba1* *(npq2, los6)*	zeaxanthin epoxidase (ZEP)	Karssen et al., 1983; Marin et al., 1996; Niyogi et al., 1998; Xiong et al., 2002
	nced3 *nced6* *nced9*	9-*cis*-epoxycarotenoid dioxygenase (NCED)	Iuchi et al., 2001; Lefebvre et al., 2006
	aba2 *(sis4, gin1, isi4, sre1, sañ3)*	short chain dehydrogenase / reductase (AB-SDR) (Xanthoxin oxidase)	Laby et al., 2000; Quesada et al., 2000; Rook et al., 2001; Cheng et al., 2002; González-Guzmán et al., 2002; Merlot et al., 2002
	aba3 *(los5, gin5)*	Defect in molybdenum cofactor (MoCo) sulfurase (MCS) required for abscisic aldehyde oxidase function	Schwartz et al., 1997; Xiong et al., 2001b; Cheng et al., 2002
	aao3	abscisic aldehyde oxidase (AB-AO)	Seo et al., 2000a, 2000b
Lycopersicon esculentum	*notabilis*	NCED	Burbidge et al., 1997a, 1999
	sitiens	abscisic aldehyde oxidase	Taylor et al., 1988; Okamoto et al., 2002
	flacca	defect in MCS	Marin and Marion-Poll, 1997; Sagi et al., 2002
Nicotiana plumbaginifolia	*aba1*	defect in MCS	Leydecker et al., 1995 Akaba et al., 1998

Table 1.2. Continued.

Species	Mutant	Gene function (enzyme)	References
Nicotiana plumbaginifolia	*aba2*	ZEP	Marin et al., 1996
Oryza sativa	*osaba1*	ZEP	Agrawal et al., (2001)
Zea mays	*vp14*	NCED	Tan et al., 1997
Hordeum vulgare	*nar2a*	MoCo synthesis	Walker-Simmons et al., 1989
Solanum phureja	*Droopy*	defect in MoCo required for abscisic aldehyde oxidase function	Duckham et al., 1989

vp = viviparous; aao = Arabidopsis aldehyde oxidase; nar = nitrate reductase; npq = nonphotochemical quenching; los = low expression of osmotically responsive genes; sis = sugar insensitive; gin = glucose insensitive; sre = salt resistant; isi = impaired sucrose induction; sañ = salobreño

ABA contents in *N. plumbaginifolia aba2* leaves are 23-48% of the wild-type; which is noteworthy since *NpZEP* is present as a single-copy gene. This suggests either the existence of a less specific enzyme for zeaxanthin epoxidation, leakiness of the *aba2* allele, or an alternate pathway for ABA synthesis (Marin et al., 1996). Expression studies with *NpZEP* and *AtZEP* reveal that transcripts are detected ubiquitously with higher basal levels in stems and leaves compared to roots and seeds (Audran et al., 1998; Xiong et al., 2002). In leaves, *NpZEP* expression is correlated with photosynthesis, exhibiting a diurnal fluctuation due to the role it plays in photosynthesis and protection against photooxidative damage (Audran et al., 1998; Liotenberg et al., 1999). A similar oscillation of *ZEP* is observed in tomato and *Arabidopsis* (Thompson et al., 2000a; Audran et al., 2001). During drought stress *ZEP* transcripts are not induced in *Arabidopsis*, cowpea (*Vigna unguiculata*), tomato, or *N. plumbaginifolia* (Audran et al., 1998; Audran et al., 2001; Iuchi et al., 2000; Thompson et al., 2000a) and it has been suggested that the circadian control of *ZEP* in photosynthetic tissues seems to produce enough violaxanthin to sustain ABA accumulation during drought stress (Taylor et al., 2005). In fact, decreased *ZEP* transcript levels in *Arabidopsis* and *N. plumbaginifolia* and no difference in protein levels in *Arabidopsis* have been observed during drought stress (Audran et al., 1998; North et al., 2005). More evidence comes from transgenic *N. plumbaginifolia* constitutively over-expressing *ZEP*, which do not display higher ABA

contents or water stress tolerance compared to wild-type (Borel et al., 2001). However, contradictory results were reported in *Arabidopsis* where *ZEP* was induced by drought, salt, and polyethylene glycol treatment (Xiong et al., 2002). In roots, expression of *ZEP* is induced by drought in *Arabidopsis*, tomato, and *N. plumbaginifolia* (Audran et al., 1998; Audran et al., 2001; Iuchi et al., 2000; Thompson et al., 2000a). In seeds, *NpZEP* mRNA abundance has been shown to peak just before mid-development, at about the time when ABA concentrations are maximal (Audran et al., 1998). Localization of *AtZEP* during seed development by i*n situ* hydridization showed expression in the embryo cells from the globular stage to the dessication phase; in contrast, in the seed coat, *AtZEP* expression was specific to the maturation phase (Audran et al., 2001).

Thus, in non-photosynthetic tissues, where the concentration of epoxy-carotenoids is lower, expression of *ZEP* seems to have regulatory role and ZEP may be a rate-determining step for ABA and epoxy-carotenoid synthesis under these conditions (i.e. roots and seeds). Supporting the contention that ZEP may be rate-determining in seeds, over- or antisense expression of *Np*ZEP generated plants with heightened or reduced seed dormancy, respectively (Frey et al., 1999).

Several studies have indicated that *AtZEP* may be regulated by ABA by positive feedback regulation. In *Arabidopsis* seedlings, 100 μM ABA is able to induce expression of *AtZEP*. In addition, *AtZEP* also appears to be regulated by endogenous ABA. In the ABA-deficient mutants *aba1-1*, *aba2-1*, and *aba3-1*, the transcript level was significantly lower under stress conditions; although the basal level remains unchanged under non-stress conditions (Xiong et al., 2002). Supporting this, Audran et al. (2001) found that *AtZEP* was not significantly changed in leaves of *aba2-1*, *aba3-1*, *abi1-1*, and *abi2-1* plants. However, the *abi1* mutation (but not the *abi2* or *era1*) also seems to lead to significantly reduced *AtZEP* transcript levels under ABA treatment (Xiong et al., 2002). *AtZEP* is also up-regulated by glucose application (Cheng et al., 2002).

Synthesis of neoxanthin from violaxanthin and formation of the *cis* isomers of neoxanthin and violaxanthin from their *trans*-orientated cousins are ambiguities in the later stages of the ABA pathway that remain to be clarified. Putative neoxanthin synthase (*NSY*) genes have been isolated from tomato and potato (Al-Babili et al., 2000; Bouvier et al., 2000) but the tomato *NSY* was later found to be a *LCY-b*, affecting lycopene to β-

cartone synthesis (Ronen et al., 2000). However, recently, mutants lacking neoxanthin isomers where identified in *Arabidopsis* and tomato; the *Arabidopsis* gene has been cloned (Nambara and Marion-Poll, 2005). Thus, we should know, in the not too distant future, whether the putative NSY in *Arabidopsis* encodes a protein capable of producing all-*trans*-neoxanthin or both neoxanthin isomers. It is interesting that the enzymes responsible for the *trans-cis* isomerization of neoxanthin and violaxanthin have not been discovered yet and it may be that other known enzymes are responsible for this conversion – e.g. the CRTISO enzyme, responsible for the transition to all-*trans*-lycopene (see Appendix, Fig. A3).

1.5.1.2 Xanthophyll cleavage

Cleavage of the C_{40} molecules, 9-*cis*-violaxanthin and 9'-*cis*-neoxanthin, to form C_{15} xanthoxin and a C_{25} apo-aldehyde is carried out by the enzyme 9-*cis*-epoxycarotenoid dioxygenase (NCED) (Fig. 1.4). The first NCED gene was cloned from the maize (*Zea mays*) mutant *vp14* by insertional mutatgenesis (Schwartz et al., 1997b; Tan et al., 1997). Cloning of *VP14* was an important milestone not only to ABA research. It was the prototypic carotenoid cleavage dioxygenase (CCD) from plants and animals and has subsequently allowed the discovery of similar carotenoid oxygenases involved in vitamin A synthesis (Bouvier et al., 2005; Moise et al., 2005). In subsequent years, NCED/CCDs have been identified as a gene family in many plant species (see Schwartz et al., 2003a; Bouvier et al., 2005). In the *Arabidopsis* genome, 9 NCED/CCD sequences have been identified and 5 are presumed to function in ABA biosynthesis (*AtNCED2, 3, 5, 6,* and *9*) (Iuchi et al., 2001; Tan et al., 2003). Of recent interest is the characterization of *AtCCD7* and *AtCCD8*, which are involved in the synthesis of a novel carotenoid-derived signaling molecule that inhibits lateral branching (axillary bud growth) (Schwartz et al., 2004).

The NCED dioxygenase cleavage reaction takes place between positions 11 and 12 (11',12') in a *cis*-xanthophyll molecule; however, it is unknown if neoxanthin or violaxanthin is the predominant *in planta* substrate. *In vitro*, a recombinant GST-fusion VP14 protein was able to cleave both 9-*cis*-violaxanthin and 9'-*cis*-neoxanthin but not *trans*-xanthophyll isomers and required iron and oxygen to form xanthoxin (Schwartz et al., 1997b). However, several lines of evidence point to 9'-*cis*-neoxanthin as the *in planta*

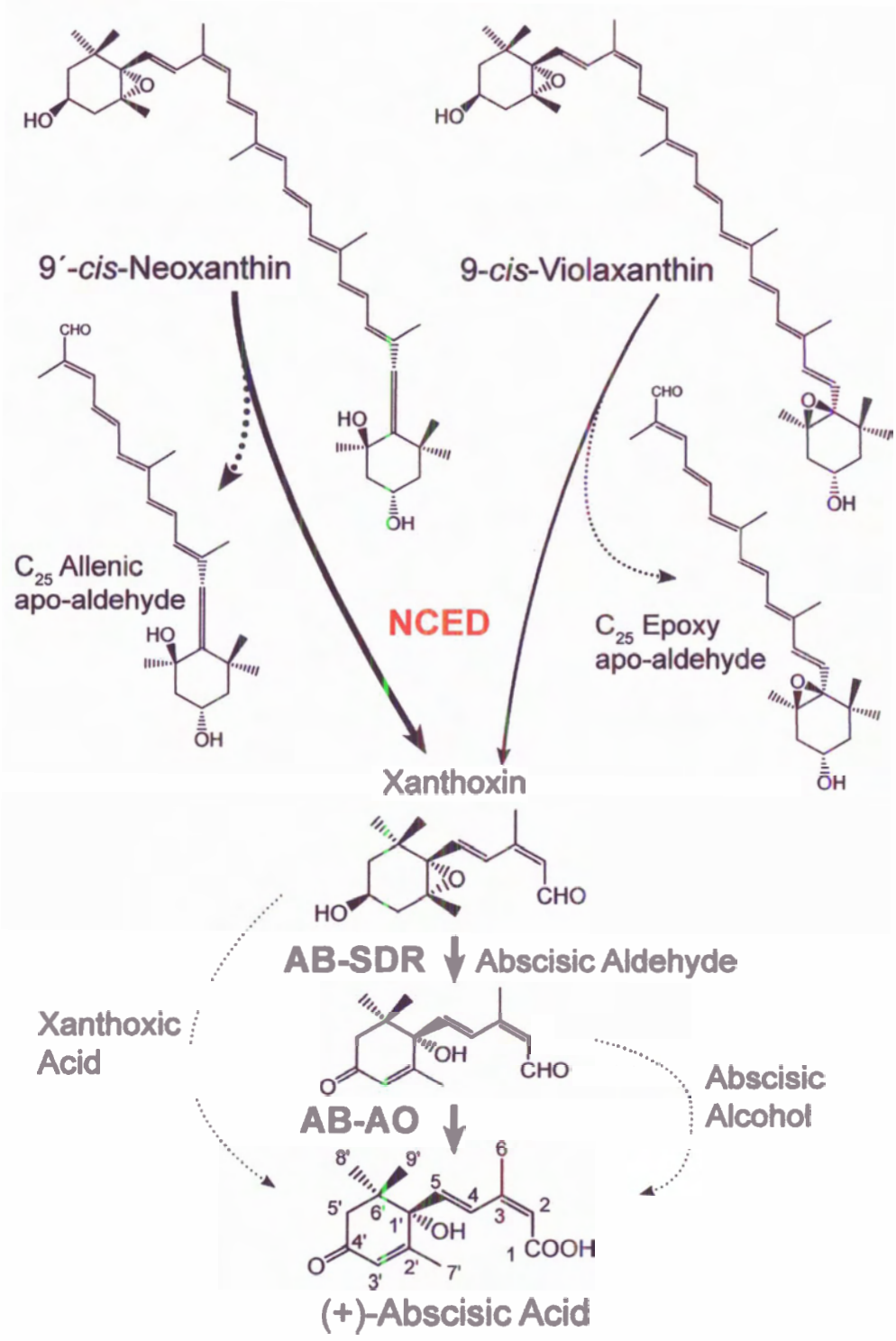


Fig. 1.4. The carotenoid cleavage reaction and formation of abscisic acid (ABA). The major pathway is though to occur through 9'-*cis*-neoxanthin, xanthoxin, and abscisic aldehyde. Other minor paths include the formation of xanthoxic acid and abscisic alcohol. The fate of the C_{25} cleavage products are unknown. NCED, nine-*cis*-epoxycarotenoid dioxygenase; AB-SDR, abscisic short-chain dehydrogenase / reductase (xanthoxin oxidase); AAO,abscisic aldehyde oxidase.

substrate. In spinach (*Spinacia oleracea*), neoxanthin exists predominately as the 9'-*cis*-isomer (>90%), whereas <10% of violaxanthin exists as the *cis*-isomer (Strand et al., 2000). The recombinant enzymes, *Pv*NCED1 from bean (*Phaseolus vulgaris*) and VP14, have lower K_m values for neoxanthin as a substrate rather than 9-*cis*-violaxanthin (Qin and Zeevaart, 1999; Schwartz et al., 2003b). However, as pointed out by Schwartz et al. (2003a), definitive identification of the endogenous substrate for NCED would require the purification of the C_{25} by-product, which has been difficult perhaps due to rapid degradation or recycling (Parry and Horgan, 1991).

As with other enzymes involved in the MEP and carotenoid biosynthesis pathways, NCED proteins from various species have been found to be targeted to chloroplasts. *Pv*NCED1, *Vu*NCED1 from cowpea (*Vigna unguiculata*), and VP14 all contain sequences targeting NCED to the chloroplast (Qin and Zeevaart, 1999; Iuchi et al., 2000; Tan et al., 2001). In fact, *Pv*NCED1 and VP14 have been specifically shown to interact with the thylakoid membrane (Qin and Zeevaart, 1999; Tan et al., 2001). About 35% of the VP14 in chloroplasts was found to be associated with the thylakoids, while *Pv*NCED1 was found to be strictly thylakoid-bound (Qin and Zeevaart, 1999; Tan et al., 2001). In *Arabidopsis*, the 5 NCEDs presumed to be involved in ABA synthesis are differentially located between the stroma and thylakoid-bound fractions and this most likely represents an additional layer of regulation for NCED activity as NCEDs epoxy-carotenoid substrates are exclusively found in the membranes (Tan et al., 2003). *At*NCED2, *At*NCED3, and *At*NCED6 were located in both the stroma and thylakoid fractions; *At*NCED5 was exclusive to the thylakoid fraction; whereas *At*NCED9 was exclusive to the stroma fraction (Tan et al., 2003).

The cleavage reaction accomplished by NCED is though to be the key regulatory point as it is the first step officially committed to ABA-biosynthesis; key especially with respect to ABA accumulation under drought stress (Schwartz et al., 2003a). Drought stress treatments were shown to induce gene expression in leaves of maize *VP14*, tomato *LeNCED1*, bean *PvNCED1*, *Arabidopsis AtNCED3*, cowpea *VuNCED1*, and avocado *PaNCED1* (*Persea americana*) (Tan et al., 1997; Qin and Zeevaart, 1999; Chernys and Zeevaart, 2000; Thompson et al., 2000a; Iuchi et al., 2001; Tan et al., 2003). In *Arabidopsis*, although *AtNCED3* is the major stress-induced form, smaller relatively

minor increases in *AtNCED2*, *AtNCED5*, and *AtNCED9* occur during stress. *AtNCED3* expression is induced within 10 min after leaf detachment. Consistent with NCEDs role in stress and its rapid response ability, it has been suggested that the intronless structure of NCED genes may be a mechanism for enhancing/preserving NCED expression and ABA synthesis under stress (Tan et al., 2003). NCED expression can also been induced when roots are dehydrated; as has been demonstrated in tomato and bean (Qin and Zeevaart, 1999; Thomson et al., 2000a). Little is known about the regulation of the NCED protein; however detailed studies with an antibody to *Pv*NCED1 reveal that protein levels closely reflect the increase in *PvNCED1* mRNA in dehydrated bean leaves and roots (Qin and Zeevaart, 1999).

It has been suggested that the above mentioned drought-induced NCED genes are orthologues (Taylor et al., 2005); however, it is clear that many of the NCED genes from the different species lack clear orthologous relationships. For example, although *VP14* and *AtNCED3* both show saturable binding to thylakoid membranes, are drought-induced in leaves, and are expressed in roots, there are clear differences. *AtNCED3* is not the dominant *NCED* expressed during seed development; *AtNCED5* and *AtNCED6* show the strongest expression in mid to late stages of seed development (Tan et al., 2003). However, *vp14* plants have 70% lower ABA content in embryos and 35% lower in water-stressed leaves (Tan *et al.*, 1997). Thus, in addition to a role in drought-stress, *VP14* also plays a significant role in ABA synthesis in seeds.

During seed development in *Arabidopsis*, two distinct peaks in ABA are observed, a sharp rise midway though development from maternal origin followed by a second less pronounced peak involved in the establishment of dormancy derived from ABA synthesis in the embryo (Karssen et al., 1983). Although, it has been postulated that the first peak of "maternal" ABA may come from *de novo* synthesis in developing embryos and that maternal ABA is only needed as a signal to trigger embryo/endosperm ABA synthesis (Xiong and Zhu, 2003). *AtNCED3*, *AtNCED5*, *AtNCED6*, and *AtNCED9* are expressed during seed development in *Arabidopsis* siliques. As mentioned above, *AtNCED5* and *AtNCED6* show the strongest expression in seeds during mid to late development and may contribute to embryo ABA levels. *AtNCED3* is expressed predominantly in maternal tissues such as the funiculus (Tan et al., 2003). *AtNCED3* also seems to play a stress-

regulatory role in seeds. *Arabidopsis sto1*, a salt-stress tolerant mutant that is allelic to *nced3*, can germinate when seeds are imbibed in a high osmotic solution (145 mM NaCl) suggesting that ABA facilitates a developmental arrest in wild-type seeds (see section 1.8) (Ruggiero et al., 2004). Similarly, other ABA-deficient mutants such as *aba1* (ZEP), *aba2* (SDR, see 1.5.3.3.1 below), and *aba3* (MCS enzyme, see 1.5.3.3.2 below) are also tolerant to salt-stress during germination (Léon-Kloosterziel et al., 1996).

Recently, during microarray analysis of *Arabidopsis Cvi* seeds, *AtNCED6* and *AtNCED9* mRNAs were found to be more than 10-fold higher in dormant seeds compared to non-dormant seeds (Taylor et al., 2005, references therein). In addition, *AtNCED6* and *AtNCED9* have been further characterized and shown to be involved in dormancy induction (Lefebvre et al., 2006). The Lefebvre et al. (2006) study provides the first evidence that ABA synthesized in both the endosperm and embryo is important for dormancy imposition. Through RT-PCR analysis of RNA from tissue collected at 10 days after pollination, *AtNCED3* was detected in both empty silique and seed tissues while *AtNCED5*, *AtNCED6*, and *AtNCED9* were only detected in seed tissues. In contrast to Tan et al. (2003), Lefebvre et al., 2006 show that *AtNCED6* and *AtNCED9* are the most abundant *NCEDs* in seed tissues. Further localization of *AtNCED6* and *AtNCED9* through promoter GUS/GFP fusions and *in situ* hydridization showed differences in spatial and temporal expression. *AtNCED6* was specifically expressed in endosperm tissues from before fertilization and throughout endosperm development. *AtNCED9*, in contrast, was expressed in both embryo and endosperm tissues. In accordance with expected gene redundancy in the *NCED* family, seeds from single mutants of *Atnced6* or *Atnced9* displayed similar degrees of dormancy compared to wild-type. *Atnced6* / *Atnced9* double mutants showed increased germination rates compared to wild-type; but still showed an intermediary rate of germination, between that of wild-type and the *aba3-1* mutant (see below). Resistance to paclobutrazol during germination was enhanced for both single and double mutants but was greater for double mutants. This may indicate that less GAs were required for *Atnced6*, *Atnced9*, and *Atnced6* / *Atnced9* germination since paclobutrazol is a cytochrome P450 monooxygenase inhibitor that inhibits GA biosynthesis (although other alternatives exist; see section 1.6 for further discussion) (Rademacher, 2000; Lefebvre et al., 2006). Interestingly, *AtNCED6* or *AtNCED9* mutants

do not display visible vegetative phenotypes (e.g. response to rapid dehydration), which suggests the role of these genes may be restricted to seed or other specific undetermined developmental stages (Lefebvre et al., 2006).

Other localized patterns of *AtNCED* gene expression exist, revealing a coordinated control over ABA biosynthesis in various tissues and at various development stages (Tan et al., 2003; Lefebvre et al., 2006). For example, *AtNCED2* and *AtNCED3* are expressed in roots, with expression localized in root tips, pericycle, and the cortical cells at the base of lateral roots (Tan et al., 2003). The observed *NCED* expression in predicted sites of lateral root initiation correlates well with the implication of ABA in the regulation of lateral root growth and initiation (De Smet et al., 2003; Signora et al., 2001). De Smet et al. (2003) have reported that ABA can inhibit lateral root development. However, it is also known that ABA accumulation can maintain primary root elongation under osmotic stress (Sharp et al., 1994). Tan et al. (2003) suggest that ABA may play a dual role in roots, by promoting or inhibiting lateral root development depending on ABA level and suggest a threshold model. Interestingly, loss of function alleles of the ABA-insensitive transcription factor *ABI3* confer reduced lateral root responsiveness in the presence of auxin and an auxin transport inhibitor. In contrast, a mutant hypersensitive to ABA, *era1*, has increased numbers of lateral roots (Brady et al., 2003).

Studies up-regulating the expression of *NCED* either inducibly or constitutively provide more evidence that NCED is a rate-determining enzyme. Over-expression of *LeNCED1* in transgenic tomato plants resulted in increased leaf and seed ABA contents and increased seed dormancy (Thompson et al., 2000b). Similarly, over-expression of *PvNCED1* in tobacco resulted in increased ABA contents and also accumulation of the catabolite phaseic acid (PA, see below). The increase in leaf ABA was observed in plants constitutively over-expressing *PvNCED1* with the 35S promoter and plants expressing *PvNCED1* under an inducible DEX promoter (Qin and Zeevaart, 2002). In the presence of DEX, seeds showed a 4 d delay in germination indicating ABA levels may have been increased (although ABA was not measured) (Qin and Zeevaart, 2002). Over-expression of *AtNCED3* in *Arabidopsis* resulted in an increase in endogenous ABA level and promoted transcription of drought- and ABA-inducible genes such as *RD29B*, *KIN1*, and *RAB18* (Iuchi et al., 2001). By contrast, anti-sense plants downregulating *AtNCED3* had

lower ABA levels, increased transpiration rates, and no induction of *RD29B* during drought-stress in aerial tissues of 3 week old plants (Iuchi et al., 2001). In addition to drought tolerance and increased seed dormancy, other physiological changes in associated with over-expression of *NCED* include an increased tendency to guttate at high humidity presumably because of ABA-enhanced root pressure (Thompson et al., 2000b; Taylor et al., 2005).

Whether the amount of ABA, present within a given tissue, can affect the regulation of ABA biosynthesis through a feedback mechanism is particularly relevant with regards to NCED, since NCED has been suggested to be a key rate-determining step in the pathway (Xiong and Zhu, 2003). In tomato and cowpea, *LeNCED1* and *VuNCED1* expression did not respond to exogenous ABA application (10 and 100 μM respectively) (Iuchi et al., 2000; Thompson et al., 2000a). In addition, *LeNCED1* expression was not altered in leaves of the tomato ABA-deficient mutant *sitiens* under drought stress (Thompson et al., 2000a). However, ABA has been shown to regulate *AtNCED3*. In *Arabidopsis* Landsberg and C24, 100 μM triggered small increases in *AtNCED3* but 10 μM ABA did not stimulate expression in *Arabidopsis* Columbia (Xiong et al., 2002). Cheng et al. (2002) also reported that *AtNCED3* was stimulated by 100 μM ABA in both shoots and roots of *Arabidopsis* Landsberg. Finally, *AtNCED3* transcripts were reduced under salt and osmotic stress in the ABA-deficient mutants *los5* (*aba3*) and *los6* (*aba1*) as compared to wild-type seedlings (Xiong et al., 2001b; Xiong et al., 2002). These results suggest there may be a positive feedback regulation of ABA biosynthesis by ABA; however, definitive evidence is needed from species other than *Arabidopsis*.

Recently, a synthetic chemical inhibitor of NCED has been developed; termed abamine, it is based on modification of the lipoxygenase inhibitor nordihydroguaiaretic acid (Han et al., 2004). Abamine inhibits recombinant NCED activity, inhibited stomatal closure in spinach leaves under osmotic stress, increased radicle elongation in cress (*Lepidium sativum*) seeds, and decreased the drought-induced 16-fold increase in ABA by 50% (50-100 μM abamine). Consequently, *Arabidopsis* plants treated with abamine were more sensitive to drought stress and showed significant decreases in drought tolerance compared to controls (Han et al., 2004). The development of abamine or other more specific ABA inhibitors are likely to be very valuable in testing the role ABA plays

in various developmental processes (e.g. seed dormancy) and stress responses – especially where genetic analysis (e.g. through mutants) is not possible. Currently, fluridone is the most commonly used ABA-biosynthetic inhibitor but since it inhibits PDS activity and the development of chlorophyll, it can be hard to determine ABA effects from the costs of depleting earlier carotenoids (Taylor et al., 2005).

1.5.1.3 The C15 pathway in the cytosol

ABA is produced from *cis*-xanthoxin in the cytosol via the intermediate abscisic aldehyde (Fig. 1.4). It has been suggested that the name xanthoxal be used in place of xanthoxin (Milborrow et al., 1997); although this has not been adopted in recent literature. That C_{15} xanthoxin is a precursor to ABA is demonstrated by: (i) conversion of xanthoxin to ABA in cell-free extracts from leaves and roots of bean and from leaves of pea, maize, *Cucurbita maxima*, and *Vigna undulata* (Sindu and Walton, 1987), and (ii) the ABA-deficient *aba2* mutant of *Arabidopsis* lacks xanthoxin oxidase activity (gene later identified as SDR, see below) and hence accumulates xanthoxin (Léon-Kloosterziel et al., 1996; Schwartz et al., 1997a). Similar to the path from zeaxanthin to xanthoxin, the conversion of xanthoxin to ABA may involve several routes (Fig. 1.4; Cutler and Krochko, 1999). It has been suggested that xanthoxin can be converted to xanthoxic acid before ABA (Milborrow, 2001). For example, ^{14}C-labeled xanthoxic acid was converted to ABA in ripening avocado fruit extracts *in vitro* (Milborrow et al., 1997). However, Finkelstein and Rock (2002) point out that the evidence for xanthoxic acid as an immediate precursor to ABA is clearly circumstantial but do note that, at least in certain species, a metabolic matrix of xanthoxic acid and abscisic aldehyde may exist. Another, minor shunt pathway to ABA from xanthoxin is the formation of abscisic alcohol. This pathway only appears important when abscisic aldehyde oxidase (AAO, see below) activity is impaired but does operate in potato, barley, and tomato at minor levels (Rock et al., 1991; Rock et al., 1992; Finkelstein and Rock 2002). However, *in planta* it is generally assumed that the major path is through abscisic aldehyde (Fig. 1.4; Cutler and Krochko, 1999; Nambara and Marion-Poll, 2005). Most mutants deficient in this step of the pathway accumulate abscisic aldehyde (or its byproduct abscisic alcohol, which may

be an alternate route for ABA synthesis as noted above – Fig. 1.4) and include the *sitiens* and *flacca* mutants of tomato (see below; Finkelstein and Rock, 2002).

1.5.1.3.1 Xanthoxin to ABA-aldehyde... AB-SDR action

The conversion of xanthoxin to abscisic aldehyde is accomplished by a short-chain dehydrogenase / reductase (SDR) family enzyme (AB-SDR) (Fig. 1.4). AB-SDR was identified by map-based cloning of *ABA2* in *Arabidopsis* and represents the only species in which a gene encoding an enzyme with xanthoxin oxidase activity has been identified (Cheng et al., 2002; González-Guzmán et al., 2002). Mutants at the *aba2* locus were originally selected based on a screen for germination in the presence of the GA biosynthesis inhibitor paclobutrazol and also in a screen for NaCl tolerance (Léon-Kloosterziel et al., 1996). Schwartz et al. (1997) later identified that *aba2* plants are blocked in the conversion of xanthoxin to abscisic aldehyde and thus lack xanthoxin oxidase activity.

Mutants allelic to *aba2* have been identified in numerous screens. In fact, Rook et al. (2001), selecting seedlings that were able to establish on high concentrations of sucrose, were the first group to discover that *ABA2* belongs to the SDR family (although little characterization of SDR/*ABA2* was performed). Mutants of *AtABA2* were also uncovered in additional screens based on seed germinabilty or seedling growth on high concentrations of glucose, sucrose, and NaCl and using infrared thermal imaging to isolate mutants defective in stomatal regulation (Laby et al., 2000; Quesada et al., 2000; Cheng et al., 2002; González-Guzmán et al., 2002; Merlot et al., 2002).

*At*ABA2 protein (or xanthoxin oxidase) is presumed to be encoded by a single gene in the *Arabidopsis* genome based on the fact that loss-of-function leads to severe ABA deficiency, of 56 SDR family members in *Arabidopsis* the closest SDR paralogue is only 62% similar, and no other SDR genes have been identified in various mutant screens (Cheng et al., 2002; Nambara and Marion-Poll, 2005). However, null *aba2* mutants *gin1-3* and *aba2-11* contain approx. 20-30% of the ABA of wild-type plants suggesting alternate pathways for ABA biosynthesis (e.g. formation of xanthoxic acid) or non-specific residual xanthoxin oxidase activity (Cheng et al., 2002; González-Guzmán et al., 2002). It is also plausible that although the given 1-D identities are low between

Arabidopsis family members, the 3-D structures could be very similar, as found by Oppermann et al. (2003).

The AB-SDR *At*ABA2 enzyme is a cytosol-localized NAD-dependent oxidoreducatase catalyzing the oxidation of the 4'-hydroxyl to a ketone, desaturation of the 2' 3' bond, and opening of the 1' 2' epoxy ring (Cheng et al., 2002; González-Guzmán et al., 2002). In addition, SDR enzymes usually form dimers or tetramers (Jörnvall et al., 1995). The multimeric nature of *At*ABA2 was shown by intragenic complementation between different mutant alleles (e.g. *aba2-1* with either *aba2-11* or *aba2-12*) (Merlot et al., 2002).

Expression of *ABA2* was detected in aerial tissues and roots but was lower in dry seeds and rosette leaves; within rosette tissues it was preferentially localized to the petioles rather than leaf blades. Further localization using *ABA2* promoter:GUS fusions reveals expression in areas such as vascular bundles. GUS-localized expression of *AtABA2* and *AtNCED*s in vascular bundles leads us to the suggestion that ABA is synthesized in vascular tissues (Cheng et al., 2002; Tan et al., 2003; see section 1.5.3.3.2 for further discussion). *ABA2* was not induced by drought or 50-100 μM ABA treatment (Cheng et al., 2002; González-Guzmán et al., 2002). Thus, in contrast to other ABA synthesis genes such as NCED, it appears *AtABA2* is not a major regulatory step in the production of stress induced ABA – at least not drought stress. However, *AtABA2* is regulated by glucose, leading us to the conclusion that *ABA2* is a key regulator of sugar induced ABA-accumulation and may act as a modulator for glucose-signaling and sensing during various developmental phases (i.e germination and seedling development) (Cheng et al., 2002).

1.5.1.3.2 The final step, ABA-aldehyde to ABA... AB-AO and MCS

The last step in the ABA biosynthetic pathway converts abscisic aldehyde to ABA and is accomplished by the enzyme abscisic aldehyde oxidase (AB-AO) (Fig. 1.4). Deficiency of AB-AO activity results from either mutations in the apoenzyme itself (e.g. tomato *sitiens*) or in a molybdenum co-factor (Moco) required for its activity (as is the case for tomato *flacca* and *aba3* of *Arabidopsis*) (Schwartz et al., 2003; Taylor et al., 2005). Similar to NCED, AB-AO exists as part of a multi-gene family of aldehyde

oxidases in *Arabidopsis* and tomato (Seo et al., 2000a; Taylor et al., 2005, references therein). The different isoforms of aldehyde oxidases are not necessarily specific to ABA biosynthesis itself but have a range of functions (and substrates). For example, among four aldehyde oxidases, the *Arabidopsis abscisic-aldehyde-oxidase-1* (*AAO1*) gene product is thought to participate in indole-3-acetic acid synthesis (Seo et al., 1998; Akaba et al., 1999). However, it has recently been shown that the *AAO3* gene product, a homodimer, efficiently oxidizes abscisic aldehyde and thus was deemed an abscisic aldehyde oxidase (AB-AO) (Seo *et al.*, 2000a; Seo *et al.*, 2000b). Initial findings indicate that the *AAO3* gene is expressed in leaves at steady-state levels but is rapidly increased upon dehydration (yet neither the AAO3 protein or its activity seemed to increase in dehydrated leaves) (Seo *et al.*, 2000a). However, the *aao3-1* mutant does exhibit a wilty phenotype in leaves (Seo *et al.*, 2000b). ABA contents in turgid leaves of the *aao3-1* mutant were about 35% of the wild-type and when water-stressed the mutant had ABA levels that were 20% of the wild-type. However, in mature dry seeds, the ABA contents were approx 60% of wild-type seeds; contrast this to the *aba3-2* mutant (in which the Moco activity is impaired) with seed ABA contents approx 12% of the wild-type. As such, seed dormancy is markedly affected in the *aba3-2* mutants but not *aao3-1* mutants (Seo *et al.*, 2000b). Seo et al. (2000a) suggested that *AAO3* is an unlikely candidate for ABA biosynthesis in seeds. However, as Nambara and Marion-Poll (2005) point out, the mutation in an intron-splicing site of *AAO3-1* suggests that the *aao3-1* mutant allele may be leaky. Indeed, the recent identification of the salt-resistant mutants *sre2-1* and *sre2-2* (renamed *aao3-2* and *aao3-3*) as null-alleles of *aao3* show that AAO3 plays a major role in ABA biosynthesis in seeds. *aao3-2* and *aao3-3* mutants seeds displayed osmotolerance in germination assays and reduced seed dormancy; however, dry seeds of *aao3-2* and *aao3-3* still contained approx. 1/3 the ABA of wild-type suggesting that other AO enzymes may participate in ABA synthesis (González-Guzmán et al., 2004). Indeed, Seo et al. (2004) found that AAO1 and AAO4 could partially contribute to ABA biosynthesis in seeds as ABA levels are further reduced in double mutants (by approx. 50%). However, the authors note that considering the enzymatic properties of AAO1 and 4 enzymes (i.e. their ability to catalyze abscisic aldehyde to ABA) the contribution from these two enzymes is probably negligible (Seo et al. 2004).

The *sitiens* locus of tomato also encodes an AB-AO enzyme and forms mainly the inactive *trans* isomer of abscisic alcohol (Linforth et al., 1987). Interestingly, *sitiens* mutants have decreased ABA content in the seed and decreased seed dormancy (Taylor et al., 1988; Groot and Karssen, 1992). More recently, Okamoto et al. (2002) showed that the *AAO3* gene from *Arabidopsis* was able to complement the *sitiens* mutation. However, none of the 5 identified tomato aldehyde oxidases map to the *sitiens* locus and *sitiens* remains to be cloned (Taylor et al., 2005, references therein).

AAO3 is up-regulated by 100 μM ABA, 300 μM NaCl, and by 2 and 6% glucose (Cheng et al., 2002; Xiong et al., 2002). Expression of *AAO3* is reduced in *aba1*, *aba2*, and *aba3* mutants but only during stress treatment (Cheng et al., 2002; Xiong et al., 2003) and suggests ABA may regulate its own accumulation (see below). In addition, expression of *AAO3* is contingent upon presence of active *ABI1* gene (but not *ABI2*) as transcript levels were reduced in an *abi1-1* mutant background (Xiong et al., 2002). Recently, AAO3 protein and *AAO3* transcripts were localized within *Arabidopsis* tissues using a GFP-fusion protein construct, immunohistochemistry, and *in situ* hybridization (Koiwai et al., 2004). GFP fluorescence was detected in root tips, lateral root primordium (similar to NCED promoter GUS localization noted above), vascular bundles of roots, etiolated hypocotyls, inflorescence stems, leaf veins, and guard cells. Using an AAO3-specific antibody, the AAO3 protein was localized to phloem companion cells, xylem parenchyma cells, and leaf guard cells (Koiwai et al., 2004). As Nambara and Marion-Poll (2005) point out, it is interesting that both *NCED* and *AAO* genes have been localized to guard cells, suggesting that ABA synthesized within the guard cells themselves may trigger stomatal closure (Tan et al., 2003; Koiwai et al., 2004). However, it is also possible ABA or ABA precursors are transported to guard cells or other signals are used to relay the (drought) stress signal.

Aldehyde oxidase requires a molybdenum cofactor (MoCo) for its catalytic activity. Mutations in MoCo synthesis in plants affect the expression of a number of enzymes including nitrate reductase, xanthine dehydrogenase (XD), aldehyde oxidase (AO), and sulfite oxidase (Mendel and Hänsch, 2002; Walker-Simmons et al., 1989; Eilers et al., 2001). Both XD and AO require a mono-oxy MoCo co-factor with a terminal sulfur group. It is a MoCo sulferase (MCS) that replaces one oxygen in a dioxo-molybdenum

molecule with that of sulfur (Yesbergenova et al., 2005, references therein). Thus, since AB-AO is an aldehyde oxidase mutations in MoCo biosynthesis genes lead to ABA deficiency. Consistent with this, *MCS* mutations in tomato *flacca* and *Arabidopsis aba3* confer ABA-deficient phenotypes (Bittner et al., 2001; Xiong et al., 2001b; Sagi et al., 2002). Consistent with its role in ABA biosynthesis, *AtABA3* is upregulated by drought and salt stress, 100 μM ABA, and glucose (Xiong et al., 2001b; Chang et al., 2002). And similar to *ZEP* and *AAO3*, *AtABA3* is reduced in ABA biosynthesis mutants during stress treatment (Xiong et al., 2001; Xiong et al., 2002).

In summary, *ZEP*, *MCS*, and *AB-SDR*, suggested to be single-copy genes in *Arabidopsis*, demonstrate the utility of these genes within the ABA biosynthetic apparatus. Mutations in *ZEP* and *MCS* can result in very low levels of ABA (e.g. 10% of wild-type in seeds); contrastingly, null mutations in *AB-SDR* lead to leaky levels of ABA (20-30% wild-type in seedlings) suggesting either alternate pathways to ABA (e.g. through xanthoxic acid) or other less specific SDR gene family members capable of oxidizing xanthoxin. The presence of *NCED* and *AAO* as gene families in various species (the most striking example being *Arabidopsis*) reveal the plasticity present within plants as each gene can show developmental and tissue specificity and overlap their functions to create functional redundancies. NCED is a major rate-determining step in ABA biosynthesis in vegetative tissues (e.g. drought stressed leaves) yet this does not preclude other ABA biosynthetic loci from helping determine ABA homeostasis especially in non-photosynthetic tissues (e.g. over-expression of *ZEP* increases seed dormancy in tobacco, Frey et al., 1999). One intriguing detail that has emerged is the possibility that ABA can regulate its own synthesis and catabolism as many of the ABA biosynthetic genes are regulated by ABA (see below for discussion on catabolism). The one caveat to this is *AtNCED3*, which may be why NCED has more of an impact as a rate-determining step (Xiong et al., 2003).

1.6 ABA Catabolism

A reduction in the ABA concentration of a cell can be due to a decrease in the biosynthetic rate, an efflux out of the cell due to passive or carrier-mediated transport, and/or catabolic inactivation (Cutler and Krochko, 1999). Catabolic inactivation of ABA

can occur through either oxidation or conjugation. The importance of the two different routes of inactivation varies between species and even between different tissues (Zeevaart, 1999, references therein). The predominant pathway by which the natural enantiomer S-(+)-ABA is oxidized in leaves, developing seeds, and seedlings is through hydroxylation at the 8'-methyl group to form the unstable intermediate (+)-8'-hydroxyABA which subsequently cyclizes to phaseic acid [(-)-PA] (Milborrow, 1969; Milborrow, 1970; Krochko et al., 1998). The cyclization to PA occurs spontaneously *in vitro* although it has been suggested that this process occurs enzymatically *in vivo* (Milborrow et al., 1988). (-)-PA can be reduced (at the 4' position) to dihydrophaseic acid [(-)-DPA] (Fig. 1.5; Walton et al., 1973). The reduction from PA to DPA is thought to occur via an unidentified soluble reductase (Gillard and Walton, 1976). Other minor pathways include the formation of (+)-7'-hydroxyABA and reduction of the ketone at the 4' position to produce 1',4' diols of ABA (Dathe and Schneider, 1982; Okamoto et al., 1987; Hampson et al., 1992). Conjugation occurs when the glucose ester (at 1 position) or glucoside (1' or 4' position) of ABA or its metabolites form (Fig. 1.5) (Koshimizu et al., 1968; Neill et al., 1983). Recently, a new metabolite, (+)-9'-hydroxy ABA and its cyclized form neo-PA were discovered in *Brassica napus* siliques (Zhou et al. 2004). Additional breakdown products of ABA through the 8'-hydroxylation pathway have also been discovered. Through feeding of deuterium-labeled ABA to corn suspension cultures, Zaharia et al. (2004) found new metabolites arising from further oxidation at the 8'-position. Formation of (+)-8'-oxoABA and its subsequent cyclized forms 8'-hydroxyPA, 8'-hydroxyDPA, and 8'-oxoDPA suggest the 8'-oxidation pathway is more complex than previously thought (Zaharia et al., 2005b).

Early research with radioactively-labeled isotopes of ABA (i.e. ^{14}C-ABA) lead to discover of the major catabolic routes, via PA or the glucose-conjugates, shortly after the discovery of ABA and before much of the biosynthetic pathway was discerned. However, subsequent research proceeded rather slowly, in part due to the lack of mutants deficient in ABA catabolism (Cutler and Krochko, 1999). Zeevaart and Creelman (1988) make an invaluable point that should not go unnoticed: often it is the racemic mixture of ABA used in experiments. Therefore, one must be careful to distinguish between those metabolites that are naturally occurring (i.e. metabolized from (+)-ABA) from those that

are not (i.e. metabolized from (-)-ABA) (see Walton and Li, 1995, Zeevaart, 1999 for further discussion on the metabolism of (-)-ABA).

The metabolites of ABA have little or no biological effect except for the early oxidation products (+)-8'-hydroxy ABA, (+)-9'-hydroxy ABA, and (+)-7'-hydroxy ABA (Hill et al., 1995; Zou et al., 1995; Zhou et al., 2004; see section 3.4 for further discussion). For example, (+)-9'-hydroxy ABA inhibited germination of *Arabidopsis* seeds (Columbia) and thus was hormonally active (although less so than (+)-ABA); the closed form (neoPA) did not inhibit germination. Further, in the *B. napus* microspore-derived embryos, (+)-9'-hydroxy ABA was able to induce the 3-ketoacyl-CoA synthase gene in a fashion similar to (+)-ABA. Comparatively, neoPA was far less active (Zhou et al., 2004). PA does not bind to ABA-binding proteins identified in barley and apple fruit, however, is able to inhibit GA-induction of α-amylase in barley half-grains (Todoroki et al., 1995; Zhang et al., 2001; Razem et al., 2004). Once ABA or its metabolites are conjugated with glucose they are prone to sequestration into the vacuole (see section 1.6.2.1 for further discussion; Cutler and Krochko, 1999; Zeevaart, 1999).

Studies on ABA metabolism have also been aided by the development of more persistent, harder to metabolize ABA analogs. For instance, one such analog is S-(+)-d6-ABA, with deuterium atoms in place of the proton hydrogen isotope at the 8' and 9' positions (S-[8',8',8',9',9',9']-hexadeuteroabscisic acid). In corn suspension cells, the oxidation rates are slower for S-(+)-d6-ABA than for the natural isotope S-(+)-ABA and, in cress seed, the deuterium-labeled analog has greater biological activity in delaying seed germination (Lamb et al., 1996). Overall, ABA analogs modified at the 8'-carbon group exhibit stronger ABA-like activities compared to other substitutions (Todoroki et al., 1995; Cutler et al., 2000). For example, (+)-8'-methylene ABA is more active than (+)-ABA in inhibiting germination of cress seed and excised wheat embryos, in reducing growth of corn suspension cells, and in reducing transpiration in wheat seedlings (Abrams et al., 1997). (+)-8'-methylene ABA is a relatively weak competitive inhibitor of ABA (+)-8'-hydroxylase activity and its ABA agonist properties are partly due to the fact that (+)-8'-methylene ABA is a poor substrate for the 8'-hydroxylase enzyme and is therefore more persistent in plant tissues (Cutler et al., 2000). Other ABA analogs with alterations at the 8'- and 9'-carbon atoms can act as suicide substrates for the ABA 8'-

hydroxylase enzyme (i.e. cause enzyme inactivation). These include (+)-9'-propargyl ABA and (+)-8'-acetylene ABA (Rose et al., 1997; Cutler et al., 2000). (+)-9'-propargyl ABA and (+)-8'-acetylene ABA have K_I values of 0.27 and 19.0 μM, respectively, compared to a K_m value of 16.0 μM for (+)-ABA (K_I = concentration at which the rate of enzyme inactivation is half the maximum rate); both compounds are more potent than (+)-ABA itself in inhibiting *Arabidopsis* Columbia seed germination (Cutler et al., 2000).

Because of the lack of mutants, cDNA clones for the enzymes involved in ABA breakdown, and the lack of specific biochemical assays for the enzymes, original research into the role metabolism plays in modulating ABA levels in tissues was based upon: accumulation of PA, DPA, or the glucose conjugates, the disappearance of applied or endogenous ABA, and a reduced sensitivity to applied ABA (suggesting catabolism may be active in the tissues assayed) (Cutler and Krochko, 1999). However, the (+)-ABA 8'-hydroxylase enzyme has been characterized as an integral membrane protein with properties of a cytochrome (CYP) P450 monooxygenase (Krochko et al., 1998). One key to the characterization of (+)-ABA 8'-hydroxylase as a CYP P450 enzyme was discovery of a tissue with high levels of inducible *in vivo* enzyme activity (Cutler et al., 1997). In corn suspension cells (*Zea mays* L. cv Black Mexican Sweet), ABA 8'-hydroxylase is rapidly induced by endogenous (+)-ABA, reaching a maximum at 16 h, followed by an equally rapid decline to low levels after 24 h (Krochko et al., 1998). Characterization of the ABA 8'-hydroxylase enzyme as a CYP P450 enzyme was based on several criteria: (i) a requirement for molecular oxygen, (ii) a requirement for NADPH (iii) inhibition by CO, (iv) reversal of the CO inhibition by blue light (i.e. light with a transmittance of ~450 nm), (v) membrane association (e.g. in the microsomal fraction), and (vi) inhibition of enzyme activity by the CYP 450 inhibitor tetcyclacis (50% inhibition at 10^{-6} M) (Krochko et al., 1998). The ABA 8'-hydroxylase enzyme characterization was a pivotal discovery that subsequently lead to the cloning of ABA 8'-hydroxylases by two independent groups (see below; Kushiro et al., 2004; Saito et al., 2004).

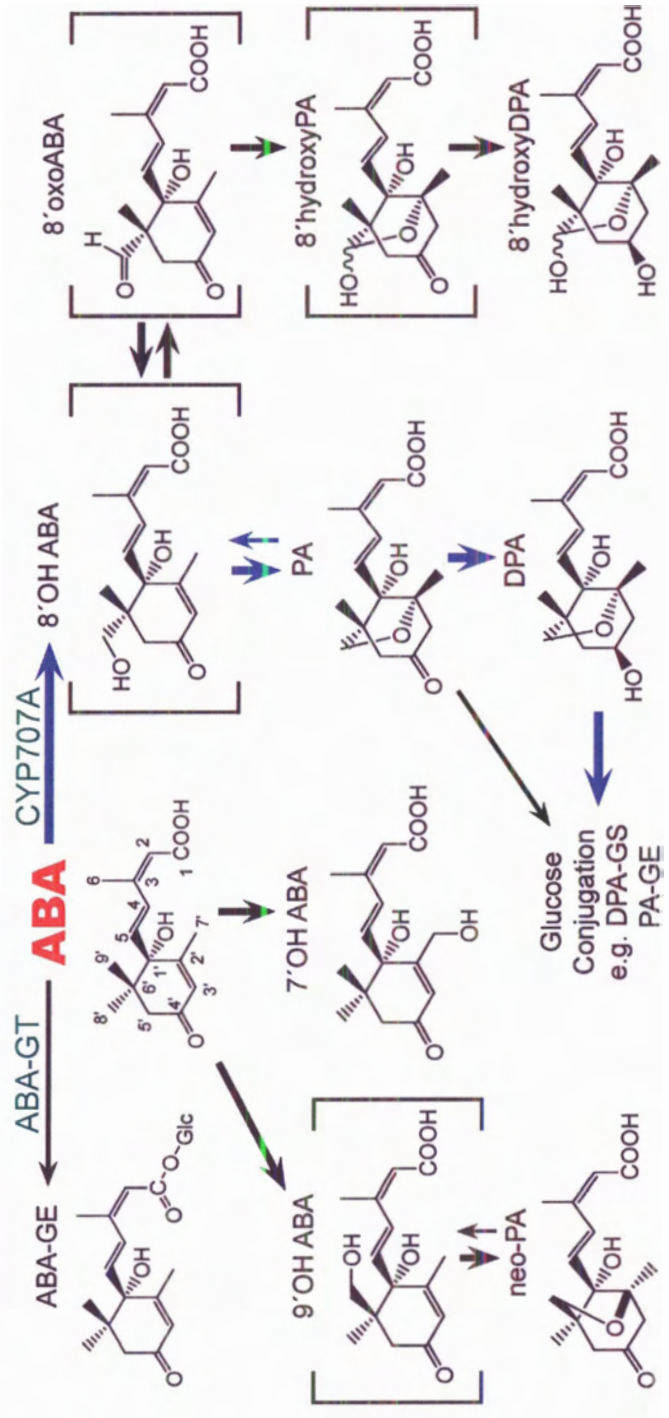


Fig. 1.5. The catabolism of abscisic acid (ABA). Oxidative pathways involve 8'-, 7'-, or 9'-hydroxylation. The major pathway (shown with blue arrows) is often through 8'OH-ABA, which cyclises to PA, which is reduced further to DPA. ABA or selected metabolites may also form glucose conjugates. Additional oxidations of 8'OH-ABA are also shown. Other paths not shown include formation of the minor products *epi*-DPA or 1',4'-diols of ABA. ABA-GE, ABA glucose ester; DPA, dihydrophaseic acid; DPA-GS, DPA glucoside; 7'OH-ABA, 7'-hydroxy abscisic acid; 8'OH-ABA, 8'-hydroxy abscisic acid; 9'OH-ABA, 9'-hydroxy abscisic acid; neo-PA, neo phaseic acid; PA, phaseic acid; PA-GE, PA glucose ester. ABA-GT, abscisic acid glycosyltransferase; CYP707A, cytochrome P450 family ABA 8'-hydroxylases.

As evidence continues to build, it is evident that the ABA content of a cell is dynamically maintained and, at times, can be in constant flux due to continued synthesis and/or degradation. And while the biosynthesis pathway has been elucidated to the point where it is now possible to monitor changes in the genes responsible for ABA synthesis and even modify their activity through mutation or over-expression (section 1.5), the same level of detail has not been obtained with regard to the degradation of ABA. However, the recent identification of the ABA 8'-hydroxylase enzyme as the CYP707A sub-family in *Arabidopsis* and two glycosyltransferases that are able to glucosylate ABA has furthered our knowledge of ABA catabolism tremendously and has opened up avenues to study the key relationship between ABA synthesis and catabolism (Xu et al., 2002; Kushiro et al., 2004; Saito et al., 2004; Lim et al., 2005).

1.6.1 Cytochrome P450s and the identification of the CYP707A subfamily as ABA 8'-hydroxylases

1.6.1.1 Cytochrome P450s

Throughout the animal and plant kingdom, cytochrome P450 monooxygenases are classically described as heme-containing mixed-function oxidase proteins that utilize NADPH and/or NADH to reductively cleave atmospheric dioxygen to produce an organic product and water (Schuler and Werck-Reichhart, 2003). P450s can mediate such reactions as aromatic hydroxylations, epoxidations, dealkylations, isomerizations, dimerizations, dehydrations, carbon-carbon cleavages, decarboxylations, nitrogen and sulfur oxidations, dehalogenations, and deaminations. However, often the products represent hydroxylated derivatives of the substrate at one of its carbon moieties – this is the case for the hydroxylation of ABA (Fig. 1.5; Schuler and Werck-Reichhart, 2003).

Cytochrome P450 nomenclature is designated as follows: related monooxygenase proteins are grouped into families with numbers (e.g. CYP**707**, CYP**88**) following the **CYP** designator used for all P450 sequences. Families usually share greater than 40% amino acid identity. Further, families are divided into subfamilies designated with alphabetical characters (e.g. CYP707**A**, CYP90**B**) that share greater than 55% amino acid identity. Subfamilies are further subdivided into individual loci designated with a second set of numbers (e.g. CYP707A**1**, CYP707A**2**, CYP707A**3**, CYP707A**4**) and allelic

variants at individual loci designated with yet another set of numbers (for instance, CYP707A1**v1** or CYP707A1**v2**) (Nelson et al., 1996;Werck-Reichhart et al., 2002; Nelson, 2005a).

Analysis of the *Arabidopsis* genome has revealed 272 cytochrome P450s, with 26 being pseudogenes, distributed into 44 families and 69 subfamilies (Werck-Reichhart et al., 2002; Schuler and Werck-Reichhart, 2003). In addition to their role in the oxidative degradation of ABA, e.g. the 8'-hydroxylase enzyme, P450s participate in an array of biochemical pathways (many of which catalyse NADPH- and O_2-dependent hydroxylation reactions). These range from the synthesis of lignins, lipids, UV protectants, pigments, defense compounds, fatty acids, and hormones such as gibberellins, brassinosteroids, and jasmonic acid (The Arabidopsis Genome Initiative, 2000; Schuler and Werck-Reichhart, 2003). For example, the closest related family to the ABA 8'-hydroxylases is the CYP88A subfamily that encode *ent*-kaurenoic acid hydroxylases, participants in early GA biosynthesis from kaurenoic acid to GA_{12} (Helliwell et al., 2001). In reference to the complexity of plant metabolism (e.g. with over 100,000 secondary metabolites), with *Arabidopsis* and rice having 272 and 458 P450 genes, respectively, *Drosophila melanogaster* has 94, *Caenorhabditis elegans* has 73, and yeast (*Saccharomyces cerevisiase*) has only 3 (The Arabidopsis Genome Initiative, 2000; Schuler and Werck-Reichhart, 2003).

P450 enzymes are divided into two main classes, A-type and non-A-type. More than 50% of *Arabidopsis* P450s are classified as A-type (e.g. ~153) and are primarily involved in plant-specific metabolism; while about 30% belong to the non-A-type (e.g. ~93) and are involved in house-keeping functions and in conserved functions essential for development and signaling such as the biosynthesis and catabolism of sterols, oxygenated fatty acids, and phytohormones (Werck-Reichhart et al., 2002). Within the non-type A group are 4-6 clades and among them is an 85-clan clade. This 85-clan clade contains several P450s involved in the synthesis of brassinosteroids, gibberellins, and this is also where the ABA 8'-hydroxylases or CYP707A subfamily resides (Szekeres et al., 1996; Choe et al., 1998; Helliwell et al., 2001; Shimada et al., 2001; Saito et al., 2004).

Most conventional plant P450s are usually anchored in the endoplasmic reticulum (ER) membrane with a single N-terminal transmembrane helix, while the rest of the

protein protrudes into the cytosol. Further, P450s maybe associated with comparably anchored NADPH-dependent P450 reductases to facilitate electron transfer from NADPH into the catalytic site of the P450 (Werck-Reichhart et al., 2002). These two properties have not been demonstrated for the CYP707A ABA 8'-hydroxylases (Kushiro et al., 2004; Saito et al., 2004).

1.6.1.2 ABA 8'-hydroxylases CYP707A subfamily

Recently, as mentioned, the *CYP707A* subfamily in *Arabidopsis* was identified, by a reverse genetic approach, as the ABA 8'-hydroxylase gene family (Kushiro et al., 2004; Saito et al., 2004). Saito et al. (2004) sought to characterize more genes in the 85-clan in the non-A-type and the first subfamily that was selected was CYP707A because of its relatedness to CYP88A (*ent*-kaurenoic acid hydroxylase). Kushiro et al. (2004), on the other hand, focused on the discovery of the ABA 8'-hydroxylase enzyme and used a phylogenetic and microarray approach to narrow the candidate genes to several possibilities (i.e. *CYP97A3, CYP97C1, CYP707A1, CYP707A3*, and *CYP714A1*). Subsequently, both Saito et al. (2004) and Kushiro et al. (2004) used functional expression in insect cells and yeast, respectively, to show that the CYP707A subfamily (CYP707A1 to A4) are ABA 8'-hydroxylases.

The recombinant CYP707A proteins convert ABA to PA *in vitro*, however other hydroxylated catabolites such as 7'- or 9'-hydroxy ABA were not detected under the assay conditions used. Further, CYP707As do not appear to be involved in cyclization of 8'-hydroxy ABA to PA since ABA is primarily converted to 8'-hydroxy ABA in short incubation periods and then 8'-hydroxy ABA is autoisomerized to PA (Kushiro et al., 2004; Saito et al., 2004). It is interesting that neither 7'- or 9'-hydroxy ABA were detected in the assay conditions used; however, it is possible different assay conditions would favour the production of 7'- or 9'-hydroxy catabolites. Alternatively, other CYP enzymes may exist that perform these hydroxylations. Either scenario is a possibility since it is not uncommon for a group of different P450s to metabolize a single substrate at alternate positions or for a single P450 to metabolize multiple substrates (Schuler and Werck-Reichhart, 2003). However, it may be that in species that show significant amounts of either 7'- or 9'-hydroxy ABA, CYP707As present may be 7'- or 9'-hydroxylases. For

example, CYP71D13 and CYP71D15 from peppermint (*Mentha piperita*) hydroxylate limonene at the C3 position while CYP71D18 from spearmint (*Mentha spicata*) modifies limonene at the C6 position (Karp et al., 1990; Lupien et al., 1999; Haudenschild et al., 2000).

Numerous inhibitors of P450 activity have been reported. Many of these have been developed as growth retardants and impact GA biosynthesis but also can inhibit aspects of brassinosteroid metabolism (Rademacher, 2000; Asami et al., 2001). As reported by Kushiro et al. (2004), the activity of CYP707A1 was inhibited by the P450 inhibitor tetcyclasis but not by another P450 inhibitor metyrapone. Thus, inhibition of CYP707As is distinguishable between different P450 inhibitors. Therefore, Nambara and Marion-Poll (2005) suggest, "it might be possible to develop a specific inhibitor of this enzyme in the future". In a more recent study, the ability of 9 cytochrome P450 inhibitors with a triazole group was tested using tobacco Bright Yellow-2 cells and CYP707A3 recombinant enzyme *in vitro* assays (Kitahata et al., 2005). Only uniconazole-P (at 3 μM) inhibited ABA catabolism in cultured tobacco Bright Yellow-2 cells. Further analysis of various structural analogs of uniconazole-P revealed that a fungicide known as diniconazole was a more potent inhibitor of 8'-hydroxylation. One obvious point that should be noted is the rather general and non-specific nature of these inhibitors. Uniconazole-P and tetcyclasis, for example, are both potent inhibitors of GA biosynthesis (steps from *ent*-kaurene to *ent*-kaurenoic acid) (Rademacher, 2000). Further, Kitahata et al. (2005) do mention diniconazole slightly retarded rice stem elongation, which could be rescued by GA treatment. In addition, diniconazole inhibited ABA catabolism during rehydration (after drought) but not during the drought treatment itself (ABA levels actually increased). Thus, these results provide insights into the mechanisms of P450 inhibition on ABA 8'-hydroxylation but perhaps do not lead us in the appropriate direction for creation of a specific inhibitor for the CYP707As.

Develop of a specific CYP707A inhibitor has also been attempted with various analogs of ABA. Using recombinant CYP707A3 protein, Ueno et al. (2005a) tested the ability of 45 structural analogs of ABA to inhibit CYP707A3 and ABA bioassay activity. Substrate recognition by CYP707A3 required the 6'-CH_3 groups (C8' and C9'), however, these groups were unnecessary for ABA activity in rice elongation and lettuce seed

germination assays. On the contrary, elimination of the 3-CH_3 (C6) and 1'-hydroxy groups, which significantly affected ABA bioactivity, had little effect on the ability of analogues to competitively inhibit CYP707A3 (Ueno et al., 2005a). In a subsequent study, the compound (1'*S**,2'*S**)-(±)-6-Nor-2',3'-dihydro-4'-deoxo-ABA and its 8'-difluorinated derivative were tested as potent and specific inhibitors of the CYP707A3 enzyme. The K_I values were 0.40 μM and 0.41 μM, respectively, which are lower than the K_M value for (+)-ABA (1.3 μM). Further, in rice seedling elongation assays, the IC_{50} (i.e. 50% growth inhibition) values of both compounds were more than 300 μM, whereas that of (±)-ABA was 2 μM. The IC_{50} (50% germination inhibition) value of the fluorinated compound in lettuce seed germination assays was 150 μM, the non-fluorinated > 300 μM, and that of (±)-ABA 12 μM. Taken together, these results suggest these compounds are effective inhibitors of CYP707A3 activity and but do not confer the ABA signal as well as ABA itself. However, as the authors suggest there is still room for improvement in the development of a potent and specific inhibitor of ABA 8'-hydroxylase (Ueno et al., 2005b).

CYP707A related-sequences are present in many species. In addition to the 4 *Arabidopsis* CYP707As, 2 CYP707As are present in the rice genome (CYP707A5 and CYP707A6), and two tomato CYP707As (A7 and A8) have been submitted to the nomenclature committee (Nelson, 2005a; nomenclature committee = D. Nelson: DNELSON@utmem1.utmem.edu). Recent sequencing of the poplar (*Populus trichocarpa*) genome has identified another possible 7 CYP707As (CYP707A9, A10, A11, A12v1, A12v2, A13, A14, A15) (Nelson, 2005b). Sequences have also been reported in wheat, lettuce, potato (DQ206632), tobacco (DQ116560), and bean and ESTs have been identified in soybean and maize (Nambara et al., 2005, references therein; Yang and Zeevaart, unpublished).

As suggested above, ABA may regulate its own biosynthesis through a feed-back mechanism (section 1.5); this may be the case for catabolic genes too, although the evidence is less substantial. All CYP707As were upregulated by 1, 30, and 100 μM ABA in 2 week-old *Arabidopsis* Columbia plants (Kushiro et al., 2004; Saito et al., 2004). In *Arabidopsis* Columbia seeds, *CYP707A2* was induced 1.5 fold by 30 μM (+)-ABA after 12 h imbibition compared to a water control (Kushiro et al., 2004). Other hormones

regulate at least one of the CYP707As, 1 μM GA and the brassinosteroid, brassinolide (1 μM), elevated *CYP707A3* transcripts in 2 week-old *Arabidopsis* Columbia plants (Saito et al., 2004). *CYP707A3* was also found to be regulated by ethylene (1 mM ethephon), drought, and 100 μM ABA during microarray analysis of selected *Arabidopsis* P450 genes (Narusaka et al., 2004).

Expression of all *CYP707A* genes in *Arabidopsis* (Columbia) has been detected in most organs and the ratio of the *CYP707A* transcripts varies according to the tissue and treatment (Kushiro et al., 2004; Saito et al., 2004). During mid-seed development, when ABA levels are higher, *CYP707A1* and *CYP707A3* are expressed abundantly; as ABA levels fall during late embryo development *CYP707A1* and *CYP707A3* are downregulated (Kushiro et al., 2004). CYP707A2 plays a major role in the decrease in ABA levels observed during early seed imbibition in non-dormant seeds, when corresponding increases in PA and DPA are seen (Kushiro et al., 2004). *CYP707A2* transcripts are high in the dry seed and increase a peak within six hours after the start of imbibition; in comparison, only small increases in the CYP707A1 and A3 are seen and occur after 18-24 h imbibition (Kushiro et al., 2004). Further, when comparing *cyp707a2* and *cyp707a3* mutant seeds, only *cyp707a2* seeds show a hyper-dormant phenotype; this correlates with increased ABA levels in the mature seeds of *cyp707a2* plants (over 6-fold compared to wild-type seeds) (Kushiro et al., 2004).

During stress treatments, CYP707As are induced by drought, 250 mM NaCl, and osmiticum (400 mM mannitol) (Kushiro et al., 2004; Saito et al., 2004). However, increased CYP707A expression is especially observed during rehydration treatments following drought stress (Kushiro et al., 2004). Thus, CYP707A expression correlates somewhat with ABA and metabolite levels. As ABA levels increase during stress and ABA biosynthesis is upregulated (e.g. through increased NCED expression, see section 1.5) so too is catabolism as increases in PA and sometimes DPA are observed (Qin and Zeevaart, 2002; Kushiro et al., 2004; Nambara and Marion-Poll, 2005). When dehydrated plants are rehydrated ABA levels decrease and further increases in PA are observed (Harrison and Walton, 1975; Zeevaart, 1980; Kushiro et al., 2004). Thus, the emerging picture is one of coordinated regulation of between ABA biosynthetic and catabolism

genes, which in turn help dictate ABA homeostasis during development and in response to environmental stimuli.

1.6.2 The glucosylation of ABA

1.6.2.1 Glycosyltransferases

The UDP-Glycosyltransferase (UGT) family 1 in *Arabidopsis* is a multigene family of ~100 UGTs (Lim et al., 2003). UGTs function to transfer a sugar from a donor molecule such as UDP-glucose to an acceptor molecule that can be a hormone, secondary metabolite, or xenobiotic. Several phytohormone UGTs have been identified including those that conjugate auxin, cytokinin, salicylic acid, and ABA (Lim et al., 2003; Lim and Bowles, 2004). In reference to ABA, 8 UGTs were identified that can glucosyalte the hormone, but only one showed a distinct preference for (+)-ABA (Lim et al., 2005; see section 1.6.2.2).

UGTs can catalyze the formation of either a glucose ester or a glucoside. Glucose esters are high-energy compounds that can act as biosynthetic intermediates in which the aglycone (substrate) can be further transferred onto a second acceptor. Glucosides, on the other hand, are thought to represent detoxification compounds (such as those of pesticides and herbicides), although monolignol glucosides have been associated with lignin biosynthesis (Lim et al., 2003, references therein). Both glucose ester and glucoside forms are thought to allow access to membrane-bound transporters and exit pathways from the cytosol, such as to the cell wall or to the vacuole (Jones and Vogt, 2001; Lim et al., 2003). These statements are of interest with regards to glucosylation of ABA.

Several conjugates of ABA and also its metabolites PA and DPA have been reported, although conjugation at the C1 position to form ABA-GE appears to be most wide-spread (Cutler and Krochko, 1999; Zeevaart, 1999). Conjugates of ABA are thought to be physiologically inactive with the conjugates being sequestered in the vacuole (Kleczkowski and Schell 1995; Zeevaart 1999). However, it has recently been suggested that ABA-GE can act as a transport form of ABA through the xylem (and to a limited extent in the phloem) (Sauter et al. 2002). The molecular mechanism of the transport of ABA-GE is unknown (as is the transport of ABA itself); however, it has been suggested

to be mediated by ABC transporters (Sauter et al. 2002, references therein). In addition, release of ABA from ABA-GE via β-D-glucosidases has been detected in barley, sunflower, and wheat (Lehmann and Vlasov, 1988; Dietz et al., 2000; Sauter et al. 2002), although glucosidases that hydrolyse ABA-GE remain to be identified (i.e. none have been purified, cloned, and characterized to date) (Sauter et al. 2002).

1.6.2.2 Identification of ABA glucosyltransferases

A UGT gene encoding ABA glucosyltransferase, termed AOG, was identified from Adzuki bean (*Vigna angularis*) as the first reported gene for ABA catabolism (Xu et al., 2002). The product from racemic ABA and UDP-D-glucose was identified to be ABA-GE in recombinant assays. Unlike CYP707As and the UGT described below, AOG exhibits wide substrate specificity. Recombinant AOG (rAOG) converted 2-*trans*-(+)-ABA better than (+)-ABA, (-)-ABA, and *trans*-cinnamic acid. However, rAOG was not able to conjugate UDP-glucose to PA (Xu et al., 2002). The AOG gene is induced by 50 μM ABA, drought, and wounding in adzuki bean hypocotyls but not in leaves (Xu et al., 2002).

Recently, the *Arabidopsis* UGT 71B6 was identified as an ABA glucosyltransferase that forms ABA-GE from (+)-ABA (Lim et al., 2005; Priest et al., 2005). In a detailed analysis of the structural requirements for UGT activity, Priest et al. (2005) found that 71B6 does not glucosylate PA, DPA, or modified analogs of 7'- or 9'-hydroxy ABA and both the 8'- and 9'-C are important for the ability of 71B6 to glucosylate ABA at C1. Further *in planta* analysis of 71B6 awaits.

1.7 Regulation of genes involved in ABA metabolism by other signaling factors

As detailed above (in section 1.5, 1.6) ABA may regulate its own accumulation by modulating the biosynthetic and catabolic pathways. However, the specifics of ABA biosynthetic and catabolic gene regulation remains mostly unknown and detailed studies of the promoters of these genes and factors that may bind to them have not been reported. Several studies have uncovered factors that either directly or indirectly regulate ABA levels (although direct regulation by any factor has yet to be shown).

As mentioned previously, seeds of the phytochrome-deficient (*pew1*) mutant of *Nicotiana plumbaginifolia* accumulated higher levels of ABA in the mature seeds – suggesting that ABA metabolism is controlled, at least in part, by a phytochrome-mediated light signal (Kraepiel et al., 1994). In addition, seeds are hyper-dormant and plants drought resistant. Further, double mutant analysis with the *Npaba1* (AB-AO deficient) mutant suggested that the effect of the *Nppew1* mutation does not affect ABA biosynthesis (at least earlier steps in the ABA biosynthetic pathway from AB-AO). The phytochrome- and ABA-deficient double mutants (*pew1-aba1*) are non-dormant due to ABA deficiency but accumulated no more *trans*-ABA-alcohol glucoside (the accumulation product in *Npaba1* mutants) than *Npaba1* alone. The results suggest that the endogenous function of the *Np*PEW1 protein is to modulate ABA levels by up-regulating ABA catabolism rather than by down-regulating ABA biosynthesis (Kraepiel et al., 1994).

Recently the FUS3 transcription factor was shown to be a nexus of hormone action during *Arabidopsis* embryogenesis (Gazzarini et al., 2004). Firstly, *FUS3* expression was influenced by auxin but more relevant to this review is the fact that FUS3 positively regulates ABA synthesis while negatively effecting GA synthesis. In loss of function *fus3* mutants, ABA levels decrease while GA levels increase; conversely, *FUS3* misexpressors showed the opposite trend, an increase in ABA and decrease in GA levels (Gazzarini et al., 2004). Gazzarini et al. (2004) suggest that it is this manipulation of the ABA/GA level ratios by FUS3, and a positive/negative feedback regulation of FUS3 by ABA/GA respectively, that helps determine developmental events during seed development. Interestingly, maternally derived ABA can inhibit the viviparous germination of *fus3* mutants (Raz et al., 2001) supporting the contention that FUS3 maintains embryo growth arrest 'indirectly' by impinging on the ABA pathway.

ERA (Enhanced Response to ABA) mutations 1 to 3 of *Arabidopsis* are characterized by their inability to germinate in low ABA concentrations not inhibitory to wild-type (Cutler et al., 1996). The most prominent example of the *era* mutations is *era1* whose gene encodes a β-subunit farnesyltransferase (Cutler et al., 1996; for further details see Nambara and McCourt, 1999; Finkelstein and Rock, 2002). Recently, the *era3* locus has been shown to be allelic to *ethylene insensitive2* (*ein2*) locus (Ghassemian et al., 2000).

The *EIN2* gene encodes a novel integral membrane protein with homology to the Nramp metal-ion transporter family (Alonso et al., 1999). Mutations in this pathway lead to an over accumulation of ABA suggesting that *ERA3* (*EIN2*) is a negative regulator of ABA synthesis. In fact *ZEP* transcripts are increased in *ein2* mutants (Ghassemian et al., 2000). In addition, mutations that decrease ethylene sensitivity (such as *ein2*) increase the sensitivity of seeds to ABA. Conversely, application of the ethylene precursor ACC or mutants with a constitutive ethylene response (*ctr1* mutants) decrease the sensitivity of the seed to ABA. In a similar study Beaudoin et al. (2000) screened for mutations that either enhanced or suppressed the ABA-resistant seed germination phenotype of *Arabidopsis aba1-1*. Alleles of the constitutive ethylene response mutant *ctr1* and *ein2* were recovered as enhancer and suppressor mutations, respectively (Beaudoin et al., 2000). Thus, ethylene appears to function in a similar way to GA – ethylene is antagonistic to ABA during development and seed germination. However, it is not known how the *ein2* mutation impacts ABA biosynthesis. It could be a direct specific regulation of ABA biosynthesis or due to the complex web of interactions between the different hormones.

Genetic analysis of the *Arabidopsis sad1* (supersensitive to ABA and drought) mutant indicated that ABA biosynthesis is also regulated at the level of mRNA stability. The *SAD1* locus encodes a polypeptide similar to multifunctional Sm-like snRNP proteins required for mRNA splicing, export, and degradation (Xiong et al., 2001a). The *sad1* mutant shows reduced levels of ABA and PA especially under drought stress. Expression and feeding analyses further demonstrated that the SAD1 protein is a positive regulator of the ABA biosynthetic genes *AAO3* (*AB-SDR*) and *AtABA3* (*MCS*) but not *AtABA1* (*ZEP*) (under exogenous ABA treatment) (Xiong et al., 2001a). It will be interesting to see if future studies reveal whether the regulation is direct and whether other genes involved in ABA biosynthesis or under different growth conditions or in different tissues (e.g. in seeds) are also regulated by *SAD1*.

1.8 How does ABA specifically relate to dormancy in mature seeds? What factors may be involved?

There is conclusive evidence that ABA present during seed development plays a significant role in regulating dormancy induction during development (section 1.3 and 1.4). The ABA present during development helps keep the seed in an anabolic or development mode (or a 'status quo' hormone) while suppressing events that would otherwise lead to premature activation of the germinative (growth) program (counteracting promotive hormones such as GA and ethylene). By maintaining the developmental program the seed is able to complete maturations events such as deposition of its storage reserves and preparation for desiccation (e.g. accumulation of LEAs). Also, because the seed is able to complete its maturation program, those structures or elements (e.g. the seed coat, see section 1.2) that contribute to dormancy imposition in the mature seed are fully developed. Thus, ABA may contribute to dormancy directly, by regulating dormancy-specific genes in imbibed seeds, and indirectly, by simply keeping the seed in a developmental mode, allowing the seed to complete maturation.

However, the correlation between ABA levels and dormancy is not always high enough to demonstrate that ABA maintains the dormant state. It could simply be a by-product of the dormant state, perhaps maintaining an environment conducive to surviving during a dormant state (i.e. more as a stress hormone). Some of the transcripts that are more prevalent in dormant or ABA-treated embryos (seeds) are proteins associated with the survival of stress or are normally expressed during seed development (Bewley, 1997; Li et al., 2005). For example, in wild-oat glutathione peroxidase was recovered in a differential screen to detect transcripts associated with the dormant state (Johnson et al., 1995). In Douglas fir seeds, a dehydrin and two other LEA-like proteins were recovered in a differential screen to detect transcripts whose expression is enhanced during moist chilling (Jarvis et al., 1996, 1997). Toorop et al. (2005) recovered four proteins in a differential screen for genes associated with either germination or dormancy in *Arabidopsis* Landsberg and Cvi ecotypes. Two cDNAs encoding ribosomal proteins associated with germination were recovered and suggested to be associated with growth (e.g. of meristematic tissue and lateral roots). Two cDNAs encoding a caleosin-like

protein and a protein similar to a low-temperature-induced protein were expressed in both dry and dormant-imbibed seed (Toorop et al., 2005). However, it remains to be seen how and if these latter two proteins participate in the dormancy mechanism.

Two recent studies have looked at more wide-scale changes in gene expression through microarray and cDNA-amplified fragment length polymorphism (cDNA-AFLP) analyses. Transcript profiling of imbibed canola (*Brassica napus*) during polyethylene glycol (PEG) and ABA analog (PBI 429, which is more persistent than ABA itself) treatment revealed groups of up-regulated and down-regulated genes (compared to germinating canola seed). For example, homologs of *RAB18*, 12S seed storage protein, oleosin, and late embryogenesis abundant (LEA) genes were induced by both PEG and ABA analog treatment. Li et al. (2005) suggest that expression of LEAs and other seed development protein genes in PEG- and ABA analog-treated seeds reflects a continuation of expression typical of seed development when germination is blocked and probably helps to maintain embryo viability during osmotic stresses encountered by the imbibed seed. Li et al. (2005) also examined changes in several transcription factors associated with seed germinability (i.e. *FUS3, LEC1, ABI5, ABI3*) and found that *ABI5* and *ABI3* were up-regulated by PEG and ABA analog treatment (although ABI3 was initially down-regulated in PEG compared to germinating control seeds) (see below for further discussion on ABI3 and ABI5). In addition, *PICKLE* (*PKL*), a CHD3-chromatin-remodeling factor that prevents expression of the embryonic developmental state (e.g. expression of *FUS3* and *LEC1*) during *Arabidopsis* germination and is probably induced by GA, was down-regulated by PEG and ABA-analog treatment (Ogas et al., 1999; Li et al., 2005). Thus, the Li et al. (2005) example illustrates that during an induced quiescent state in mature imbibed seed there are active processes to encourage seed survival and that a great proportion of these are modulated by ABA.

In a comparison of dormant and after-ripened *N. plumbaginifolia*, Bove et al. (2005) identified that 7% (i.e. 1020 out of 15000 cDNAs) were differentially expressed between dormant, dry seed, and early imbibition compared to non-dormant seed. Out of the 1020 cDNAs, 412 were sequenced and 83 cDNA fragments showed homology to identified genes. Thus, many 'unknown proteins' potentially involved in the dormancy mechanism remain to be characterized. Of the 83 identified cDNAs, 40 with identity to kinases,

serine threonine phosphatases, phospolipases, transcriptional regulators, G protein subunit, WD-repeat proteins, calcium transporting ATPase, and components of regulated proteolysis were recovered. Sixteen cDNAs predominantly repressed in dormant seed were assigned to proteasome components; 31 cDNAs, possibly involved in post-transcription, translation, post-translation and vesicular trafficking, were also repressed during dormancy maintenance perhaps illustrating translational and protein transport control. One hypothesis suggested by Bove et al. (2005) is that in fresh mature seeds, ABA down regulates, at the RNA level, the transition towards protein synthesis (for proteins involved in germination) as a mechanism to maintain dormancy. It is also noteworthy that Bove et al. (2005) showed that changes in gene expression occur during after-ripening (i.e. transcripts are differentially regulated even in the dry seed!). Recently, during after-ripening of tobacco, both transcription and translation of class I β-1,3-glucanase (βGlu I) were detected in low-hydrated seeds (Leubner-Metzger, 2005). βGlu I is associated with testa rupture (an effect conferred during after-ripening) and also the ABA-controlled decrease in mechanical restraint of the endosperm during germination (Leubner-Metzger, 2005, references therein). Thus, Leubner-Metzger (2005) proposed that *de novo* gene (and protein) expression is a novel molecular mechanism for the release of coat-imposed dormancy during oilseed after-ripening.

Several individual genes have been identified in seeds of beechnut (*Fagus sylvatica*) that may function in regulation of the dormant-imbibed state. FsGTP1 GTP-binding protein, FsPK1 and FsPK2 protein kinases, and FsPP2C1 and FsPP2C2 protein phosphatase 2C genes are ABA inducible in seeds and are expressed in dormant or dormancy-terminating seeds (Nicolàs et al., 1998; Lorenzo et al., 2001, 2002, 2003; Gonzáles-García et al., 2003). For example, expression of *FsPP2C1* is detected in dormant seeds and increases after ABA treatment, but decreases during dormancy release through moist chilling or GA treatment (Lorenzo et al., 2001). Further, stable over-expression of *FsPP2C1* in *Arabidopsis* results in plants that are more ABA-insensitive than wild-type and have reduced seed dormancy, ABA-resistant root growth, and increased resistance to mannitol, NaCl, and paclobutrazol (Gonzáles-García et al., 2003). The authors suggest that FsPP2C1 is an important regulator modulating the ABA signal

in dormant beechnut seeds – operating through a negative feedback loop (Gonzáles-García et al., 2003).

Another example of a putative regulator of dormancy in mature seeds is the 'ABA-associated' ABI3 (VP1) family of transcription factors. In both wild oat (*Avena fatua*) and common ice plant (*Mesembryanthemum crystallinum*), the expression of *VP1*-homologues is correlated with the degree of dormancy present in mature seeds during imbibition (Jones et al., 1997; Fukuhara et al., 1999). Further, in wild oat, if dormancy is reintroduced (secondary dormancy) *AfVP1* transcripts reappear (Jones et al., 1997; Holdsworth et al., 1999). Further, *VP1* genes in select wheat cultivars have splicing defects and that this contributes to their increased susceptibility to preharvest sprouting or precocious germination (McKibbin et al., 2002). Because ABI3 has been shown to have repressor (and activator) functions during seed development and during seedling growth (see section 1.3), it is interesting to postulate that ABI3 has similar functions during dormancy maintenance in the mature seed – regulating post-imbibition dormancy-related processes. However, conclusive evidence for ABI3 awaits and is actually quite hard to demonstrate experimentally since ABI3 exerts its major influence during seed development (i.e. stable over-expression would have more impact during seed development – but perhaps transient or inducible expression alternatives exist that can test this hypothesis). More recently, maintenance of dormancy in yellow-cedar (*Chamaecyparis nootkatensis*) appears to involve ABI3 (Zeng et al., 2003). A decline in *Cn*ABI3 protein abundance is correlated with dormancy termination; *CnABI3* transcripts also correlate with dormancy termination and their disappearance occurs during germination. Further, it has been suggested that regulation of *CnABI3* is not restricted to the transcriptional level and may occur at the level of the protein translation (e.g. protein stability) (Zeng et al., 2003). As alluded to above, one key transition that may occur during the dormancy to germination transition is the up-regulation of proteasome components (which are perhaps induced to degrade proteins responsible for maintaining the dormancy mechanism?). Interestingly, *AIP2* (*ABI3-Interacting Protein 2*), which was originally uncovered in a yeast 2-hybrid screen for proteins that interact with ABI3 encodes an E3-ubiquitin ligase (Kurup et al., 2000; Zhang et al., 2005). E3-ubiquitin ligases target proteins for degradation via the 26S proteasome pathway (Vierstra, 2003).

AIP2 can polyubiquinate ABI3 *in vitro* and, in transgenic plants, induced AIP2 expression leads to decreased ABI3 protein levels. Further, *Arabidopsis aip2-1* null mutants show higher ABI3 protein levels compared to wild-type after seed moist chilling, are hypersensitive to ABA, and mimick an ABI3-overexpression phenotype. Conversly, *AIP2* overexpressors contain lower levels of ABI3 protein compared to wild-type and are more resistant to ABA, phenocopying *abi3* (Zhang et al., 2005). Thus, AIP2 is a negative regulator of ABA responses, helping regulate ABI3 protein levels (Zhang et al., 2005).

ABI5, a member of bZIP transcription factor family (section 1.3), has been implicated in ABA-regulated gene transcription mediated by ABI3 (VP1) proteins and direct physical interactions between ABI3 and ABI5 have been demonstrated (Nakamura et al., 2001). ABI5 binding to ABA responsive elements (ABRE) in the promoter of target genes may facilitate the interaction of ABI3 with its target element, the RY *cis* motif, and the two transcription factors may act in tandem to help determine ABA-regulated gene expression (Finkelstein et al., 2002, references therein). In fact, ABI3 and ABI5 seem to act together to regulate a post-germinative development arrest, which sets in after breakage of seed dormancy but prior to autotrophic growth (Lopez-Molina et al., 2002). This arrest is transient in nature and ABI5 accumulation is induced by ABA only within a short interval of about 60 h following moist chilling, during which ABA and ABA-dependent ABI5 activity are essential to initiate the growth arrest of germinated embryos. The arrested, germinated embryos remain viable but quiescent, while tolerant of osmotic stress (Lopez-Molina et al., 2001). ABI3 is also induced by ABA within this short developmental window, acts upstream to ABI5, and is essential for ABI5 protein expression (Lopez-Molina et al., 2002). An intermediary signal, between ABA and ABI5, has also been reported during post-germinative embryo arrest – a mitogen activated protein kinase (MAPK) signaling pathway (Lu et al., 2002). Similar to ABI3, an ABI five-binding protein (AFP) is thought to target ABI5 for degradation via the ubiquitin-proteasome pathway (Lopez-Molina et al., 2003). Similar to *AIP2*, *afp-1* and *afp-2* mutants are hypersensitive to ABA, whereas transgenic plants overexpressing *AFP* are more sensitive. Thus, similar to AIP2, AFP is a negative regulator of the ABA response by modulating ABI5 protein levels.

In summary, *de novo* ABA synthesis has been implicated in the dormancy mechanism of many species (section 1.2, chapter 5); however, the direct link between endogenous ABA (its biosynthesis and catabolism) and promotion of factors maintaining dormancy in the imbibed seed is somewhat lacking. Many signaling factors, phosphatases, kinases, and transcription factors such as ABI3 and ABI5, have been implicated in the dormancy mechanism (as noted in the examples above). Yet our understanding of the precise role these proteins play during dormancy maintenance is superficial at best. It is interesting to speculate that ABI3 and ABI5 participate in a program to inhibit the phase transition between the dormancy and germination. Although it has been shown ABI3 and ABI5 are induced / expressed during PEG treatment in canola (Li et al., 2005), further analysis of factors inducing a 'germinative arrest' (such as NaCl stress) in wild-type and ABA biosynthetic mutants would further reveal further how ABI3 and ABI5 contribute to dormancy (secondary dormancy in this case).

As mentioned, analysis of the transcripts from dormant and non-dormant seeds indicates that several 'developmental programs' are 'repressed' in the dormant seed (Bove et al., 2005). Interestingly, using a massively parallel signature sequencing approach, Hoth et al. (2002) discovered some of the same gene families were ABA-regulated (although these experiments were done in *Arabidopsis* seedlings). For example, ABA-regulated genes included genes encoding ribosomal proteins that stabilize the tertiary structure of ribosomes and control the dynamics of protein synthesis (~70% were down-regulated) and genes involved in regulated proteolysis (~75% upregulated) (Hoth et al., 2002). This suggests ABA may be involved in regulating some of the same pathways during seed dormancy and acts to prevent the phase transition to germination while promoting factors involved in seed and seedling survival (many of these survival factors and upstream signaling factors, e.g. LEAs and ABI3, are also expressed during seed development, thus we see a partial continuity between late seed development and dormancy in the imbibed seed). As noted above, this may be one factor that has hampered discovery of factors involved in dormancy maintenance (i.e. factors may exert major influences during seed development and thus implication in subsequent events such as dormancy maintenance is harder to prove).

1.9 Comparing the dormancy situation in *Arabidopisis* to that of the conifer western white pine

1.9.1 Arabidopsis seed dormancy

Mature *Arabidopsis* seeds exhibit modest primary dormancy when shed from the mother plant. Conditions that break dormancy in *Arabidopsis* include: moist-chilling (stratification) (e.g. ~3 days at ~4°C for Landsberg erecta cultivar (Ler)), after-ripening (storage for a month for Ler), or GA (50 μM for Ler) (Koornneef and Karssen, 1994; Russell et al., 2000). However, the degree of dormancy does vary among the different *Arabidopsis* ecotypes. For example, while one month of after-ripening is sufficient to break dormancy in the Ler and Columbia ecotypes, Enkheim and Wassilevskija ecotypes require further after-ripening or chilling treatments (e.g. a further 5 days chilling) (Debeaujon et al., 2000).

The Cape Verdi Islands (Cvi) ecotype has recently been suggested to be an excellent model for dormancy in *Arabidopsis* (Ali-Rachedi et al. 2004). The seeds show a well-developed degree of primary dormancy in mature dry seeds. Dormancy can be broken by 5-7 months of after-ripening at ~20°C or by moist-chilling at 4°C for 4-7 days (Ali-Rachedi et al. 2004; Feurtado et al., unpublished). Unlike Ler seeds, GA is not sufficient to break dormancy; however, compounds such as the ABA-biosynthesis inhibitor fluridone (section 1.5) and nitrate (see below) are sufficient to break dormancy (Ali-Rachedi et al. 2004; see chapter 5 for further discussion). Further work with this ecotype may uncover previously unidentified genes (mechanisms) involved in dormancy maintenance.

Recently, nitric oxide and nitrate have been shown to break dormancy in *Arabidopsis* (Bethke et al., 2004; Alboresi et al., 2005). Bethke et al. (2004) showed that the NO donor sodium nitroprusside (SNP) could relieve dormancy in the ecotypes C24 and Columbia-1 in a concentration dependent manner (i.e. 25 μM was promotive but 250 μM was inhibitory). In addition, the NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3 oxide (cPTIO), was able to strengthen the dormancy of unstratified and partially stratified seeds (but not non-dormant seeds) (Bethke et al., 2004). High nitrate (50 mM) fed to *Arabidopsis* Columbia mother plants produced less dormant seed populations than 10 mM (normal nitrate); in turn, 10 mM produced less

dormant seeds than 3 mM (nitrate starved) fed plants. Secondly, it seems that the nitrate accumulated within the seeds affected seed dormancy and was not solely a nutritional affect (although it is unknown whether the accumulated nitrate effects dormancy induction during developmental or whether the accumulation is important during imbibition). However, exogenously applied nitrate (1 and 10 mM) released dormancy of mature wild-type seeds (Alboresi et al., 2005). Alboresi et al. (2005) suggest that nitrate acts to positively regulate dormancy / germination by altering GA and ABA synthesis or signaling. Supporting this is the fact that high-nitrate-produced seeds displayed a greater resistance to the GA-biosynthesis inhibitor paclobutrazol suggesting the GA requirement for germination was less. Interestingly, nitrate (7 mM) can also release dormancy in the more dormant ecotype Cvi; NO_3^- speeds the decrease in ABA levels during imbibition of dormant Cvi seeds (similar to the ABA biosynthesis inhibitor fluridone) (Ali-Rachedi et al. 2004).

Studies have shown that both the testa and embryonic ABA seem to play a role in *Arabidopsis* seed dormancy and its maintenance. *Arabidopsis* seed coat mutants impaired in testa pigmentation and/or structural characteristics often exhibited reduced dormancy (Debeaujon et al., 2000). One of the main defects in the testa pigmentation mutants is a reduction or absence of phenolic compounds. The presence of phenolic compounds restricts the permeability of the seed coat and may contribute to dormancy by limiting water entry, oxygen availability, the exit of endogenous inhibitors (ABA), and/or the mechanical resistance of the testa (see Chapter 4 for further discussion). In dormant wild-type seeds, removal of the testa resulted in germination (Debeaujon et al., 2000). The ABA biosynthesis inhibitor norflurazon was partially effective in releasing wild-type and testa-mutant seeds from dormancy, suggesting *de novo* ABA synthesis may play a role in dormancy (Debeaujon and Koornneef, 2000). Further, Debeaujon and Koornneef (2000) provide evidence that GA is necessary to overcome the germination constraints imposed by both the seed coat and ABA-related embryo dormancy.

1.9.2 Western white pine seed dormancy

Unlike *Arabidopsis* seeds, seeds of western white pine (PW) exhibit a pronounced and deep physiological dormancy. Moist-chilling (stratification) of PW seeds for a period

of at least 90 days yields the highest and most synchronous germination (Hoff, 1987). And even with this lengthy treatment, the germination of some seedlots is uncertain and inconsistent perhaps due to inadequate dormancy-breakage. Some seedlots require up to 120 days of moist-chilling to procure higher germination capacities (Gansel, 1986). Attempts have been made to remove dormancy and elicit adequate germination in PW seed through a variety of methods. These include: freezing, alternating warm and cold stratification for 30 days each, exposure to infrared radiation, and various chemical or mechanical treatments to diminish the hard seed coat (Larsen, 1925; Anderson and Wilson, 1966; Works and Boyd, 1972; Pitel and Wang, 1985). Yet none of these methods has achieved enough success to replace the traditional cold stratification. Current dormancy-breaking procedures for white pine still rely on a lengthy period of moist-chilling to break dormancy – a 2-day water soak, drain, 12-day water exchange, followed by moist-chilling (~ 4°C) for 98 days (Leadem, 1996; Feurtado et al., 2003).

The dormancy mechanism of PW seems to be mainly coat-imposed. PW seeds have a thicker outer seed coat layer consisting of thin and leathery and thick and hard portions (Hoff, 1987). Surrounding the megagametophyte is a thin nucellar membrane commonly referred to as the papery-layer (Hoff, 1987; Dumroese, 2000). Removal of a portion of the hard coat on the side of the seed resulted in 16% germination without a stratification treatment (Hoff, 1987). Further treatments that remove either part of the hard seed coat or the entire seed coat but also disrupt the papery-layer resulted in ~50% germination with no stratification treatment (Hoff, 1987). Hoff (1987) suggests that the megagametophyte and embryo contribute to the remaining dormancy present in PW seeds. However, excised embryos are able to elongate in culture (Dumroese, 2000; see chapter 4). Dumroese (2000) suggests that the papery-layer acts a barrier to gas exchange rather than a barrier to water uptake and that this is an important aspect that helps keeps PW seeds dormant. Thus, both the seed coat, including the papery nucellar membrane, and the megagametophyte contribute to a coat-imposed (coat-enhanced) dormancy in PW seeds. Whether the seed coat and megagametophyte act as a mechanical restraint, limit gas exchange (as Dumroese (2000) suggests), and/or prevent the leaching of inhibitors have yet to be fully clarified. However, recently it was found that the puncture force required to penetrate the megagametophyte and testa was reduced following moist chilling, while

the growth capacity of the embryo was increased (see chapter 4). Additionally, work has demonstrated that ABA may play a role in maintaining the dormant state in PW seeds. ABA concentrations in the embryo and megagametophyte decrease substantially as stratification proceeds (see chapter 3). Thus, the coat-enhanced dormancy mechanism in PW is complex with numerous interacting factors, one of which could be the prevention of oxygen diffusion into the megagametophyte and embryo, thus hindering oxidative catabolism of ABA (see chapter 4).

1.9 Final thoughts, Conclusions, and Research Objectives

ABA mutants, especially in *Arabidopsis*, maize and tomato, have implicated ABA as a major player in seed development, dormancy induction, and possibly even dormancy maintenance. The ABA biosynthetic pathway, now virtually deciphered, has revealed numerous redundancies in the various enzymes involved and that several of the substrates may take alternate routes to ABA. The finding that the major enzymes involved in the formation and breakdown of ABA (e.g. NCED, CYP707A) exist as multi-gene families only complicates the picture but is not surprising since ABA is implicated in so many physiologically and developmentally diverse processes from seed development to various environmental stress responses (e.g. to drought, cold, and salt). However, more progress needs to be made with regards to ABA catabolism, which undoubtedly contributes a great deal to the fluxes in ABA we see in a particular tissue.

One of the more important aspects that deserves further attention is how ABA levels are regulated. There is a need for a comprehensive analysis of the ABA biosynthetic and catabolic genes identified to date. This is hindered during microarray analysis because of the low expression of some of these genes (e.g. CYP707As); a more thorough approach could be attempted using a more sensitive technique such as real-time PCR expression analysis. Further, developmental factors that govern ABA levels during seed development include both negative (e.g. *pew1* and *ein2*) and positive (e.g. *fus3*) regulators. However, how these *Arabidopsis* loci exert their functions remains to be determined. It will be necessary to analyze the promoters of given biosynthetic and catabolic genes to more fully determine possible regulatory points. For example, since

FUS3 is a putative transcription factor does it directly regulate transcription of ABA biosynthetic and catabolic genes?

Another connection that needs to be made is how ABA regulates the transcription of various factors putatively involved in dormancy maintenance (e.g. ABI5 and ABI3). Factors that regulate the amounts of ABI5 and ABI3 protein have been identified but how *ABI5* and *ABI3* are regulated at the level of transcription remains unclear. Could ABA act through a similar mechanism as auxin; auxin binds to transport inhibitor response protein 1 (TIR1) (or related auxin-binding factors, ABFs) which subsequently targets Aux/IAA proteins for proteolosis subsequently modulating transcription of auxin-regulated genes (Dharmasiri et al., 2005). Recent identification of the ABA-binding protein FCA, a floral regulatory gene, suggests hormone-mediated protein binding is a mechanism for ABA gene regulation. In this case, FCA, in combination with FY, modulate Flowering Locus C (FLC) mRNA levels; when ABA binds to FCA the combinatorial action of FCA-FY is inhibited and FLC transcripts increase causing a delay in flowering (Razem et al., 2006).

Many questions remain as to how dormancy is maintained and how ABA participates in this process. Undoubtedly, the biggest gap in our knowledge is with respect to ABA catabolism and that is where this thesis study will take root. The focus will be on the conifer western white pine as a model for seed dormancy and, in particular, how ABA metabolism participates in this process. The work presented here will centre on: (i) the mechanistic aspects of factors necessary for dormancy termination (i.e. what factors can improve/optimize dormancy breakage?); and (ii) the intrinsic factors involved in the dormancy mechanism of western white pine with particular attention paid to ABA (i.e. how is ABA involved in the dormancy mechanism?). Part (i) of the thesis will discuss the conditions necessary for synchronous efficient germination of western white pine (Chapter 2). Part (ii) focuses on 3 introductory studies involved in cementing ABA as a causal factor in the dormancy mechanism (Chapters 3 to 5). As a continuation of part (ii), genes involved in ABA metabolism in western white pine were cloned and their expression monitored through dormancy breakage (moist chilling) (Chapter 6). The underlying hypothesis to this thesis, as it began, was: "The inherent dormancy of western white pine seeds is due to the complex relationships between several causal factors,

including structures surrounding the embryo which create mechanical restraint and the presence of germination inhibitors which may include the phytohormone abscisic acid."

CHAPTER 2

Increasing the temperature of the water soak preceding moist-chilling promotes dormancy-termination of seeds of western white pine (*Pinus monticola* Dougl.)

2.1 Introduction

At maturity, seeds of western white pine exhibit a pronounced primary dormancy. Dormancy is generally classed as either embryo or coat-enhanced dormancy (Bewley and Black, 1994). For western white pine, it is primarily coat-enhanced being imposed by the seed coat and to lesser extents the megagametophyte and nucellar membrane. A papery-membrane (the endotestae), located between the hard outer layers of the seed coat and the megagametophyte and nucellar cap, is a structural feature of particular importance in the coat-enhanced dormancy (Owens and Molder, 1977; Hoff, 1987; Dumroese, 2000; see Chapter 4). Elucidating the mechanisms of this coat-enhanced dormancy of western white pine seeds has proven difficult; factors implicated include restriction of water and gas (O_2) entry into the seed and prevention of the release (and/or catabolism) of germination inhibitors (Hoff, 1987; Dumroese, 2000; Ren, Abrams and Kermode, unpublished results). For example, an effective dormancy-breaking treatment (that includes prolonged moist chilling) leads to a five-fold decrease in abscisic acid (ABA) within the embryo and megagametophyte and it is very likely that enhanced ABA catabolism plays a key role in this decline (see Chapter 3). Treatments that terminate the dormancy of western white pine seeds must alleviate the block to germination (radicle protrusion) imposed by seed tissues surrounding the embryo. It is during moist-chilling that the mechanical restraint imposed by the seed coat and megagametophyte decreases while the growth potential (turgor) of the embryo increases (see Chapter 4).

Attempts to elicit effective dormancy breakage and hence adequate germination of western white pine seed have employed a variety of methods. These include: (i) freezing; (ii) alternating warm and cold moist periods (30 d each); (iii) exposure to infrared radiation and (iv) various chemical or mechanical treatments that diminish the hard seed coat (Larsen, 1925; Anderson and Wilson, 1966; Works and Boyd, 1972; Pitel and Wang, 1985). However, none of these methods has achieved enough success to replace traditional moist chilling at 2-4°C. Moist chilling (cold stratification) of western white pine seeds for a period of at least 90 d yields the highest and most synchronous germination (Hoff, 1987). Yet even with this lengthy treatment, the germination of some seedlots is uncertain and inconsistent perhaps due to inadequate dormancy-breakage.

Some seedlots require up to 120 d of moist chilling to procure higher germination capacities (Gansel, 1986). Thus, at present, dormancy-breaking procedures for western white pine still rely on a lengthy period of moist-chilling to break dormancy and the general current practice in the British Columbia (B.C.) forest industry is to use a prolonged pre-soak in water for 14 d followed by moist chilling for 98 d (Leadem, 1996; D. Kolotelo, pers. commun.).

Our objectives in this study were to shorten the time needed to obtain adequate germination and further, to elicit a rapid rate and improved synchronicity of germination of western white pine seed populations. To achieve these objectives, both the conditions of the pre-treatment soak and the methodology of moist chilling were modified. The initial water soak in which seeds imbibe water, generally at 15-20°C, prior to moist chilling varies significantly between forest nurseries and other sectors of the industry from 1-14 d in duration (Gansel, 1986; Hoff, 1987; Leadem, 1996; D. Kolotelo, personal commun.). To optimize this pre-soak treatment, we tested the effects of varying the duration of the soak, the temperature of the soak and the inclusion of chemicals known to promote dormancy-breakage of other species. For example, certain chemical treatments (1-propanol, GA_3 and KNO_3) either alone or in combination with a previous water soak at elevated temperature are effective in increasing the efficiency of dormancy termination of seeds of yellow cedar (*Chamaecyparis nootkatensis*) (Xia and Kermode, 2000). Notably, these treatments are able to replace warm stratification and reduce the time required for moist chilling. Current techniques for moist chilling (cold stratification) also differ. Here seeds are often moist-chilled in plastic bags or between layers of Kimpak (absorbent cellulose paper, Kimberly-Clark, Wisconsin, USA) within Petri dishes, seed aging boxes or plastic bags (the latter is commonly referred to as cold stratification) (Dumroese, 2000; D. Kolotelo, personal commun.). Traditionally, tree seeds have also been mixed in either a sand or sphagnum-peat matrix during moist-chilling (also termed cold stratification) (Derkx, 2000). To address the factor of the moisture content of seeds during moist chilling we implemented solid matrix priming (SMP) (Taylor et al., 1988). SMP is similar to osmotic priming, allowing the seed to attain a threshold moisture content and pre-germinative metabolic activity but preventing radicle emergence. However, it has the

advantages of allowing aeration, incorporation of biological agents to combat soil-borne pathogens, and improved ease of handling (Taylor et al., 1988). Controlling the water content to below that of a fully-imbibed seed improves the germination of many conifer species, particularly *Abies* species which are prone to germination during stratification (Edwards, 1996; Poulsen, 1996; Wu et al., 1999; Wu et al., 2001; Ma and Kermode, unpublished). Thus, in the present study, we sought to develop methods that optimize the moisture content of western white pine seeds during moist chilling, particularly through the use of SMP.

2.2 Materials and Methods

2.2.1 Seed materials

Five seedlots of varying harvest year, germination capacity and degree of dormancy were obtained from the B.C. Ministry of Forests Tree Seed Centre (Surrey, B.C., Canada) (Table 2.1). Seeds were stored at –20°C before use.

Table 2.1. Western white pine seedlots used for dormancy-breakage studies.

Seedlot number	Year collected	Location Lat. (N)	Long. (W)	Elevation	Germination capacity (%)[a]
03727	1978	50°07'	121°53'	1400	77
05066	1985	51°09'	118°11'	1000	72
08006	1981	51°31'	119°12'	909	87
43590	1995	49°40'	124°15'	600	77
61022	1997	50°00'	118°00'	750	86

[a]British Columbia Ministry of Forests, Tree Seed Centre data of germination percentages obtained after 28 d in germination conditions (30°C d, 20°C, nights; eight-hour photoperiod) following a "G55" dormancy-breaking protocol: running water soak for 14 d at 10°C followed by a 98 d moist chilling at 2°C. Germination criteria are met when the length of the radicle is four times the length of the seed.

2.2.2 Seed treatments

2.2.2.1 Chemical treatments and increased temperature soaks

For chemical treatments, increased temperature soaks, and their associated controls seedlots 03727 and 08006 were used. Seeds were soaked in running de-ionized H_2O at room temperature (~21-22°C) or in a 17 L-capacity-circulating-water bath at 25-28°C for 6 to 12 d. Chemical incubations implemented at the end of the water soak, were conducted with agitation (70 rpm) at 25°C in parafilm-sealed Petri dishes (25 x 100 mm) containing 30 mL solution and 100-150 seeds). Chemical treatments included a 1 d incubation in 1-propanol (20-100 mM; Anachemia, Montreal, Que., Canada) followed by a 2 d incubation in 200 mg/L GA_3 (Sigma Co., St. Louis, MO, USA). As a control for the chemical treatments, seeds were incubated in water with agitation (70 rpm) at 25°C. Following the chemical or control treatments, seeds were briefly rinsed and blotted and then placed on Whatman 3MM paper supported by 24-ply Kimpak in a clear plastic seed box (11.2 x 11.2 x 3.6 cm, Hoffman Manufacturing Company, Albany, Oregon, USA), in which the Kimpak and filter paper were moistened with 25 mL ddH_2O. Seeds were then chilled at 4°C (in a constant temperature incubator; Conviron Model TC 16, Winnipeg, Man, Canada) for the desired time (60, 75, or 96 d). Seeds were then transferred to germination conditions (25°C d, 15°C nights with an eight hour photoperiod) maintained in a controlled growth chamber (Conviron Model E15, Winnipeg, Manitoba, Canada). For germination, the seeds were handled in the same manner as for moist chilling, except the Kimpak and filter paper were moistened with 50 mL ddH_2O. Germination data are based on four replicates of 50 to 60 seeds each.

2.2.2.2 Solid-matrix priming (SMP) treatments

For SMP and SMP controls seedlots 05066, 43590, and 61022 were used. Seeds were soaked for 12 d at room temperature (~21-22°C) in aerated de-ionized H_2O, drained, sterilized with 3% H_2O_2 for 30 min and rinsed several times with ddH_2O. Following a brief surface drying under a flow-hood for approximately 5 min (until seed surface-moisture disappeared), seeds were transferred to moist-chilling conditions. Solid matrices

for SMP were sand and Agro-lig Greensgrade (humic acids); for the SMP control, seeds were placed on Whatman 3MM paper supported by 24-ply Kimpak that had been moistened with approx. 25 mL ddH_2O in a clear plastic seed box (Hoffman Manufacturing). SMP treatments during chilling used 30 mL play-sand (Target Products Ltd., Burnaby, British Columbia, Canada) or 30 mL Agro-lig Greensgrade (American Colloid Company, Reeder, North Dakota, USA) that were enclosed in 18-ounce sterile sample bags (Fisher Scientific, Nepean, Ontario, Canada); for the duration of chilling, the bags were tied loosely at the top with a twist tie. Three different moisture contents were used for each matrix. For play-sand, 5% water content (w.c., based on dry weight) (2.5 mL ddH_2O), 15% w.c. (7.5 mL), and 25% w.c. (12.5 mL) were used and for Agro-lig Greensgrade, 40% w.c. (9.4 mL), 70% w.c. (16.45 mL), and 100% w.c. (23.5 mL) were used. After 45 to 98 d of moist-chilling at 2°C in the dark, seeds were rinsed with water through a 14 mesh-size screen in order to remove the matrix material before being transferred to germination conditions (same as above). Germination data were based on three replicates of 50 seeds each (see below for germination details).

2.2.3 Germination tests and analyses

Germination was defined as 1 cm of radicle growth and was counted at 3 d intervals over 30 d. For the SMP studies, in addition to monitoring germination capacity (GC) over 30 d, the germination rate (GR) was also calculated based on the formula: $GR = [(T \times G_1) + (T-1) \times (G_2-G_1) + (T-2) \times (G_3 - G_2) + \ldots\ldots (1 \times (G_T - G_{T-1}))]/T$, where T is the duration of the germination test in d (i.e. T=30) and G_1, G_2, G_3,..... G_T are the total germination percentages on d 1, 2, 3, ... and T (Xu, 1990; Ren and Kermode, 2000).

2.2.4 Seed moisture contents during SMP

Seed moisture contents (m.c.) were calculated using the equation: seed m.c. % = [(fresh weight – dry weight)/fresh weight] x 100. To calculate fresh weights, seeds were weighed at various times including before soaking (storage m.c.), after soaking (fully imbibed seed), and after moist chilling (for 45, 60, 75, or 98 d). After moist-chilling seeds had to been removed from the water-moistened matrix with a brief rinse in

deionized-H_2O. Before weighing, seeds were blotted to remove surface moisture with a paper towel. Dry weight calculations were based on the average of three replicates of 30 seeds (for each seedlot) following drying first at 104°C for 20 h and then over silica gel in a desiccator for two h.

2.2.5 Tetrazolium chloride (viability) test

To assess seed viability after a 98 d-moist-chilling period in the SMP control samples (chilling of seeds conducted on water-moistened filter paper/Kimpak), seeds were stained with tetrazolium chloride. Seeds were halved longitudinally through the center of the embryo – one half of the seed was discarded while the other half was soaked in tetrazolium chloride for approx. 30-45 min in Petri dishes containing a 0.5% solution of 2,3,5-triphenyl-tetrazolium chloride (Sigma-Aldrich Canada, Oakville, Ontario). Viable seeds were denoted by the appearance of red staining in the embryo and megagametophyte.

2.3 Results

2.3.1 Effectiveness of 1-propanol, GA_3, and higher temperature soaks in promoting dormancy breakage

Chemical treatments that included 1-propanol and/or GA_3 following a 21, 25, or 27°C water soak (prior to moist chilling) did not improve dormancy breakage (germination) over the water controls (Table 2.2; compare treatment 5 with treatments 8 and 9 and treatment 4 with treatment 7). However, increasing both the temperature and the duration of the water soak did have a positive effect on dormancy breakage in seedlot 08006 (Table 2.2; compare treatment 1 with treatment 2 for soak duration and treatments 2 vs. 3 and 4 vs. 5 for soak temperature). For example, seeds that were moist-chilled for 60 d following a 21°C pre-soak showed a significant improvement in their germination percentage, when the soak was increased from 6 to 12 d. Increasing the temperature at which the seeds were soaked over 12 d from 21°C to 25°C also improved germination (which increased from 48 to 82.8%; Table 2.2); a further increase in the soak temperature to 27°C led to a further improvement in germination, which reached 90.3% (Table 2.3,

seedlot 08006), the maximum germination capacity of seedlot 08006 (Table 2.1). A closer examination of the effects of water soak temperature on the germination of seedlots 03727 and 08006, revealed that the optimum soak temperature was 27°C and that optimum soak time was 10 to 12 d (Table 2.3; data not shown). Marginally increasing the soak temperature to 28°C for the 12 d soak preceding 60 d of moist chilling decreased the germination capacity of all seedlots (Table 2.3).

Testing an increased water soak temperature over a range of different moist chilling times proved that increased temperature soaks (27°C optimum) could effectively reduce the moist-chilling requirement of western white pine seeds (Fig. 2.1). There were virtually no differences in the germination capacity or rate of seeds moist-chilled for 60, 75, or 96 d following a previous 10 d soak at 27°C. However, there was a clear effect of the duration of moist chilling on the germination of seeds that had received a previous 10 d soak at 21°C (Fig. 2.1).

Table 2.2. Germination capacities of western white pine seedlot 08006 after being subjected to various pre-treatments prior to 60 d of moist chilling. Experiment performed and data collected by Jian-Hua Xia.

Soak treatment[a]			Soak (d)	Germination Percentage[b]
Step 1	Step 2	Step 3		
1. 6 d soak (21°C)[c]			6	36.0 ± 8.1
2. 12 d soak (21°C)			12	48.0 ± 4.2
3. 12 d soak (25°C)			12	82.8 ± 4.4
4. 6 d soak (21°C)	3 d soak (25°C)		9	63.5 ± 8.4
5. 6 d soak (27°C)	3 d soak (25°C)		9	77.2 ± 5.6
6. 6 d soak (21°C)	1 d soak (25°C)	2 d soak GA_3[e]	9	66.0 ± 6.8
7. 6 d soak (21°C)	1 d soak, propanol[d]	2 d soak GA_3[e]	9	57.6 ± 9.0
8. 6 d soak (27°C)	1 d soak (25°C)	2 d soak GA_3[e]	9	75.1 ± 6.2
9. 6 d soak (27°C)	1 d soak, propanol[d]	2 d soak GA_3[e]	9	80.2 ± 6.8

[a]Unless noted otherwise, soaks were performed in de-ionized H_2O.
[b]Germination after 30 d is based on the average of four replicates of 50-60 seeds each (± S.D.).
[c]All temperatures are accurate to 1°C.
[d]Conducted at 25°C with 1-propanol at 40 mM.
[e]Conducted at 25°C with GA_3 at 200 mg/L.

Table 2.3. Optimal soaking temperature for dormancy-breakage of western white pine seedlots 08006 and 03727[a]. Experiment performed and data collected by Jian-Hua Xia.

Seedlot	D[b]	Water soak temperature (°C)[c]			
		21	25	27	28
03727	15	28.6 ± 10.2	42.4 ± 4.4	58.5 ± 10.4	33.7 ± 12.4
03727	30	34.6 ± 3.4	65.0 ± 6.8	77.8 ± 3.4	62.5 ± 11.8
08006	15	40.9 ± 2.2	46.5 ± 8.0	76.1 ± 9.8	41.6 ± 2.6
08006	30	48.0 ± 4.2	82.8 ± 4.4	90.3 ± 4.8	85.0 ± 7.2

[a]Seeds were soaked at indicated temperatures for 12 d followed by a 60 d moist-chilling period. Germination data are based on the average (± S.D.) of four replicates of 50-60 seeds each.
[b]D in germination conditions
[c]Temperatures are accurate to 1°C.

2.3.2 Effectiveness of solid matrix priming during moist chilling and the control over seed moisture

Solid matrix priming (SMP) was conducted on 3 seedlots (05066, 43590, and 61022) during moist chilling as a means of controlling more precisely the moisture content (m.c.) of western white pine seeds during chilling. Sand and Agro-lig Greensgrade were chosen as matrices since they could be easily separated from the seed after moist chilling. A range of moisture contents was tested to optimize the seed m.c. during dormancy breakage. However, SMP during moist chilling was ineffective in achieving adequate dormancy-breakage compared to controls (i.e. seeds chilled on water-moistened filter paper/Kimpak) and the germination capacities (GCs) for the different seedlots were below those predicted in Table 2.1 (Table 2.4; Fig. 2.2). For example, following a 98 d SMP/moist chilling treatment, germination of seedlot 61022 was 54.1% in sand (25% water content, w.c.) and 46.0% in Agro-lig Greensgrade (100% w.c.), while the germination of control seeds was 96.7% (Fig. 2.2C). (Germination capacity for this seedlot reported by the Tree Seed Centre is 86%; Table 2.1). Germination percentages following 45- and 60 d SMP/chilling treatments (conducted with sand and Agro-lig Greensgrade at the different moistures) were generally similar and most were similar to the controls (Fig. 2.2).

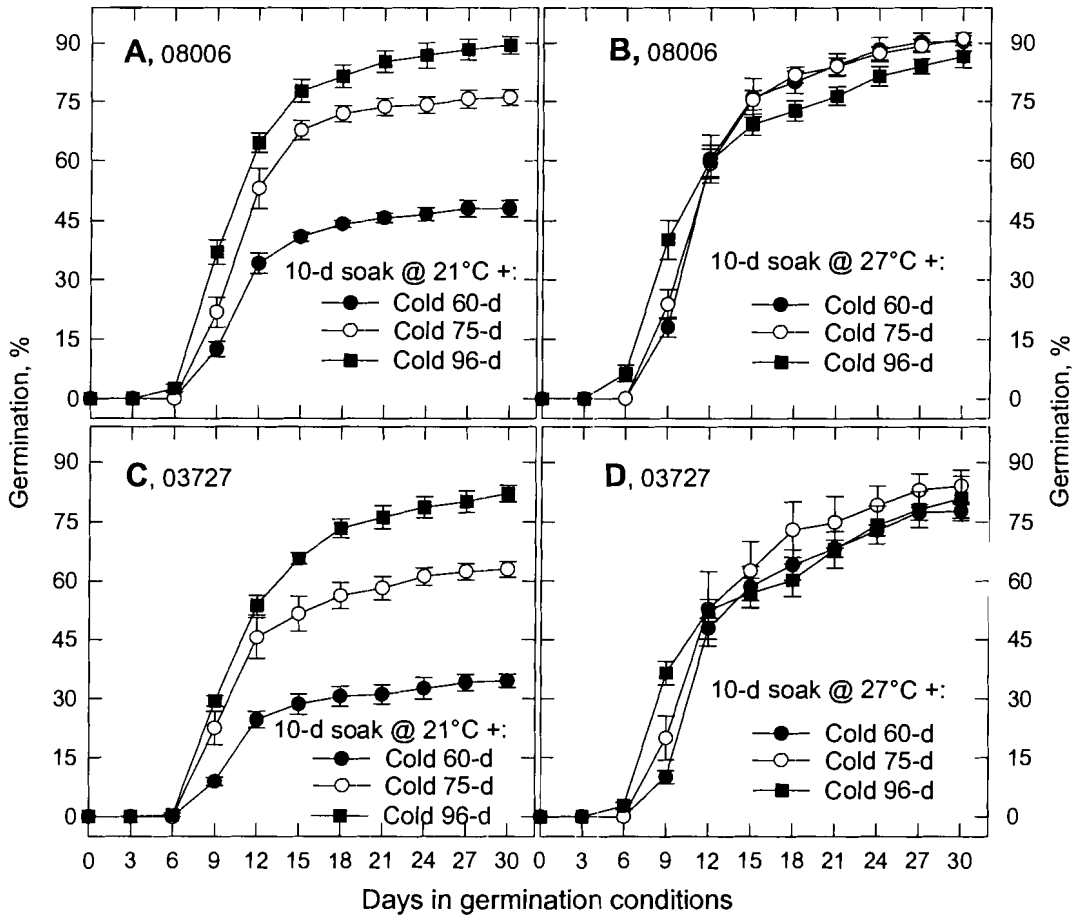


Fig. 2.1. Germination of western white pine seedlots 08006 (**A**, **B**) and 03727 (**C**, **D**) following a water soak and moist chilling. Seeds were either soaked at 21°C (**A**, **C**) or 27°C (**B**, **D**) for 10 d, followed by 60, 75, or 96 d of moist chilling on a wet substratum, before transfer to germination conditions for 30 d. Data are based on the average of four replicates of 50-60 seeds each (± S.D.). Experiment performed and data collected by Jian-Hua Xia.

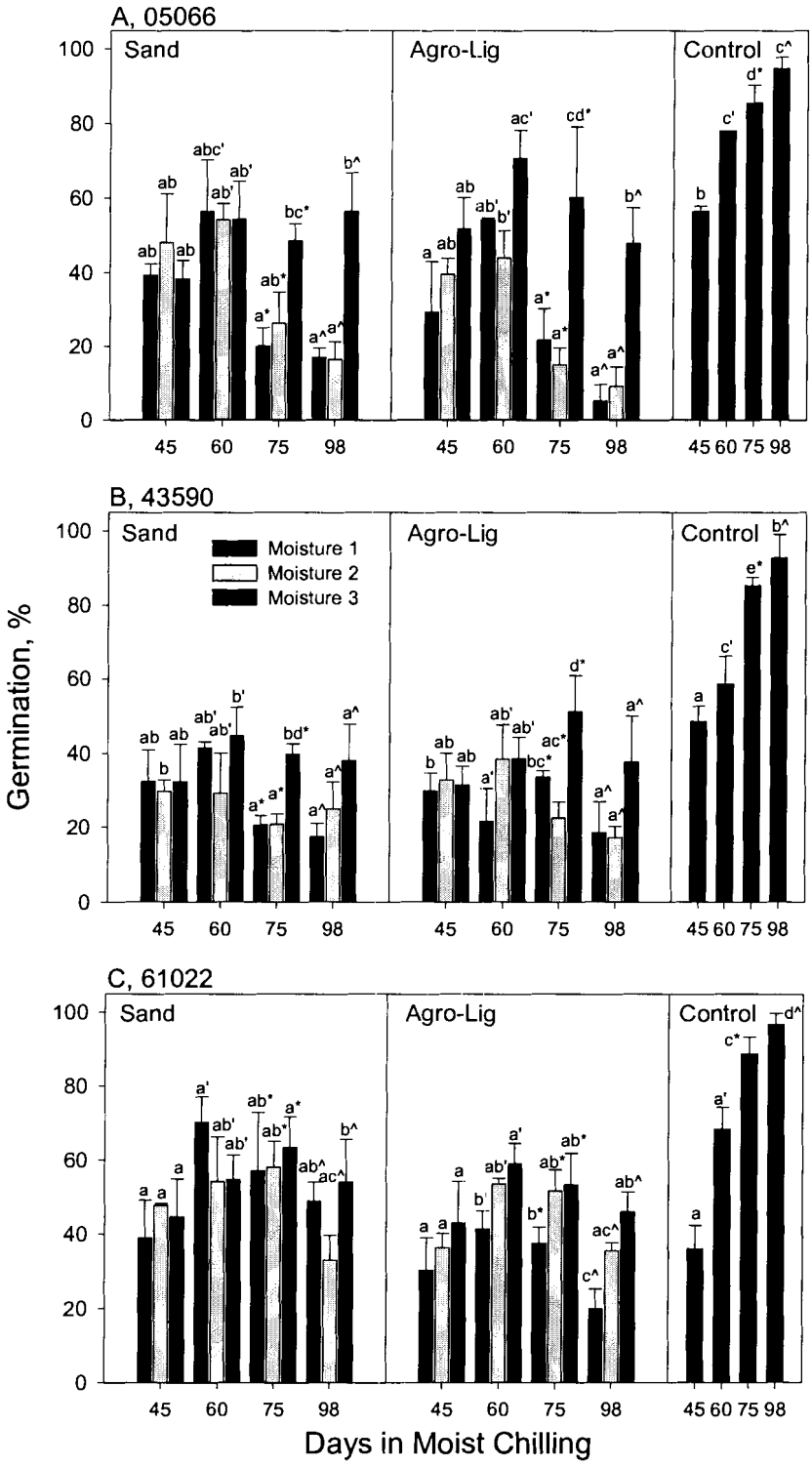


Fig. 2.2. Germination of western white pine seedlots 05066 (A), 43590 (B), and 61022 (C) following a 12 d water soak and control-moist-chilling or SMP-chilling at three moisture contents. Seeds were moist- chilled (45 to 98 d) in either sand (sa) or Agro-lig Greensgrade (al) (solid matrix priming) or on a wet substratum – filter paper/Kimpak (control, ct = control-moist-chilling). Data are based on the average of three replicates of 50 seeds each (± S.D.) after 30 d in germination conditions. Significant differences between treatments within each moist-chilling period are represented with different letters (Tukey test, $P < 0.05$).

Table 2.4. Germination capacity (GC), germination rate (GR), and average moisture content (m.c.) of western white pine seedlots following various periods of moist chilling conducted on water-moistened filter paper/Kimpak (control for the SMP/chilling studies).

Seedlot	Chilling time (d)[a]	GC[b] (%)		GR[b]		Average m.c.[b] (%)	
05066	45	56.5 ± 1.4	ae	42.4 ± 1.5	ab	34.8 ± 1.6	a
	60	78.0 ± 0.0	bg	62.2 ± 1.2	cd		
	75	85.5 ± 4.8	bc	68.7 ± 4.5	de		
	98	94.7 ± 3.0	c	78.5 ± 2.1	e		
43590	45	48.6 ± 4.1	ef	32.5 ± 3.4	bf	29.2 ± 1.3	b
	60	58.7 ± 7.5	ae	44.5 ± 5.8	ab		
	75	85.3 ± 2.3	bc	64.8 ± 2.3	cd		
	98	92.7 ± 6.4	c	72.9 ± 8.7	de		
61022	45	36.1 ± 6.1	f	26.1 ± 4.5	f	29.9 ± 0.8	b
	60	68.4 ± 5.9	ag	55.0 ± 5.3	ac		
	75	88.7 ± 4.5	bc	69.7 ± 4.4	de		
	98	96.7 ± 3.0	c	80.9 ± 2.2	e		

[a]Seeds were soaked for 12 d in running de-ioninzed H_2O at 22 ± 1°C, sterilized in 3% H_2O_2 for 30 min, rinsed several times with ddH_2O and surfaced dried before moist chilling at 2 ± 1°C.
[b]GC and GR are based on the average ± S.D. of three replicates of 50 seeds each. Significant differences are denoted by different letters (Tukey test, $P < 0.05$). Moisture content data are based on the average ± SD of four replicates of 50 seeds each.

Germination following SMP/chilling peaked when the treatment was conducted for 60 or 75 d but the germination percentages were well below the predicted germination potential (Table 2.1) and were also below that which can be achieved when control moist chilling (on water-moistened filter paper/Kimpak) is conducted for 98 d. In contrast, the germination of control moist-chilled seeds continued to increase as the duration of the treatment was increased (Fig. 2.2; Table 2.4). After 98 d of this control-moist-chilling treatment, the overall germination achieved was superior to that predicted in Table 2.1 (B.C. Ministry of Forests, Tree Seed Centre data) and far exceeded that derived from the SMP/chilling treatments (Fig. 2.2; Table 2.4). A tetrazolium chloride test performed on these control-moist-chilled seeds (after the 30 d in germination conditions) revealed that the percentage of ungerminated seeds that were still viable depended on the seed lot (e.g. for seedlot 05066, 4.6% were alive vs. 0.7% dead; for seedlot 43590, 3.3% were alive vs. 4.0 % dead and for seedlot 61022, 0% were alive and 3.3% were dead).

Moisture contents of the three seedlots varied little during moist chilling in the control (water) treatments and were close to that of fully imbibed seeds (Table 2.4; data not shown). There was also little variation in seed m.c. during the sand or Agro-lig

Greensgrade SMP-moist-chilling treatment between 45 and 98 d, within each of the three different matrix water contents (Table 2.5; data not shown). However, germination capacities and rates of the three seedlots did not correlate with the m.c. of the seeds during chilling – this becomes especially evident in comparing the effects of control-chilling vs. SMP-chilling (Table 2.5).

Table 2.5. Germination capacities (GCs) and rates (GRs) in comparison to the moisture content (m.c.) of seeds after 45 and 98 d of moist chilling in sand vs. on a wet substratum (control)[a,b,c].

Moist Chilling	Matrix	Matrix Water	GC after 30 d		GR		m.c. (%) of seeds after chilling	
45 d	Sand	5%	32.5 ± 8.4	abc	23.6 ± 6.4	ab	30.9 ± 1.7	ab
		15%	29.7 ± 3.1	abc	21.4 ± 1.7	ab	31.3 ± 1.1	ab
		25%	32.4 ± 10.1	abc	22.0 ± 6.4	ab	33.6 ± 1.0	a
	Control		48.6 ± 4.1	a	32.5 ± 3.4	a	29.3 ± 0.0	b
98 d	Sand	5%	17.6 ± 3.4	c	11.6 ± 3.0	b	31.4 ± 2.6	ab
		15%	25.1 ± 7.2	bc	17.1 ± 5.6	ab	32.0 ± 1.6	ab
		25%	38.1 ± 9.8	ab	29.1 ± 7.5	a	33.3 ± 0.6	a
	Control		92.7 ± 6.4	d	72.9 ± 8.6	c	30.7 ± 0.0	ab

[a]Seeds were soaked for 12 d in running de-ionized H_2O at 22 ± 1°C, sterilized in 3% H_2O_2 for 30 min, rinsed several times with ddH_2O and surfaced dried before moist chilling at 2 ± 1°C.
[b]GC, GR, and m.c. are based on the average ± S.D. of three replicates of 50 seeds each. Significant differences are denoted by different letters (Tukey test, $P < 0.05$).
[c]For the control, seeds were chilled on water-moistened filter paper/Kimpak.

2.4 Discussion

Western white pine seeds exhibit profound primary dormancy when shed from the parent tree. At natural stands, this dormancy renders seed populations virtually ungerminable unless the appropriate conditions are met. Under controlled laboratory conditions, prolonged periods of moist chilling of up to 120 d seem to satisfy the conditions needed for adequate dormancy-breakage. Yet, even with a 120 d dormancy breaking treatment, not all seedlots or seeds from a particular population germinate in a rapid and synchronous manner (Gansel, 1986). This in turn results in a loss of efficiency of forest nursery operations and increased costs as multiple seeding becomes necessary to compensate for less-than-optimal germination and post-germinative growth. Maximizing both the germination capacity and rate of a particular seedlot is the only means of

ensuring optimal seedling emergence; these factors will become increasingly important to the forest tree seed nurseries as energy costs continue to mount.

Germination of western white pine seeds (i.e. radicle protrusion) occurs when the restraints imposed by the tissues surrounding the embryo are 'weakened' to such an extent that radicle elongation can commence (see Chapter 4). This 'weakening' not only decreases the force that normally serves to impede radicle extension from the embryo within a dormant seed, but may also enhance other processes. For example, it may facilitate water and gas exchange and enhance the release and/or metabolism of inhibitors (Dumroese, 2000; see Chapter 3; Feurtado et al., 2004). A critical factor in eliciting adequate dormancy-breakage of western white pine seeds is the maintenance of high seed moisture content during moist chilling (similar to that of the fully imbibed seed) (Table 2.4, data not shown). Moist chilling of white spruce (*Picea glauca*) seeds at moisture contents of 25% or higher leads to maximum germination capacities after just one week of chilling. However, a seed moisture content below 20% during chilling result in a complete loss of germinability, regardless of the length of moist chilling. Thus, there is a threshold of moisture content in white spruce seeds (between 20 and 25%) where conditions are favoured for dormancy alleviation (Downie et al., 1998). Similarly, in yellow-cedar (*Chamaecyparis nootkatensis*) seeds, high seed moistures (of 35-40% on a fresh weight basis) are critical for effective dormancy breakage (Xia et al., 2001). For western white pine seeds, a high moisture content is not sufficient in itself to elicit adequate dormancy-breakage and germination. Solid matrix priming (SMP) (conducted in loosely sealed 18-ounce sample bags, in which seeds were dispersed between either sand or Agro-lig Greensgrade) yielded seed moisture contents that were similar to those of the control seeds (those subjected to water-moist-chilling) (Tables 2.4 and 2.5). Further, free water was still visible after 98 d of chilling in the highest matrix water contents, 25% w.c. for sand and 100% w.c. for Agro-Lig Greensgrade (data not shown). However, SMP led to germination that was far below that predicted for the different seedlots (Table 2.1) and greater discrepancies were observed between the control and SMP treatments as chilling proceeded (Fig. 2.2). The SMP treatments may have failed as a result of inadequate air exchange. Thus, not only is high seed moisture content a prerequisite for adequate

dormancy-breakage but, at least for western white pine, it is likely that the degree of aeration is also paramount to procuring high germination percentages.

Apart from the maintenance of high seed moisture and continuous gas exchange during moist chilling, a prolonged water soak period prior to chilling seems to generate higher germination percentages (i.e. greater dormancy-breakage) (Leadem, 1996; Table 2.2). Although the hard seed coat, papery-layer, nucellar cap, and megagametophyte of western white pine seeds may provide a barrier against water uptake, the seed appears to be fully imbibed after a water soak period of just 3 d (based on fresh weights) (Dumroese, 2000; Feurtado and Kermode, unpublished results). Why, then, do western white pine seeds benefit from a longer soak duration of up to 14 d? Two possibilities can be suggested: (i) Although the fresh weight data suggest that the seed is fully imbibed by 3 d, it may take longer for the seed to reach 'full imbibition', in which all the tissues of the seed are equally hydrated. Indeed there may be a redistribution of water during the longer soak duration so that the embryo and megagametophyte become fully imbibed. Confirmation of this hypothesis will await *in vivo* imaging studies of water uptake; for example the precise analyses yielded by Nuclear Magnetic Resonance microimaging (see Köckenberger, 2001 for a review). (ii) The longer soak duration, conducted at a warmer temperature than subsequent moist chilling, would allow the seed to effect metabolism that is potentially important for the ultimate dormancy-breakage of the seeds. It is not uncommon for dormancy-breaking treatments to include a warm moist period ('warm stratification'), where the seed is imbibed at warmer temperatures prior to moist chilling. Many of the tree seed nurseries in B.C. and the B.C. Ministry of Forests use dormancy-breaking protocols for conifer seeds, such as those of yellow-cedar, that implement a four-week warm moist period that precedes moist chilling (Ren and Kermode, 1999; D. Kolotelo, pers. commun.).

Until 1995, the common practice for dormancy breakage of western white pine seeds used by the B.C. Ministry of Forests included a one-month warm moist period that preceded 60 d of moist chilling; this protocol is no longer used because of problems associated with germination during moist chilling and excessive fungal growth (D. Kolotelo, pers. commun.). Our results of the effects of higher temperature soaks also

support the contention that the beneficial effects of increased soak time are related to enhanced metabolism. Increasing the soak temperature to an optimum of 27°C for 10 to 12 d (Fig. 2.1), may allow the seed to conduct metabolism to the fullest potential. These metabolic processes are likely important for the ultimate dormancy breakage of the seed; even an increase in the 'general metabolism' of the seed may prepare it for the dormancy-breaking events that occur during moist-chilling.

While it is tempting to speculate that prolonged soaking of the seed leads to an enhanced leaching/metabolism of inhibitors such as abscisic acid (ABA), a decline in seed ABA does not take place during a 13 d soak of western white pine seeds. The amount of ABA in both the embryo and megagametophyte is similar in dry seeds, seeds given a 3 d soak at 15°C, and seeds given both a 3 d soak at 15°C and a subsequent 10 d soak at 25°C (see Chapter 3). However, ABA contents do decline markedly after a subsequent 98 d moist chilling treatment (see Chapter 3). Nonetheless, there could well be an effect of elevated temperature on membranes; altering relationships between membrane components in turn may alter seed or embryo responsiveness to endogeonus hormones (Xia and Kermode, 2000).

Beneficial effects of warm stratification or water soaks conducted at an elevated temperature have been found by others for both western white pine seeds and for other conifer seeds, such as those of yellow-cedar (Xia and Kermode, 2000; Pitel and Wang, 1985; Gansel, 1986). Pitel and Wang (1985) found that both the total germination percentage and the rate of germination was improved if western white pine seeds were subjected to an eight d soak at 20°C rather than at 4°C. Gansel (1986) determined that a warm moist period of 30 d prior to a 90 d moist-chilling treatment improved germination capacities, especially for deeply dormant/low germinating seedlots of western white pine.

In summary, we have determined that increased temperature soaks prior to moist chilling can effectively reduce the requirement for moist chilling time and so speed the process of dormancy-breakage (Fig. 2.1). Seeds soaked at 27°C for 10 d prior to 60 or 75 d of moist chilling germinate at the same rate and synchronicity as those chilled for 96 d but soaked at 21°C for 10 d prior to chilling. SMP and moisture content analyses suggest that high moisture content (close to that of a fully imbibed seed) and adequate air

exchange during chilling are important determinants to achieving the best possible germination capacity and rate.

CHAPTER 3

Dormancy termination of western white pine (*Pinus monticola* Dougl. Ex D. Don) seeds is associated with changes in abscisic acid metabolism

3.1 Introduction

Once dominant to Inland Northwest forests from the coastal- to central-interior-regions of British Columbia and south into California and Montana, western white pine (*Pinus monticola* Dougl. Ex D. Don) is now a smaller component of North American forests primarily as a result of the white pine blister rust disease caused by the fungus *Cronartium ribicola* (Fins et al. 2002). However, breeding programs have developed more rust-resistant genotypes and trees are beginning to produce seed (Owens et al. 2001; Fins et al., 2002). To conserve these valuable seed stocks efforts must focus on improving current inefficiencies in the termination of seed dormancy (Dumroese 2000).

Seeds of western white pine exhibit pronounced primary dormancy at maturity. Dormancy-breaking procedures for western white pine rely on a moist-chilling treatment of approx. 90 d, which is generally preceded by a water soak of up to 14 d (Gansel 1986; Hoff 1987; Leadem 1996; D. Kolotelo personal commun.). Yet even with this lengthy treatment, the germination of some seed lots is inconsistent and additional moist chilling of up to 120 d is required (Gansel 1986). Attempts to effectively break dormancy and achieve adequate germination have employed a variety of methods, including: freezing, alternating warm and cold moist periods, exposure to infrared radiation, and chemical or mechanical treatments that help diminish the hard outer seed coat (Larsen 1925; Anderson and Wilson 1966; Works and Boyd 1972; Pitel and Wang 1985). However, none of these methods has proved superior to traditional moist chilling. Recently methods that include an increased temperature of the water soak prior to moist chilling enhance the efficiency of dormancy termination (Feurtado et al. 2003; Chapter 2); however, a lengthy moist chilling period at 2°C is still a prerequisite.

Little is known about the mechanism of dormancy maintenance in western white pine seeds and why prolonged moist chilling seems to be a requirement for effective dormancy breakage. Dormancy of seeds of this species is primarily "coat-enhanced" (see Bewley and Black 1994), imposed by the seed coat, nucellar membrane, and megagametophyte. In addition, a papery-membrane, located between the hard outer layers of the seed coat and the megagametophyte and nucellar membrane, is a structural feature of particular importance in the coat-enhanced dormancy (Owens and Molder 1977; Pitel and Wang

1985; Hoff 1987; Dumroese 2000). However, other physiological aspects of the dormancy mechanism have not been explored -- in particular the contribution of abscisic acid (ABA).

Genetic and molecular studies have clearly demonstrated a function for ABA in dormancy imposition during seed development (Karssen et al. 1983; Kermode 1995; Phillips et al. 1997; Foley 2001). Moreover, ABA is necessary for dormancy maintenance during post-imbibition (Le Page-Degivry and Garello 1992; Wang et al. 1995; Bianco et al. 1997; Le Page-Degivry et al. 1997; Yoshioka et al. 1998). For instance, when tobacco (*Nicotiana plumbaginifolia*) seeds are imbibed, there is an accumulation of ABA in dormant seeds but not in seeds that have been allowed to afterripen. The carotenoid- and ABA-biosynthesis inhibitor fluridone, when used in conjunction with gibberellic acid, is effective in breaking dormancy; exogenous application of both chemicals to seeds inhibits accumulation of ABA during imbibition (Grappin et al. 2000). However, ABA amount is not immediately indicative of changes in dormancy status; rather, the relative rates of biosynthesis and catabolism (metabolic flux) is a superior indicator of whether a seed will terminate dormancy (i.e. germinate). For example, endogenous ABA levels decline in beechnut (*Fagus sylvatica*) embryos to the same extent regardless of dormancy status, yet [^{3}H]-ABA turnover is greater in embryos subjected to a dormancy-breaking cold pre-treatment than in embryos that remain dormant because of their maintenance at 23°C (Le Page-Degivry et al. 1997). Increased ABA catabolism is associated with dormancy termination of seeds of yellow-cedar (*Chamaecyparis nootkatensis*), beechnut, Douglas fir (*Pseudotsuga menziesii*), and barley (*Hordeum vulgare*) (Le Page-Degivry et al. 1997; Schmitz et al. 2000, 2002; Corbineau et al. 2002; Jacobsen et al. 2002).

The major pathway by which ABA is catabolized is through hydroxylation at the 8' position to form 8'-hydroxy ABA, which reversibly cyclizes to phaseic acid (PA). Further reduction of PA can take place at the 4' position to form dihydrophaseic acid (DPA). ABA and ABA metabolites (PA and DPA) can also become conjugated with glucose forming an ester (PA) or an ester or glucoside (ABA and DPA). Other minor pathways include formation of 7'-hydroxyl ABA (7'OH ABA) and ABA 1',4' diols (Cutler and Krochko 1999; Zeevaart 1999; see chapter 1 for further discussion).

To begin to characterize mechanisms underlying dormancy of western white pine seeds and, in particular, to assess the role of ABA and its catabolism, we quantified ABA and selected metabolites during dormancy breakage and germination/growth. This was achieved using a newly developed HPLC-tandem mass spectrometry method using multiple reaction monitoring and internal standards incorporating deuterium-labeled ABA and metabolite analogs for PA, DPA, 7'OH-ABA, and ABA-glucose ester (ABA-GE) (Chiwocha et al. 2003; Zaharia et al., 2005a). Our specific goals were to: (1) discover if ABA plays a role in the dormancy mechanism of western white pine seeds; (2) investigate changes in ABA catabolism during dormancy breakage; and (3) examine the routes of ABA catabolism in the various seed tissues.

3.2 Materials and methods

3.2.1 Seed material

Mature seeds of western white pine seed lot 08006 were obtained from the British Columbia Ministry of Forests, Tree Seed Center in Surrey, BC, Canada. Seed lot 08006 was collected in 1981 from an elevation of 909 m at 51°31' latitude and a longitude of 119°12'. Seeds were stored at -20°C before use.

3.2.2 Dormancy-breaking treatments and germination conditions

The traditional method employed by the British Columbia Ministry of Forests to break dormancy of western white pine seeds includes a 14 d running water soak (~10°C) prior to 98 d of moist chilling at 2°C (Leadem 1996; D. Kolotelo personal commun.). We have optimized the procedure, by incorporating a warmer water soak (25-27°C) that increases the efficiency of dormancy-breakage (Feurtado et al. 2003; Chapter 2). More specifically, following a 3 d running tap water soak at 15 ± 3°C, seeds were soaked for 10 d at 25°C in aerated-de-ionized water. After sterilization with 3% H_2O_2 (v/v) for 30 min and several rinses in de-ionized distilled water (ddH_2O), seeds were surface dried until seed surface-moisture disappeared. Seeds were then placed on Whatman 3MM paper supported by 24-ply Kimpak (Kimberly-Clark, Wisconsin, USA) that had been moistened with approx. 25 mL ddH_2O in a clear plastic seed box (Hoffman Manufacturing Co., Albany, Oregon,

USA). Following 98 d of moist chilling at 2 ± 2°C in the dark, seeds were transferred to germination conditions (23°C, 16-h photoperiod, and light intensity approx. 100-150 $\mu mol \cdot m^{-2} \cdot s^{-1}$). After a brief rinse with water, seeds were maintained in the same plastic seed box except that 50 mL de-ionized distilled water was present. For some analyses, seeds were subjected to only the initial water soak (e.g. a 3 d soak or a 3 d + 10 d soak), or to a partial dormancy-breaking treatment (13 d soak, plus 30 or 45 d of moist chilling), prior to their transfer to germination conditions. As a control treatment, seeds were kept hydrated as described for the moist chilling treatment, but were placed for 30 d in moist warm conditions (23°C, 16-h photoperiod, and light intensity approx. 100-150 $\mu mol \cdot m^{-2} \cdot s^{-1}$) immediately after the 13 d water soaking with no period of moist chilling.

3.2.3 Extraction of ABA and ABA metabolites

Seeds were dissected into embryo, megagametophyte, and seed coat at various times during dormancy breakage and germination. Upon dissection and weighing, samples were frozen in dry ice and stored at –80°C until being lyophilized for 24 h, after which samples were stored over Drierite desiccant (Anachemia, Montreal, Quebec, Canada) at room temperature. For embryo and seed coat samples, 25 seed parts were used (30 and 100 mg DW, respectively). Megagametophyte samples were weighed out into 150 mg DW aliquots (25 megagametophytes ≈ 250 mg DW). After addition of 400 μL of extraction solvent (80% acetone, containing 1% glacial acetic acid and 19% water by vol.), samples were ground using 0.635-cm ceramic spheres (Q-biogene, Carlsbad, California, USA) in 2 mL screw cap microcentrifuge tubes with a Fast Prep FP120 machine (Q-biogene). The extraction solvent containing 80% acetone, 1% glacial acetic acid and 19% water (by vol.) was previously determined to be more efficient than an extraction solvent containing 99% isopropanol : 1% glacial acetic acid (v/v) (data not shown). Embryo samples were ground for 10 seconds at a speed of 4.5 $m \cdot s^{-1}$, while megagametophytes and seed coats were ground for 10 seconds at a speed of 5.0 $m \cdot s^{-1}$; grinding was repeated if necessary. Following grinding, sample volumes were made up to 1 mL with acetone extraction solvent, and 20 ng of deuterium-labeled internal standards were added to monitor recoveries through the hexane partition and solid phase extraction cleanup (see

Quantification of ABA and ABA metabolites). After an overnight incubation at 4°C in darkness, in which samples were shaken continuously (200 rpm), samples were centrifuged in a microcentrifuge at full speed (16000*g*) for 2 min at room temperature. Following collection of the supernatant, pellets were rinsed with 0.5 mL of 80% acetone extraction solvent. The combined acetone supernatants were lyophilized and then dissolved in 50 μL 99% MeOH : 1% acetic acid (v/v) and topped up with 99% water : 1% acetic acid (v/v) to 0.5 mL. Oils in the embryo and megagametophyte samples were removed by partitioning using 1 mL hexane. Remaining aqueous extracts were again lyophilized to remove any residual hexane and water before being dissolved in 100 μL of 99% MeOH : 1% glacial acetic acid (v/v) and topped up to 1 mL with 99% water : 1% glacial acetic acid (v/v). Oasis HLB 1 mL solid phase extraction cartridges (Waters Canada Ltd, Mississauga, Ontario, Canada) were conditioned with 100% MeOH and equilibrated with 10% methanol : 1% glacial acetic acid (v/v). Samples were spun 1 min at full speed in a microcentrifuge to remove any remaining particulate material prior to loading. Samples were loaded under a vacuum of 100-150 Torr (mm Hg) below atmosphere followed by a wash with 1 mL of 10% MeOH : 1% glacial acetic acid (v/v). Analytes were eluted using 80% methanol : 1% glacial acetic acid (v/v) before samples were lyophilized and stored at 4 °C (Zhou et al. 2003).

3.2.4 Quantification of ABA and ABA metabolites

Samples were dissolved by vortexing and sonication in 200 μL 85% H_2O : 15% acetonitrile (0.07 % acetic acid by vol.). After centrifugation in a microcentifuge at full speed (16000*g*), supernatants were transferred into autoinjector vials (Chromatographic Specialties, Brockville, Ontario, Canada). Ten μL samples were injected into a reversed phase-liquid chromatograph (RP-LC) inline with a tandem mass spectrometer (MS/MS) with an electrospray (ES) interface and the eluting ions measured by multiple reaction monitoring (MRM) in negative ion mode. The RP-LC ES-/MS/MS consisted of a Waters Alliance Model 2695 HPLC (Waters, Milford, Massachusetts, USA) inline with a Quattro Ultima (Waters, Milford, MA) fitted with an ES ion source (Z-spray, Waters). Samples were separated by HPLC using gradient elution with varied compositions of acetonitrile

and water with constant 0.04% (v/v) glacial acetic acid. Prior to delivery at a rate of 0.20 to 0.35 $mL \cdot min^{-1}$ to the analytical column (100 x 2.1 mm, 4 μm) (Genesis C18, Model FK10960EJ; Jones Chromatography, Hengoed, UK), eluates were passed through a guard column (12.5 x 2.1 mm, 5 μm) (Zorbax XDB-C8, Model Z821125926; Chromatographic Specialties, Brockville, Ontario, Canada) to help maintain performance of the analytical column. The HPLC program allowed separation of all compounds with their respective deuterated internal standard over a 25 min run. Mass spectrometry conditions were as follows: (a) *ES conditions*: polarity ES-, capillary 2.25 kV, cone 25 V, for ABA, PA, and 7'OH-ABA, 30 V for DPA, and 20 V for ABA-GE, aperture 0.2 V, source temperature 120°C, desolvation (N_2) gas temperature 350°C, cone gas flow 107 L/h, desolvation gas flow 590 L/h; (b) *Tandem quadrupole conditions*: low mass and high mass resolution were 12.0 for both mass analyzers, ion energies 1 and 2 were 1.5 and 2.5 V, entrance and exit potentials were 10 and 11 V, respectively, and detector (multiplier) gain was 650 V. Collision induced dissociation of deprotonated parent ions, accomplished with a collision energy of 10 (ABA), 12 (ABA-GE). 13 (PA), or 18 V (DPA, 7'OH-ABA) and a collision cell pressure of 3.5×10^{-3} mbar (achieved using Ar gas), was assessed using MRM. For ABA and ABA metabolites, and each of their respective deuterated standards, MRM was based on the transition from the deprotonated parent ion to the predominant daughter fragment ion. During the 25 min run, MRM was divided into 3 functions. These included DPA/d3-DPA in the first function (4.1 min retention times, 281 > 171 / 284 > 174 transition), ABA-GE/d5-ABA-GE, PA/d3-PA, and 7'OH ABA/d4-7'OH ABA in the second function (8.0, 8.8, and 9.7 min retention times, and 425 > 263 / 430 > 268, 279 > 139 / 282 > 142, and 279 > 151 / 283 > 154 transitions, respectively), and ABA/d4-ABA in the third function (11.8 min retention times, 263 > 153 / 267 > 156) (see Appendix, Figs. A5, A6). For quantification of samples, a standard curve was generated using the following concentrations of analytes: 0, 1, 2, 5, 10, 20, 50, 100, 200, 500, 700, and 1000 pg/μL – each with 100 pg/μL deuterated-internal standard. Two hundred μL of each concentration was prepared and divided into four 50 μL replicates. Ten μL of each aliquot was injected into the RP-LC ES/MS/MS and run under identical conditions to sample

extracts. Linear regression curves were generated and samples were quantified in MassLynx 3.5 (Waters).

(±)-ABA (Sigma Chemical Co., Mississauga, Ontario, Canada), (-)-PA (Balsevich et al. 1994), (-)-DPA, (+)-ABA-GE (Zaharia et al. 2005a), (±)-7'OH-ABA (Nelson et al. 1991) and were used to create the standard curves. Deuterium-labeled internal standards included: (-)-5,8',8',8'-d_4-ABA (Abrams et al. 2003), (-)-7',7',7'-d_3-PA, (-)-7',7',7'-d_3-DPA, (-)-5,8',8',8'd_4-7'OH-ABA and (+)-4,5,8',8',8'-d_5-ABA-GE (Chiwocha et al. 2003; Ross et al. 2004; Zaharia et al. 2005a). All chemicals, except (±)-ABA, were synthesized at the Plant Biotechnology Institute, Saskatoon, Canada.

3.3 Results

3.3.1 Termination of dormancy of western white pine seeds

Germination of western white pine seeds was monitored after different durations over the course of the dormancy breaking procedure. Following a 3 d soak at 15°C there was no subsequent germination; an additional soak of 10 d at 25°C had a promotive effect and yielded 15.8% germination (Fig. 3.1; 13 d). This lengthy pre-soak is needed for efficient dormancy breakage during subsequent moist chilling (Feurtado et al. 2003; Chapter 2). Dormancy-termination increased as the subsequent moist chilling period was increased, and seeds exhibited the highest germination capacity after 98 d of moist chilling (88.1%; Fig. 3.1).

3.3.2 Changes in ABA during dormancy breakage

ABA levels were highest in the seed coat of the dry seed, but decreased markedly to a very low amount by the end of the 13 d water soak (Fig. 3.2; note log scale). ABA remained in approximately the same amounts in the seed coat, thereafter, declining slightly during germination.

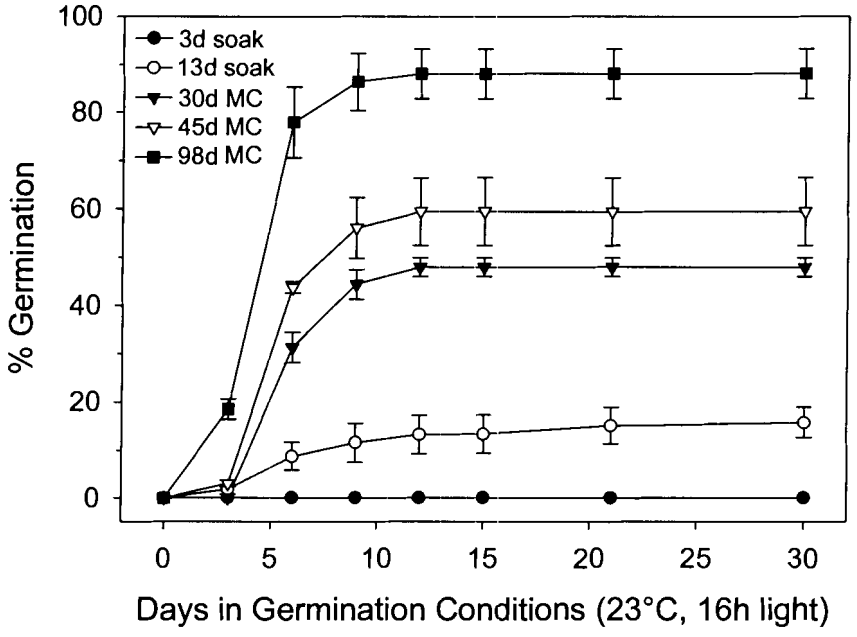
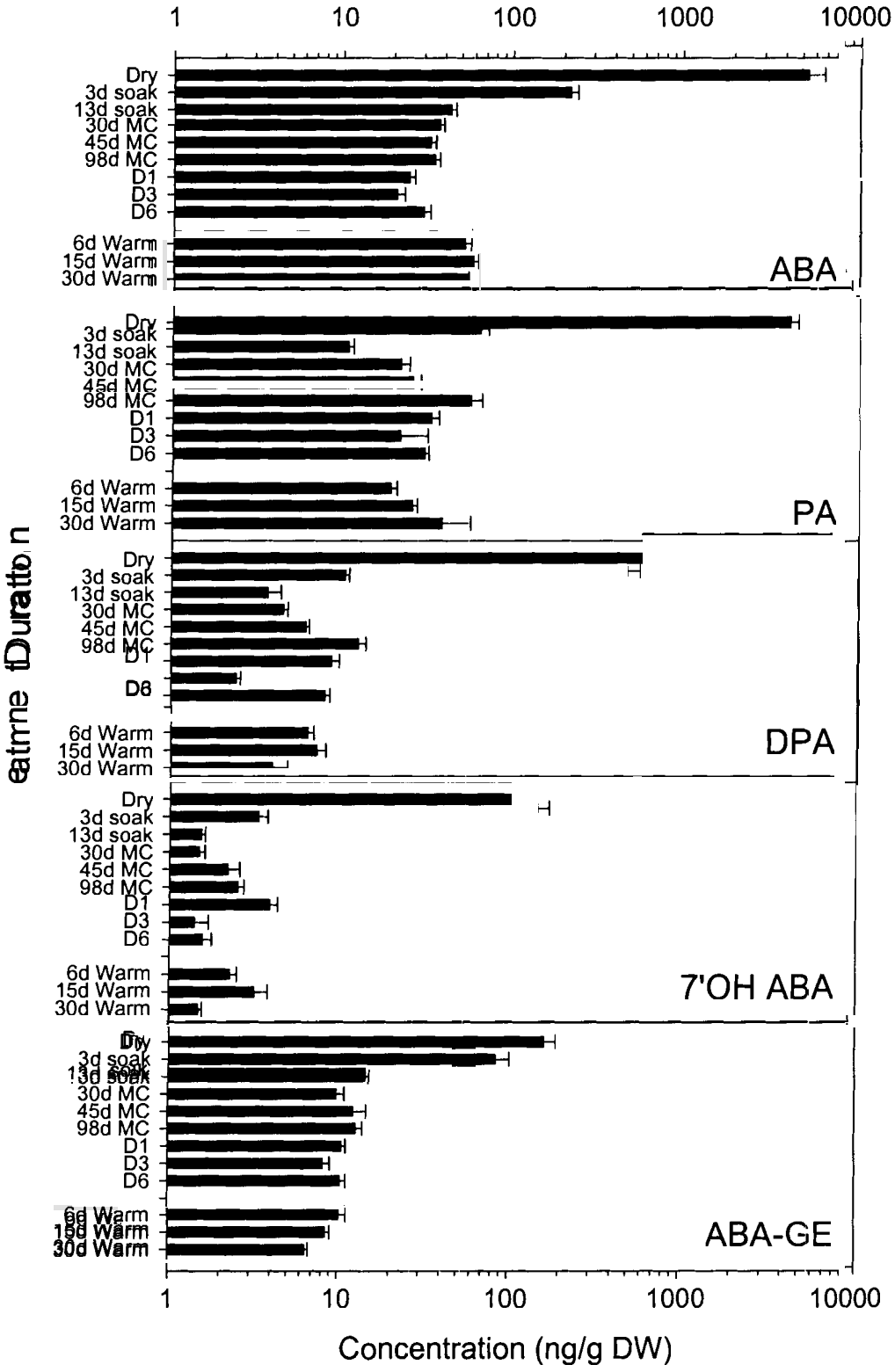


Fig. 3.1. Germination of western white pine seeds following pre-treatment water soaks and moist chilling (MC). For the full dormancy-breaking treatment, seeds were soaked at 15±3°C for 3 d, then at 25°C for 10 d followed by 98 d of moist chilling at 2±2°C. Germination was monitored for 30 d after different lengths of the dormancy-breaking treatment as indicated (i.e. after a 3 d soak, a 13 d soak, or a 13 d soak followed by 30, 45 or 98 d of moist chilling). Data are based on the average of 3 replicates of 50 seeds each (±S.E.).


1
10
100
1000
10000
Dry
3d soak
13d soak
30d MC
45d MC
98d MC
D1
D3
D6
6d Warm
15d Warm
30d Warm
ABA
PA
DPA
7'OH ABA
ABA-GE
Concentration (ng/g DW)

On a dry weight basis, ABA levels in the embryo and megagametophyte remained relatively constant over the 13 d soak period preceding moist chilling (Fig. 3.3a, compare dry, 3d soak and 13 d soak). Moist chilling, and subsequent transfer of seeds to germination conditions, had dramatic effects on ABA in the embryo and megagametophyte (Fig. 3.3 a, b). ABA levels in the embryo declined by 60% during 98 d of moist chilling and by a further 35% during germination (Fig. 3.3a; D1 and D3). Similarly, in the megagametophyte, ABA declined 58% during 98 d of moist chilling and 94% after 3 d in germination conditions (Fig. 3.3a). As the seed transitioned into a seedling (accompanied by cell division, photosynthesis, and a 92% increase in the FW/DW ratio; data not shown), the ABA level increased by over four-fold in the embyro (i.e. by 473%) (Fig. 3.3a, D6). On a fresh weight basis, similar trends were revealed, except that ABA in the embryo and megagametophyte decreases during the first 3 d of the water soak (Fig. 3.3b). During this time imbibitional water uptake by seeds results in a FW/DW ratio increase of 46% and 18% in the embryo and megagametophyte, respectively (data not shown). In addition, ABA levels in the embryo do not increase as noticeably after germination (Fig. 3.3b) due to the major FW/DW ratio increase that accompanies seedling growth.

3.3.3.Accumulation of ABA metabolites during dormancy breakage

Changes in the amounts of different ABA metabolites indicated that the flux through the pathways of ABA turnover differed in the embryo and megagametophyte of western white pine seeds during and following dormancy termination. The degradation or inactivation of ABA can occur through both oxidation and conjugation and this was revealed by changes in the amounts of PA, DPA, 7'OH ABA, and ABA-GE.

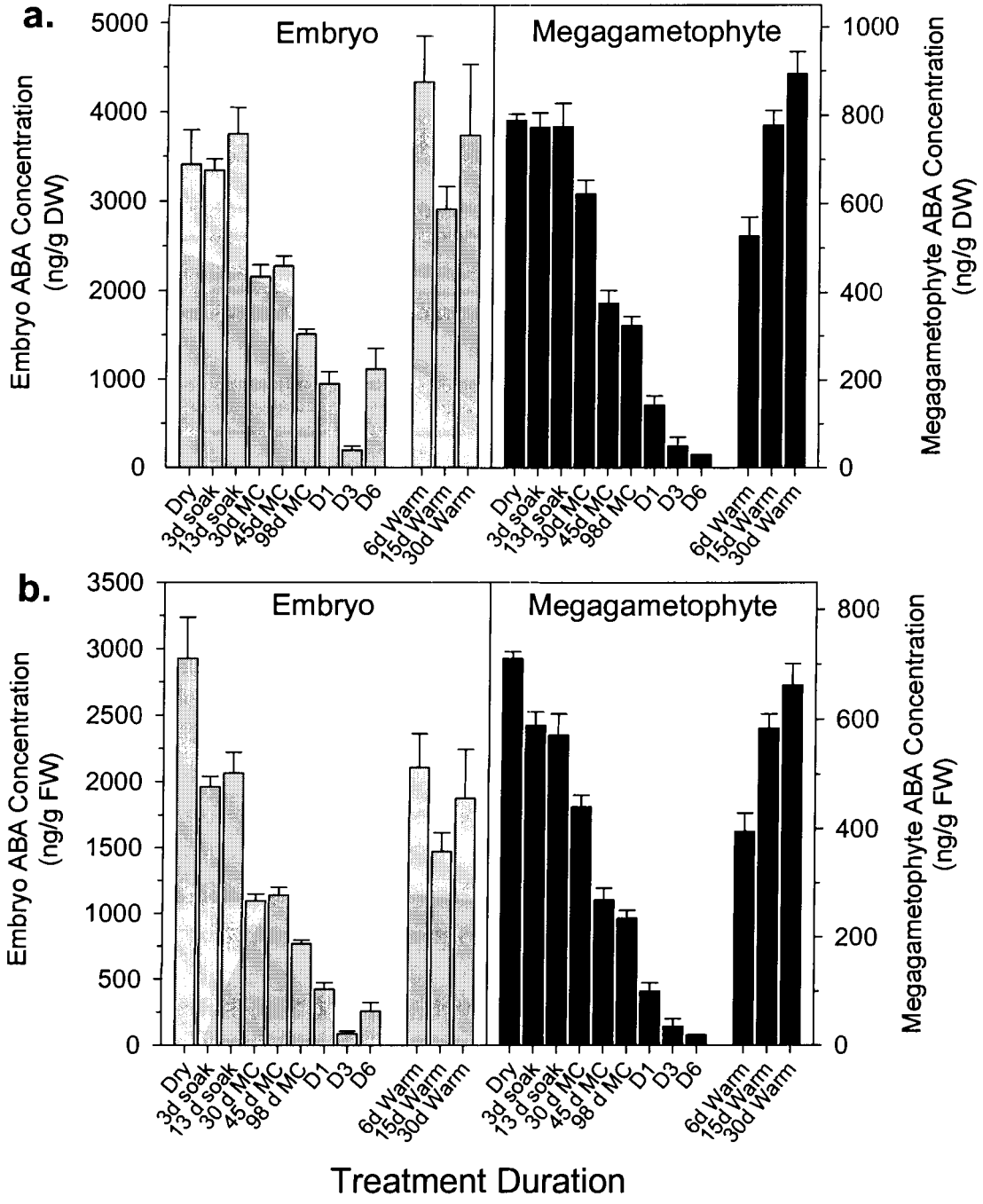


Fig. 3.3. Changes in ABA within the embryo and megagametophyte at various times during the dormancy-breaking treatment, and during and following germination (i.e. following transfer of seeds to germination conditions after dormancy-breakage). Data are expressed on either a ng per g DW basis (**a**) or on a ng per FW basis (**b**). Each bar denotes the average of 3 replicate extractions from 25 embryos or 15 megagametophytes (±SE). MC = moist chilling. D1, D3 and D6, represent d after transfer of seeds to germination conditions after being subjected to the full dormancy-breaking treatment. During D1 and D3 timepoints, seeds have not completed germination; by D6, seeds have germinated and have radicle lengths of 0.5-1.0 cm. Warm 6 d, 15 d and 30 d, are data from seeds subjected to the water soak (13 d), but transferred to warm moist conditions (for 6, 15 and 30 d), in place of moist chilling (See section 3.3).

3.3.3.1 8'Hydroxylation of ABA

During the water soaks, the amount of PA in the embryo increased slightly while in the megagametophyte the level decreased (Fig. 3.4a). DPA decreased in both seed parts during the soak (Fig. 3.4b). PA and DPA levels increased during moist chilling in all three seed parts, in the embryo and megagametophyte, and in the seed coat (Fig. 3.2 and Fig. 3.4a, b). In the latter, the increase occurred throughout moist chilling (albeit modest given the log scale of Fig. 3.2); in embryos and megagametophytes, these ABA metabolites increased primarily between 30 and 45 d of moist chilling. Transfer of seeds to germination conditions was accompanied by a decline in PA and DPA over the first 3 d, followed by an increase in the levels of these metabolites associated with the germination-to-growth transition (Fig. 3.2 and Fig. 3.4 a, b).

3.3.3.2 7'Hydroxylation of ABA

In the embryo, there was a transient increase in 7'OH ABA during the initial soaking of the seed, before it increased in amount throughout the subsequent 98 d of moist chilling. This metabolite reached a maximum at 1 d following transfer of seeds to germination conditions (D1), but it declined to a low level thereafter (Fig. 3.5a). 7'-Hydroxy ABA in the megagametophyte was present in low and generally constant amounts throughout the water soaks, moist chilling, and germination (Fig. 3.5a). In the seed coat, changes in 7'OH ABA paralleled the trend exhibited by embryos including the peak during early germination (Fig. 3.2, D1).

3.3.3.3 ABA Conjugation to Glucose

In the megagametophyte, ABA-GE did not change appreciably during the water soaks, moist chilling, or germination (Fig. 3.5b). However, ABA-GE in the embryo increased and peaked after 98 d of moist chilling, thereafter declining to levels similar to the dry seed (Fig. 3.5b). Like the other ABA metabolites (PA, DPA and 7'OH ABA), relatively high amounts of ABA-GE were present in seed coats of dry seeds. However, unlike the other ABA metabolites, which increased to some extent during moist chilling, ABA-GE remained in constant amounts after the initial soak (i.e. throughout chilling and germination) (Fig. 3.2).

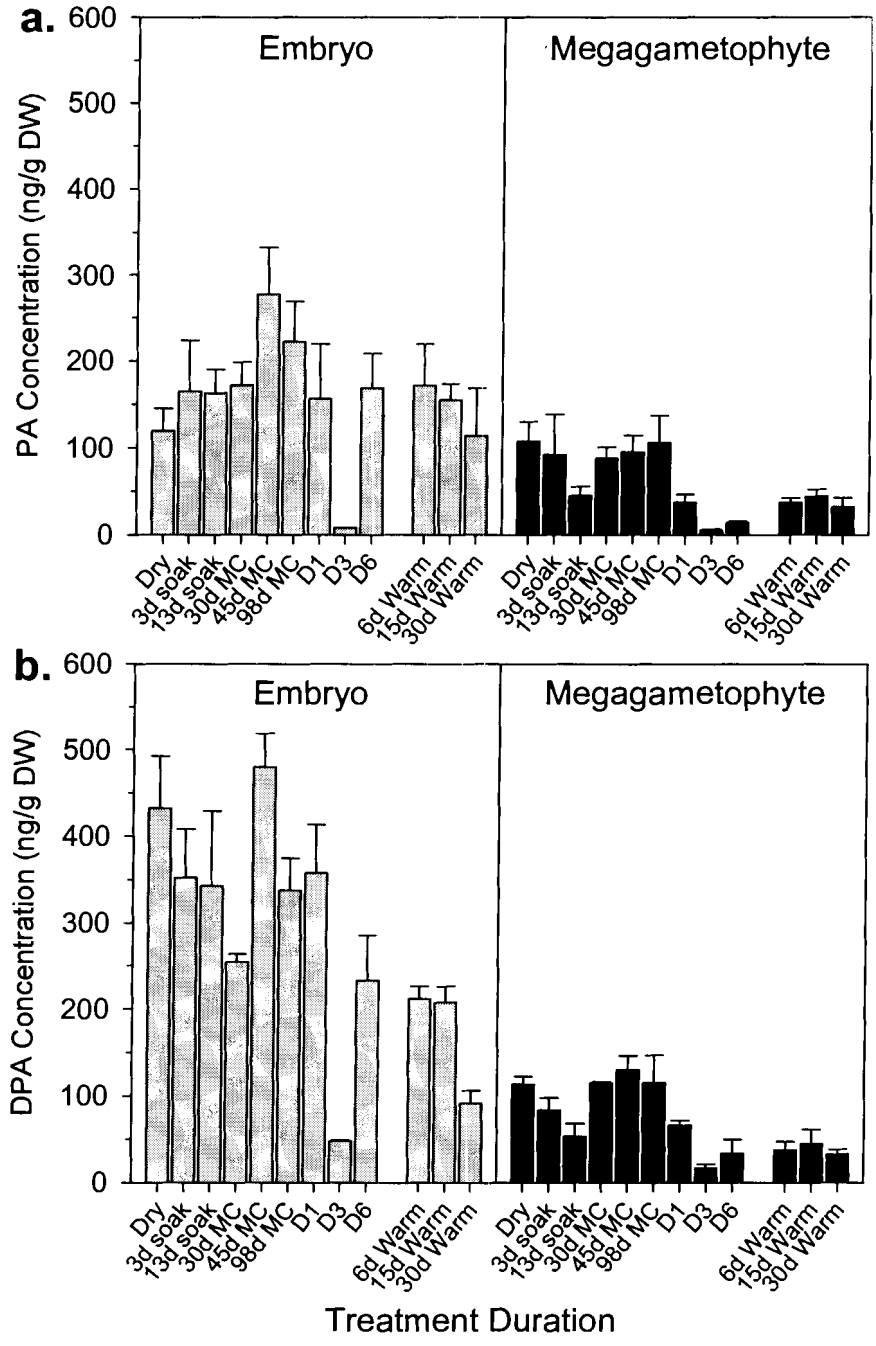


Fig. 3.4. Changes in PA (**a**) and DPA (**b**) within the embryo and megagametophyte at various times during the dormancy-breaking treatment, and during and following germination (i.e. following transfer of seeds to germination conditions after dormancy-breakage). Data are expressed on a ng per g DW basis. Each bar denotes the average of 3 replicate extractions from 25 embryos or 15 megagametophytes (±SE). MC = moist chilling. D1, D3 and D6, represent d after transfer of seeds to germination conditions after being subjected to the full dormancy-breaking treatment. During D1 and D3 timepoints, seeds have not completed germination; by D6, seeds have germinated and have radicle lengths of 0.5-1.0 cm. Warm 6 d, 15 d and 30 d, are data from seeds subjected to the water soak (13 d), but transferred to warm moist conditions (for 6, 15 and 30 d), in place of moist chilling (See section 3.3).

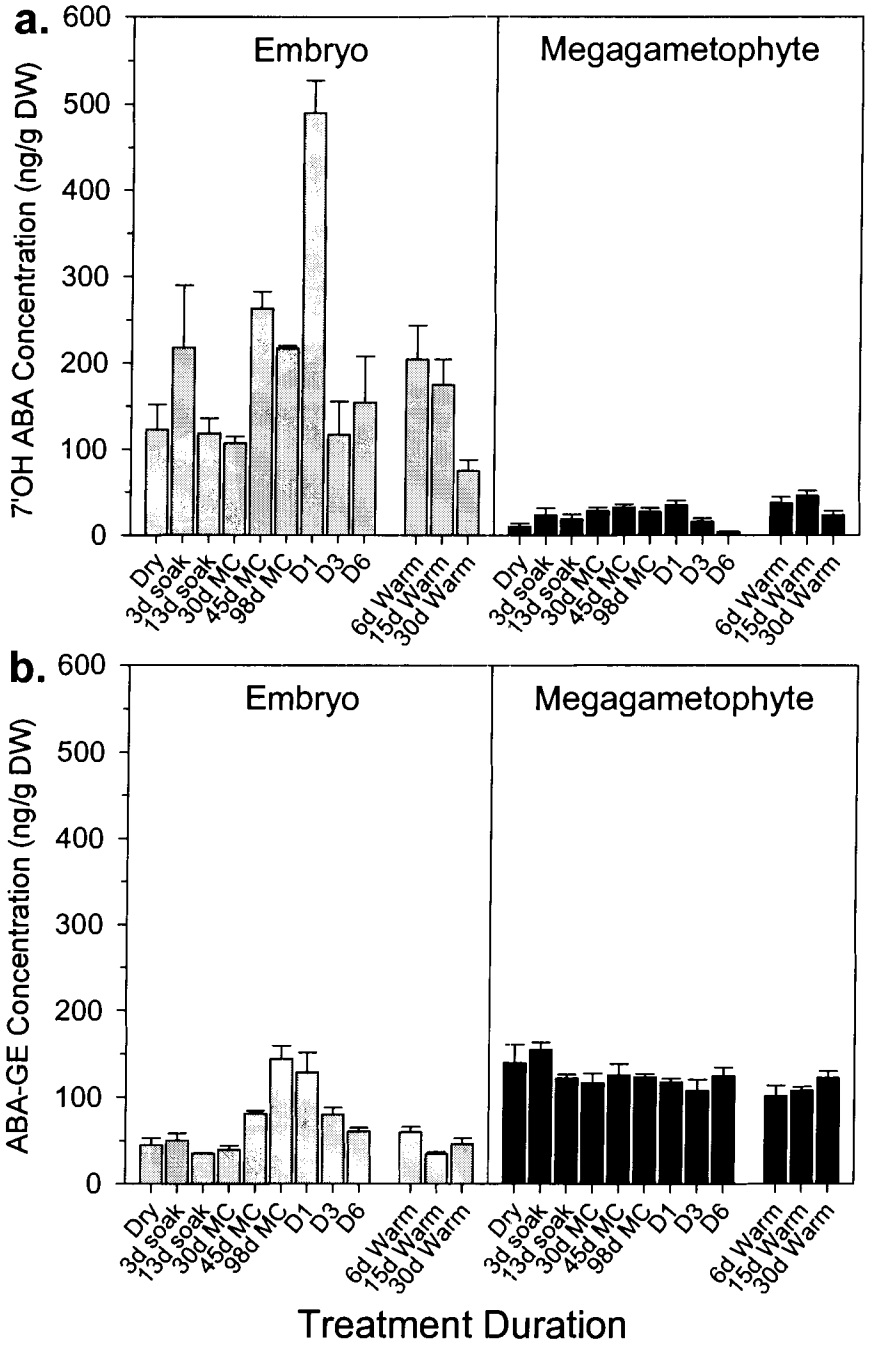


Fig. 3.5. Changes in 7'OH ABA (**a**) and ABA-GE (**b**) within the embryo and megagametophyte at various times during the dormancy-breaking treatment, and during and following germination (i.e. following transfer of seeds to germination conditions after dormancy-breakage). Data are expressed on a ng per g DW basis. Each bar denotes the average of 3 replicate extractions from 25 embryos or 15 megagametophytes (±SE). MC = moist chilling. D1, D3 and D6, represent d after transfer of seeds to germination conditions after being subjected to the full dormancy-breaking treatment. During D1 and D3 timepoints, seeds have not completed germination; by D6, seeds have germinated and have radicle lengths of 0.5-1.0 cm. Warm 6 d, 15 d and 30 d, are data from seeds subjected to the water soak (13 d), but transferred to warm moist conditions (for 6, 15 and 30 d), in place of moist chilling (See section 3.3).

3.3.4 Changes in ABA and ABA metabolites in dormant seeds maintained in warm moist conditions

As a control for moist chilling, seeds were soaked as in the standard dormancy-breaking protocol (15°C and 25°C for 3 and 10 d, respectively), but were transferred to warm moist conditions (identical to germination conditions) for 6, 15 and 30 d with no intervening moist chilling. ABA in the embryo of these ungerminated (dormant) seeds declined slightly after 15 d before increasing after 30 d in warm moist conditions to an amount equivalent to that present after the two water soaks (Fig. 3.3). Similarly, in the megagametophyte of control seeds, ABA decreased before increasing to an amount equivalent to that present in the dry seed (Fig. 3.3). In the seed coat, ABA levels were also higher than at any point during moist chilling or germination (Fig. 3.2). In general, the metabolites of ABA declined or remained at constant amounts during the 30 d in warm moist conditions (Figs. 3.2, 3.4, 3.5). However, a decline was especially evident for DPA and 7'OH ABA in the embryo (Figs. 3.4 and 3.5).

3.4 Discussion

Levels of ABA and ABA catabolites in plant cells and tissues are under constant flux as a result of the opposing forces of biosynthesis versus degradation; changes in ABA concentration within embryos and surrounding seed tissues can contribute to dormancy inception, maintenance, and termination (Cutler and Krochko 1999; Grappin et al. 2000; Schmitz et al. 2000, 2002). The degradation or inactivation of ABA can occur via oxidation and conjugation. The biological roles and activities of ABA catabolites have been largely uninvestigated; however, it is assumed that ABA glucose ester and DPA are inactive and do not have ABA-like activity. PA exhibits weak ABA-like activity in certain bioassays. For example, PA is virtually ineffective at inhibiting lettuce seed germination (>300 μM to inhibit 50% germination versus 5.0 μM for (+)-ABA) or inducing wheat Em gene expression in barley protoplasts but is able to counteract GA induction of α-amylase activity in barley-half grains (Hill et al., 1995; Todoroki et al., 1995). The hydroxy ABA catabolites, although transient, also possess bioactivity. For example, 8'-hydroxy ABA (like ABA) induces gene expression related to lipid reserve

synthesis in microspore-derived embryos of *Brassica napus* (Zou et al. 1995) and 7'-hydroxyl ABA antagonizes the GA-induction of α-amylase activity in aleurone layers of barley grains (Hill et al. 1995). To determine if ABA and its catabolism are associated with dormancy breakage in western white pine seeds we quantified ABA and the major metabolites of ABA – PA, DPA, 7'OH ABA, and ABA-GE throughout dormancy-breaking and control (warm-moist) treatments.

Moist chilling of western white pine seeds, imposed after an initial 13 d soaking of seeds, was accompanied by a significant decrease in ABA in both the embryo and megagametophyte. Moreover, the decline of ABA after different durations of moist chilling correlated well with the increased capacity of seeds to germinate following their transfer to germination conditions. Notably, the decline of ABA continued during germination. Embryo ABA decreased at a rate of 20 ng/g DW per d during moist chilling (over 98 d) and a further 400 ng/g DW per d during the first 3 d following transfer of seeds to germination conditions. In contrast, high levels of ABA were found in dormant-imbibed seeds given a warm-moist treatment in place of moist chilling (i.e. maintenance in warm moist conditions for 30 d after the initial 13 d soak). Thus, moist chilling effects a decline in ABA, by shifting the ratio of biosynthesis to catabolism such that catabolism exceeds biosynthesis as moist chilling proceeds. Perhaps equally important, subjecting seeds to an effective dormancy breaking treatment seems to increase the *capacity* for ABA catabolism and/or *reduce* synthesis of ABA when the seeds are subsequently placed in germination conditions; thus the net effect is a shift to favor catabolism. We cannot state the relative importance of the two processes to dormancy termination since the biosynthesis of ABA and rates of ABA turnover were not directly determined. Nor can we rule out other factors that mediate changes in hormone levels/availability such as the transport of ABA/ABA metabolites between tissues and within cells.

A shift in the ratio of synthesis to catabolism occurs again when the seeds are transitioning from germination to growth. In this case, ABA levels in the embryo increased 900 ng/g DW over a 3 d period (i.e. between D3 and D6). Much of this increase of ABA can be attributed to the way in which ABA concentration is expressed (i.e. on a DW basis), since there is a 92% increase in the FW/DW during the transition to seedling

growth (data not shown). However, even on a FW basis ABA increased by 200 ng/g over the 3 d. This may be somewhat surprising as ABA is generally classed as a growth inhibitor. However, ABA, at a certain endogenous concentration, is necessary for growth. The ABA-deficient *Arabidopsis* mutants *aba1-1*, *aba1-3*, and *aba1-4* are stunted in their growth due to a reduced capacity to transpire and establish turgor. When these mutants are sprayed with exogenous ABA, cell expansion and normal growth are restored (Finkelstein and Rock, 2002).

Reports on the quantification of ABA in other conifer seeds are limited; most focus on changes in ABA during development of either zygotic embryos and megagametophytes (Carrier et al. 1999; Chiwocha and von Aderkas 2002) or somatic embryos (Kong and Yeung 1995; Stasolla et al. 2002). Information regarding the involvement of ABA in the dormancy and germination of conifer seeds is more limited. ABA in gymnosperms was first identified in *Taxus baccata* (English yew) seeds (Le Page-Degivry et al. 1969) and a decline in ABA levels was correlated with dormancy termination of *Taxus* embryos cultivated in a liquid medium (Le Page-Degivry 1970). *Pinus pinea* (Stone pine) seeds washed for 24 h in water before being placed in vermiculite exhibit a higher germination capacity and rate than their non-soaked counterparts, which has been attributed to ABA being leached from the seed coat (Martínez-Honduvilla and Santos-Ruiz 1978). In *Pinus sylvestris* (Scots pine) seeds, dormancy-breaking treatments that include either white or red light, decrease ABA prior to radicle protrusion; seeds subjected to a far-red light pulse after red light, do not exhibit as great a decline in ABA, nor is dormancy relieved (Tillberg 1992). During dormancy breakage of yellow-cedar (*Chamaecyparis nootkatensis*) seeds, there is about a two-fold reduction of ABA in the embryo; in the megagametophyte, ABA does not change; however, the embryos exhibit a change in both ABA turnover and in their sensitivity to ABA (Schmitz et al. 2000, 2002). ABA may maintain dormancy of Douglas fir seeds. Dormant seeds germinate poorly at 20-23°C; under these conditions, seeds exhibit an overall increase in ABA during the culture period (Bianco et al. 1997). Most of the increase in ABA occurs in the megagametophyte and to a lesser extent in the embryo; ABA declines in the seed coat, presumably as a result of leaching. When embryos are

isolated from dormant seeds, they exhibit an approx. 28-fold decrease in their ABA level within 7 d (as compared to embryos of intact seeds) and they later 'germinate' (i.e. undergo radicle elongation) within 14 d (Bianco et al. 1997). Thus, structures enclosing the embyro (i.e. the seed coat and megagametophyte) may maintain the seed in a dormant state by limiting ABA metabolism and preventing the leaching out of ABA, and, in Douglas fir seeds, they may facilitate accumulation of *de novo* synthesized ABA. Corbineau et al. (2002) quantified ABA during moist chilling of Douglas fir seeds. After an initial increase in ABA level during moist chilling, ABA decreased in both the megagametophyte and embryo; in the former, ABA declined 4-fold during 7 weeks of moist chilling. The longer the duration of moist chilling, the faster the rate of ABA decline during subsequent germination (Corbineau et al. 2002).

As far as ABA turnover is concerned, to our knowledge, this is the first study to quantify the major ABA metabolites, PA, DPA, ABA-GE, and 7'OH ABA, during dormancy termination of a conifer seed. In angiosperm seeds, there are also few reports concerning the nature of ABA metabolites (reviewed in Cutler and Krochko 1999; Zeevaart 1999). Although the major pathway of ABA catabolism is via 8'-hydroxylation, that generates PA and in some cases DPA, the importance of this *versus* other pathways of catabolism (e.g. those involving 7'-hydroxylation of ABA) varies between species and even between different tissues and different developmental stages of the same species (Cutler and Krochko 1999; Zeevaart 1999). This was the case in western white pine seeds (Fig. 3.6). In the megagametophyte, ABA was metabolized through 8'-hydroxylation, generating PA and DPA; both metabolites increased during a 98 d moist chilling period. ABA-GE remained constant and 7'OH ABA remained at low levels. (It is not known, however, if there is any transport of the metabolites between the megagametophyte and embryo). In the embryo, ABA appears to be metabolized through both the 8'- and 7'-hydroxylation pathways as well as by conjugation to glucose. PA, DPA and 7'OH ABA levels increased in embryos during moist chilling of seeds (especially between 30 and 45 d); 7'OH ABA showed a further increase during early germination. ABA-GE levels increased in embryos throughout the entire 98 d of moist chilling and then showed a steady decrease following transfer of seeds to germination conditions.

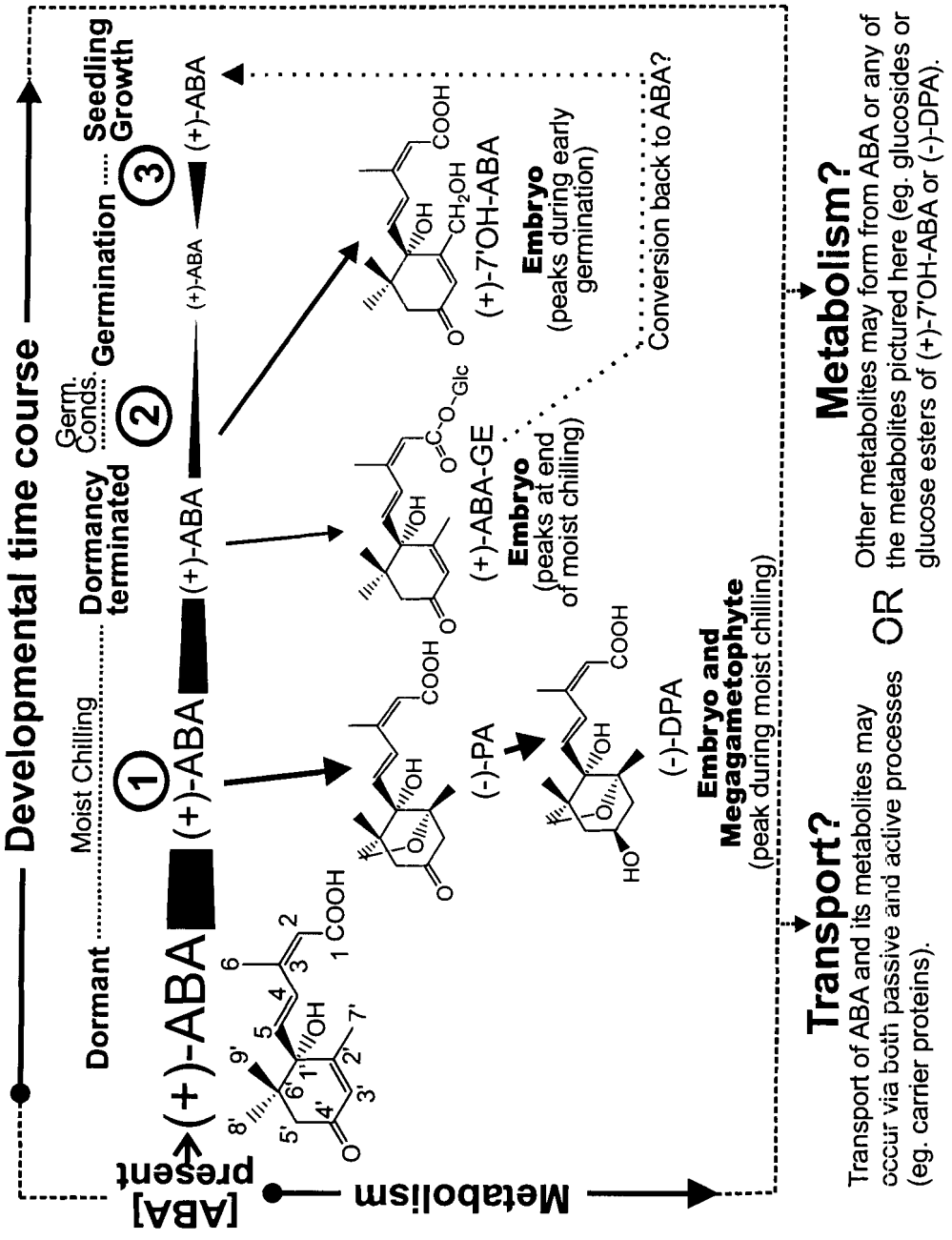


Fig. 3.6. Model of potential pathways of ABA flux in western white pine seeds during dormancy termination (moist chilling, 1), the transition to germination (2), and the transition to seedling growth (3). The decrease in ABA levels, or any of the metabolites, during moist chilling and germination are the result of shifts in the ratio of biosynthesis to catabolism (transport may also be operative; see below). ABA is metabolized through both oxidative and conjugative routes in the embryo. It is hydroxylated to form 7'OH ABA and 8'OH ABA, respectively; the latter cyclizes to PA. ABA-GE is also formed throughout moist chilling in the embryo. In the megagametophyte, the major route of metabolism is through 8'-hydroxylation (forming PA and DPA). More metabolites derived from ABA itself or from PA, DPA, 7'OH ABA, and ABA-GE may also be generated. In addition, transport of ABA and its metabolites can have an effect on the levels or pools of available compound. Transport may occur through passive and active processes; routes of transport (e.g. between the embryo and the megagametophyte or between seeds and the surrounding medium) are unknown.

The changes in the abundance of the metabolites (as compared to ABA) and the decline in DPA and ABA-GE after moist chilling and during germination are noteworthy and raise several points (Fig. 3.6). Does ABA-GE move out of the embryo during germination? Does it represent an irreversibly inactivated form of ABA, or can it later generate free ABA? It is generally assumed that ABA-GE is not a storage form of ABA and that the conjugation is irreversible, with the conjugate being sequestered in the vacuole (Kleczkowski and Schell 1995; Zeevaart 1999). The decrease in DPA, especially during germination, suggests that DPA is metabolized further, perhaps to a glucose conjugate such as DPA-glucoside. DPA-glucoside is a major metabolite in both soybean seeds and sunflower embryos (Setter et al. 1981; Barthe et al. 1993). The balance between synthesis and decline (via degradation, transport and/or subcellular compartmentation) influences the amount or pool size of available ABA; similar processes will influence the pool size of ABA metabolites. For example, the decrease in DPA in the embryo during germination (D1 to D3) may be the result of an increase in the formation of a DPA metabolite (e.g. DPA-glucoside), a decrease in the catabolism of PA, and/or loss by leakage or transport. Since PA also decreases during germination, formation of a DPA metabolite may be operative. The changes in the amounts of ABA catabolites relative to ABA suggest that the catabolites are further metabolized to unknown products in western white pine seed. Thus, as previously suggested, several of the metabolites of ABA can be conjugated to glucose, and ABA itself may be metabolized to ABA 1',4'diols, an ABA-glucoside, or yet undiscovered metabolites.

Overall, however, several suggestions can be made concerning ABA metabolism and the metabolism of PA, DPA, 7'OH ABA, and perhaps ABA-GE (Fig. 3.6). The balance of ABA biosynthesis to catabolism undergoes at least three distinct changes during the transition from dormancy to germination and growth in western white pine seeds: (i) during moist chilling, the balance is shifted so that catabolism exceeds biosynthesis (ABA decreases); (ii) during germination, when ABA decreases even more rapidly, the balance favors catabolism even more; (iii) finally, when the seeds undergo the transition from germination to growth, the ratio shifts back to favor ABA biosynthesis and ABA increases. In the absence of an effective dormancy-breaking treatment, i.e. when the seeds

are maintained in a warm moist environment for 30 d (in place of moist chilling) after the initial 13-d soak, the shift in which catabolism exceeds biosynthesis does not take place and ABA is maintained at high levels. In these dormant warm-imbibed control seeds, ABA metabolites tended to decrease suggesting that they were further metabolized and not replaced (or perhaps generated less slowly) by ABA turnover. ABA metabolites increased in moist-chilled seeds, but generally decreased during germination. We cannot necessarily attribute these changes in ABA metabolite levels to an increase in the rate of their catabolism since other factors will influence pools of available compound, including leaching, transport between tissues or sequestration within cells (subcellular compartmentation).

In conclusion, the dormancy mechanism of western white pine seeds is multi-faceted – influenced by both structural and physiological features (Hoff, 1987). One of the factors associated with dormancy in this species is ABA. However, it will be important to further address some of the important implications of this work. In addition to the identification and quantification of further metabolites, follow-up studies need to address: (1) whether ABA biosynthesis is absolutely necessary for dormancy maintenance; (2) how the enclosing seed structures such as the seed coat and megagametophyte contribute to ABA metabolism, and (3) how catabolism (metabolic flux) changes during the transition from a dormant to non-dormant state (e.g. by measuring rates of [^{3}H]-ABA turnover, the [^{3}H]-ABA metabolites produced, and elucidating potential hormone cross-talk). As the precise mechanisms are elucidated, this should facilitate ways to improve dormancy breakage of this once dominant and now emerging species for reforestation.

CHAPTER 4

The seed-coat-enhanced dormancy mechanism of western white pine (*Pinus monticola* Dougl. Ex D. Don) seeds is mediated by abscisic acid homeostasis and mechanical restraint

4.1 Introduction

The inception of primary seed dormancy, which occurs during seed development, results in mature seeds with a transient inability to germinate under conditions that are normally conducive to germination. Dormancy evolved in seed plants, in both angiosperm and gymnosperm phylogenies, in part as an adaptive trait facilitating germination when conditions for seedling and plant establishment are more ideal (Baskin and Baskin, 2001). For instance, many coniferous species require a lengthy over-wintering period of cold-moist conditions before germination occurs in more favorable spring conditions. Primary dormancy or the causes there of, can be further classified into two major types: seed-coat-enhanced dormancy and embryo dormancy. In seed-coat-enhanced dormancy the seed remains dormant because structures surrounding the embryo prevent germination through one or a combination of the following mechanisms: interference with water uptake, mechanical restraint, interference with gas exchange, supplying inhibitors to the embryo, and/or preventing the exit of inhibitors from the embryo. In embryo dormancy, it is the embryo that is dormant and the embryonic axis will not elongate even if the embryo is isolated and placed on water to germinate (Bewley and Black, 1994). In seeds of coniferous species, excised embryos often germinate when placed on water, indicating that it is the seed tissues surrounding the embryo (megagametophyte, seed coat, nucellar remnants) that play an inhibitory role and maintain dormancy in a coat-enhanced manner (Downie and Bewley, 1996; Bianco et al., 1997; Ren and Kermode, 1999).

The transition from a state of primary dormancy to germination can be induced by several means. In nature, these include: extended periods in the dry state (after-ripening), cold-moist conditions (moist chilling), light, and smoke (Egerton-Warburton LM, 1998; Toyomasu et al., 1998; Corbineau et al., 2002; Ali-Rachedi et al., 2004). In western white pine, seeds exhibit pronounced primary dormancy at maturity and require a moist-chilling treatment of approx. 90 d, which is generally preceded by a water soak of up to 14 d, to allow successful germination of seed populations (Hoff 1987; Feurtado et al. 2003). Seed dormancy in this species is classified as coat-enhanced and is imposed by the seed coat, nucellar membrane, and megagametophyte (Hoff 1987; Dumroese 2000). Factors implicated in the dormancy mechanism include: restriction of water and gas (O_2) entry

into the seed, mechanical restraint, and prevention of the release (and/or catabolism) of germination inhibitors (Hoff, 1987; Dumroese, 2000). Recently the dormancy mechanism in western white pine seed was linked to the sesquiterpenoid hormone abscisic acid (ABA) (reviewed in Kermode, 2005) (Feurtado et al. 2004; Chapter 3). ABA levels in embryos and megagametophytes decrease significantly during dormancy termination (through moist chilling) and are synchronized with an increase in the germination capacity of seeds. In the absence of conditions required to break dormancy, seeds display no net changes in ABA biosynthesis and catabolism, as ABA levels remain high (Feurtado et al. 2004; Chapter 3).

Mechanical restraint also provides the means to inhibit germination. In this case, tissues surrounding the radicle at the micropylar end of the seed, such as the megagametophyte and seed coat, mechanically resist radicle protrusion (Ren and Kermode, 1999). In order for germination to occur, the force exerted by the radicle must exceed the restraint provided by the surrounding tissues. Weakening of the structures surrounding the radicle, especially living structures such as the megagametophyte, is thought to be achieved by the action of cell wall hydrolases (Bewley, 1997). In white spruce seeds (*Picea glauca*), weakening of the micropylar end of the megagametophyte and nucellus precedes radicle protrusion, and this weakening is associated with endo-β-mannanase activity (Downie et al., 1997). Similarly, in yellow cedar (*Chamaecyparis nootkatensis*) seeds, weakening of the megagametophyte at the micropylar end occurs prior to radicle protrusion and is linked to the activity of pectin methyl esterase (Ren and Kermode, 1999; Ren and Kermode, 2000). In addition to decreased mechanical restraint, yellow cedar embryos (radicles) also display increased turgor and resistance to lower osmotic potentials following dormancy breakage compared to their dormant counterparts (Ren and Kermode, 1999). In western white pine, the megagametophyte and nucellar cap/nucellar membrane impose dormancy; the hard outer seed coat is also involved in the dormancy mechanism. It is unknown what changes, if any, occur to the radicle during dormancy breakage (i.e. if radicle turgor increases) (Hoff, 1987).

Thus, using the deeply dormant western white pine seeds as a model, we sought to determine whether a connection exists between coat-enhanced dormancy and ABA metabolism. Toward this end, the following analyses were undertaken: (1) a detailed

characterization of the seed structures that contribute to the coat-enhanced dormancy mechanism, and (2) quantification of ABA and it major metabolites (PA, DPA, 7'OH ABA, neo-PA, and ABA-GE), as a result of germination-promoting treatments involving the sequential removal of structures surrounding the embryo.

4.2 Materials and Methods

4.2.1 Determination of seed tissues that inhibit germination of the embryo

The effects of sequentially removing the seed coat and megagametophyte (the major tissues enclosing the embryo) on germinability were examined. The different treatments are annotated in Table 4.1. After dissection, unless otherwise indicated, the seed parts or embryos were placed on Whatman 3MM paper above a Kimpak in a clear plastic seed box (see section 2.2.2.1), which was then placed in germination conditions (25°C d, 15°C nights with an 8h photoperiod). Germination was monitored daily for a total of 30 d. For the treatment in which embryos were incubated in 100% relative humidity (RH), isolated embryos were placed on a supportive metal tray over water in a seed box (see section 2.2.2.1) to maintain 100% RH but avoid direct contact with water. Germination rate was determined as detailed in Xu (1990) following the formula detailed in section 2.2.3.

4.2.2 Determination of puncture force required to penetrate the megagametophyte and embryo

The mechanical resistance of the micropylar megagametophyte surrounding the radicle was measured by a FG-5.0R Digital Force Gauge (Shimpo, Lincolnwood, Illinois, USA) (Ren and Kermode, 1999). Seeds were cut into halves, transversely, and the embryo excised. The micropylar tip of megagametophyte with or without seed coat was placed on a Plexiglas holder with the tip end pointing vertically downward. A flat-ended steel needle (with a diameter of 0.5 mm, which approximated that of the radicle) was attached to the digital force gauge such that it would be lowered and penetrate through the micropylar tip at a rate of 5 mm sec^{-1}. The needle advanced without resistance through a hole in the centre of the Plexiglas sample holder. The force required to penetrate the tip was determined from the peak force of the load-deflection.

To measure the force required to penetrate the isolated embryo (a measure of the

turgidity of the cells of the radicle), the embryo was placed horizontally on the hole of Plexiglas holder. The procedure was the same as that performed on the megagametophyte, except that the needle penetrated the middle of the embryonic axis.

4.2.3 Sensitivity of the embryo to osmotic potential

Embryos were isolated from dormant seeds or from dormancy-released seeds by moist chilling, then incubated in different concentrations of PEG 8000 (Sigma) under germination conditions. The attainment of embryo growth was noted daily for 2 weeks.

4.2.4 Seed material and seed dissection treatments for ABA and ABA metabolite measurements

Details on seed materials and Pw seedlot 08006 followed those described in section 3.2.1. Dry seeds were allowed to warm to room temperature for approx. 4-6 h from storage at -20°C before sterilization. Seeds were sterilized for 5 min with 70% EtOH in a Corning® 50 mL 0.22 μM cellulose-acetate tube-top filter (Fisher Scientific). The EtOH was removed under a vacuum, seeds were further sterilized in 10% bleach for 3 min, and the bleach removed under vacuum. Seeds were then rinsed for two 5 min periods with sterile water.

Seeds were soaked with sterile water for 3 d at approx. 21°C in 50 mL Falcon tubes with gentle agitation. Sterile water was exchanged daily. Following the soak, seeds were either placed intact (denoted as Intact seed) or first dissected and then placed onto 1% agar medium (Difco, Beckton Dickinson). Dissection treatments were as follows: isolation of the embryo (denoted Embryo), removal of the entire hard outer seedcoat so that only a papery-layer surrounded the megagametophyte (denoted Intact mega), and removal of a portion of the hard outer seed coat on the dorsal side so that a small portion of the megagametophyte was exposed to air (denoted Hole in Top) (Fig. 4.1a; see Terskikh et al. (2005) for a further description of western white pine seed anatomy). Following placement on agar, seeds and seed parts were placed in germination conditions (23°C, 16-h photoperiod, and light intensity approx. 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Germination was monitored for 18 d.

4.2.5 Extraction of ABA and ABA metabolites

Seeds and seed tissues were collected at 12 h, 1 d, 3 d, and 6d after dissection and placement at germination conditions. Further dissection took place for Intact seeds, Hole in top, and Intact mega to separate the embryo and megagametophyte. Seedcoats were discarded since it was previously determined that the amounts of ABA and ABA metabolites in the seedcoat are low after a 3 d water soak (see section 3.3). After collection or further dissection, seed parts were frozen in dry ice before storage at -80°C. Following lyophilization for 24 h, embryo and megagametophyte samples were weighed to determine the dry weights (DW). Once samples had been lyophilized they were stored over Drierite desiccant (Anachemia) at room temperature. For embryo samples, 20 seed parts were used (approx. 20 mg DW). Megagametophyte samples were weighed out into 150 mg DW aliquots (20 megagametophytes approx. 200 mg DW). Further details of the extraction of ABA and ABA metabolites are described in section 3.2.3.

4.2.6 Quantification of ABA and ABA metabolites

Quantification of ABA and ABA metabolites follows section 3.2.4 with the following modifications. A new metabolite, neo-PA, a product of 9′-hydroxylation of ABA, was quantified. Synthesized (-)-neo-PA was used to generate a standard curve along with its deuterium-labeled internal standard, (-)-8′,8′,8′-d_3 neoPA (Zhou et al., 2004). Mass spectrometry conditions were as follows: (a) *ES conditions*: polarity ES-, capillary 2.75 kV, cone 25 V, for ABA, PA, 7'OH-ABA, and neo-PA, 30 V for DPA, and 20 V for ABA-GE, aperture 0.2 V, source temperature 120°C, desolvation (N_2) gas temperature 350°C, cone gas flow approx. 74 L/h, desolvation gas flow approx. 700 L/h; (b) *Tandem quadrupole conditions*: low mass and high mass resolution were 12.0 for both mass analyzers, ion energies 1 and 2 were 1.0 and 2.0 V, entrance and exit potentials were 10 and 10 V, respectively, and detector (multiplier) gain was 650 V. Collision induced dissociation of deprotonated parent ions, accomplished with a collision energy of 10 (ABA), 12 (ABA-GE). 13 (PA and neo-PA), or 18 V (DPA, 7'OH-ABA) and a collision cell pressure of approx. 5.4×10^{-3} mbar (achieved using Ar gas), was assessed using MRM. During the 25 min run, MRM was divided into 3 functions. These were: DPA/d3-DPA in the first function (4.3 min retention times, 281 > 171 / 284 > 174 parent to

daughter ion transitions), ABA-GE/d5-ABA-GE, PA/d3-PA, 7'OH ABA/d4-7'OH ABA, and neo-PA/d3-neo-PA in the second function (8.9, 9.6, 10.7 and 11.7 min retention times, and 425 > 263 / 430 > 268, 279 > 139 / 282 > 142, 279 > 151 / 283 > 154, 279 > 205 / 282 > 208 transitions, respectively), and ABA/d4-ABA in the third function (13.6 min retention times, 263 > 153 / 267 > 156) (see Appendix, Figs. A5, A6). For quantification of samples, a standard curve was generated using the following concentrations of analytes: 0, 2.5, 5, 10, 25, 50, 100, 200, 500, 750, and 1000 pg/μL – each with 100 pg/μL deuterated-internal standard (see Appendix, Fig. A7). Two hundred μL of each concentration was prepared and divided into three 65 μL replicates. Ten μL of each aliquot was injected into the RP-LC ES/MS/MS and run under identical conditions to sample extracts. Regression curves were generated and samples were quantified in MassLynx 4.0 (Waters, Milford, MA).

4.3 Results

4.3.1 Effect of sequential removal of layers surrounding the embryo on germination

Isolated embryos from dormant seeds of western white pine germinated (i.e. 100% underwent elongation and subsequent growth) when placed on wet filter paper or when incubated in 100% RH (Table 4.1, treatments 12, 13). However, in 100% RH the growth of isolated embryos was limited (note the germination rate in Table 4.1, 78 ± 2.8 and 86 ± 1.1 for embryos in 100% RH versus on filter paper, respectively). Because the embryos elongated in both treatments, this suggests that the embryo itself is not dormant and that the dormancy mechanism is coat-enhanced. The coat-enhanced mechanism was investigated further by determining which structures contribute to prevent germination (radicle protrusion).

The major tissues enclosing the embryo in western white pine are the megagametophyte, nucellar membrane, and seed coat; the nucellar cap has degenerated into a very thin membrane surrounding the micropylar end of the seed. Through analysis of germination percentage and rate after sequential excision of the enclosing tissues (Table 4.1), it was found that removal of the seed coat in the micropylar area (Table 4.1, treatments 2, 3) promotes 25% germination of the seed population, whilst removal of the seed coat and nucellar cap in the area of micropylar tip (Table 4.1, treatments 4, 5)

promoted greater germination (approx. 50%). Further removal of the megagametophyte in the micropylar tip permitted 90% germination (Table 4.1, treatment 6). After partial removal of the seed coat at the chalazal end of the seed there was no significant difference in germination between seeds with nucellar cap/nucellar membrane broken or intact (Table 4.1, treatment 7 vs. 8). A higher germination percentage was induced when the whole seed coat (chalazal end included) was removed rather than in seed populations with only the micropylar portion of the seed coat removed (Table 4.1, treatment 8 vs. 9).

Table 4.1. Germination[a] of western white pine seed after sequential removal of structures surrounding the embryo. These structures were: the seedcoat, which consists of a hard outer layer and papery membrane, the nucellar cap surrounding the micropylar end, and the megagametophyte. Data are the average of 3 replicates of 20 seeds (±SD). ANOVA and shortest range significances were used to determine significant differences at either the P=0.05 (lowercase letters) or P=0.01 levels (UPPERCASE) (Mo, 1984). Experiment performed and data collected by Chengwei Ren.

Treatment[b]		% Germination[a]		Germination rate	
1	Fully intact seeds after 3d soak (dormant control)	0	g, E	0	g, G
2	Intact seeds with hard outer layers of seedcoat cracked at the micropylar end (papery membrane and nucellar cap intact)	27±1.7	f, D	16±1.5	f, F
3	Seedcoat cracked, papery membrane and nucellar cap removed in micropylar region	50±5.8	e, C	31±4.2	e, E
4	Hard outer seedcoat removed, papery membrane and nucellar cap intact	25±2.9	f, D	16±1.9	f, F
5	Seedcoat and nucellar cap removed in the micropylar end	47±3.3	e, C	33±2.0	e, DE
6	Seedcoat, nucellar cap, and megagametophyte removed in the micropylar end	90±5.0	b, A	74±4.4	b, B
7	Chalazal end of the hard outer seedcoat removed and papery membrane intact	47±3.3	e, C	28±3.0	e, E
8	Chalazal end of the hard outer seedcoat removed and papery membrane broken	48±3.3	e, C	32±0.7	e, E
9	Whole hard outer seedcoat removed and papery membrane broken	68±4.4	d, B	42±1.6	d, D
10	Whole seedcoat removed (hard outer and papery layer) and nucellar cap	78±1.7	c, B	57±2.4	c, C
11	Identical to 10 except incubated in 100% relative humidity	52±3.3	e, C	30±2.3	e, E
12	Isolated embryos	100	a, A	86±1.1	a, A
13	Identical to 12 except incubated in 100% relative humidity	100	a, A	78±2.8	b, AB

[a] Isolated embryos did not exhibit true germination, radicles did not elongate but shoots did grow.
[b] All seed was soaked 3 d in running water before dissection. Following dissection seeds were incubated at 25°C 8h, 15°C 16 h dark for 30 d

Similarly, in the treatments analyzed for ABA and ABA metabolites, isolation of the embryo and incubation on 1% agar promoted 100% germination of the embryos (Fig. 4.1b; Embryo). When the hard seed coat was removed and the nucellar membrane cut, 77% of seeds germinated; in contrast, removal of only a portion of the hard seed coat (on the dorsal side facing the air rather than the substratum) resulted in 59% germination (Fig. 4.1b; Intact Mega and Hole in Top treatments, respectively). However, the latter treatment (Hole in Top) elicited significantly slower germination than did the former treatment (Intact Mega) (Fig. 4.1b). Germination rates for the treatments analyzed for ABA and its metabolites were 3.0 ± 0.8 (Intact Seed), 30 ± 6.4 (Hole in Top), 60 ± 7.4 (Intact Mega), and 89 ± 0.5 (Embryo). Germination of the intact seed, when incubated on 1% agar, was 7% (Fig. 4.1b).

4.3.2 Resistance imposed by tissues surrounding the embryo and growth potential of the embryo

Since the surrounding tissues in the micropylar area play a role in dormancy maintenance, the hypothesis that moist chilling may decrease the mechanical restriction of surrounding seed tissues to promote germination was investigated. This was accomplished by determining the puncture forces necessary to penetrate the seed coat and megagametophyte in the micropylar tip region during dormancy breakage (Table 4.2). The puncture force required to penetrate the micropylar tip decreased significantly after 1 d in germination conditions, following moist chilling, and decreased further during germination. After 4 d in germination conditions, the difference in puncture force required to penetrate both seed coat and megagametophyte in the tip area was not significant (as compared to 1 d in germination conditions); however, it is noteworthy that the seed coat always cracked along a suture line before radicle emergence. Thus moist chilling affected two processes – it enabled the seed coat to crack, and it weakened the mechanical strength of the nucellar cap/membrane and possibly the megagametophyte, thus providing less resistance to radicle elongation (see further discussion below). In contrast to the decrease in mechanical restraint imposed by the seed coat, nucellar cap/membrane, and megagametophyte, the opposite trend occurred for the embryo. After moist chilling, the puncture force required to penetrate the embryo increased (Table 4.2).

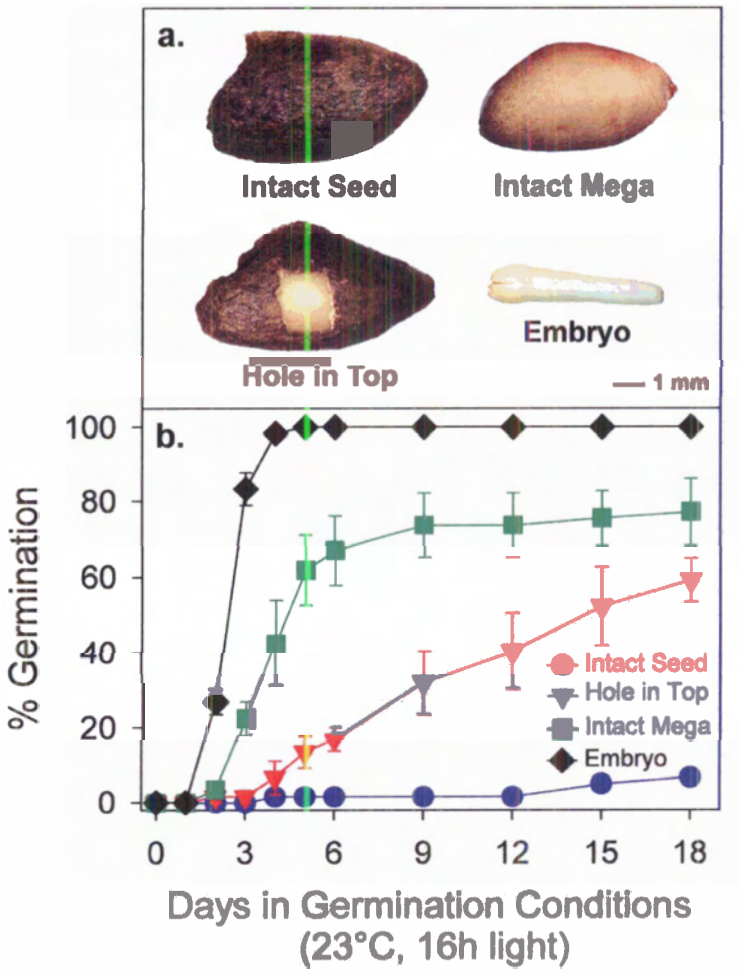


Fig. 4.1. Seed dissection treatments in western white pine seeds (**a**). Dissection treatments were as follows: isolation of the embryo (denoted Embryo), removal of the entire hard outer seedcoat (denoted Intact Mega), and removal of a portion of the hard outer seed coat on the dorsal side so that a small portion of the megagametophyte was exposed to air (denoted Hole in Top). Whole seeds (Intact Seed) were included as a control. Germination after seedcoat or megagametophyte removal (**b**). Following a 3 d soak, seeds were dissected and incubated on 1% agar in germination conditions for 18 d. Germination was scored as penetration of the radicle through surrounding structures or as elongation of the embryonic axis. Germination data are based on 3 replicates of 20 seeds each (±SE).

This could be explained by the fact that moist chilling increased embryo turgor, rendering it more capable of penetrating the surrounding megagametophyte. In agreement, the growth capacity of embryos of dormant versus non-dormant seeds differed in PEG solutions of varied osmotic potentials (Table 4.3). Embryos from both dormant and non-dormant seeds elongated in solutions with an osmotic potential up to -0.296 MPa (15% PEG). However, the germination rate was lower in embryos taken from dormant seeds. In a -0.684 MPa solution (20% PEG), 77% embryos from non-dormant seeds grew while only 57% from dormant seeds grew; the germination rate of embryos of dormant seeds was also much lower than that exhibited by embryos of non-dormant seeds.

Table 4.2. Force (N) required to penetrate the micropylar tip or embryonic axis. Data are the average of the peak force reading[a] in N of 20 seeds (±SD). ANOVA and shortest range significances were used to determine significant differences at P=0.05 (lowercase letters) (Mo, 1984). Experiment performed and data collected by Chengwei Ren.

Treatment	Force to penetrate seed coat and megagametophyte (N)		Force to penetrate megagametophyte (N)		Force to penetrate embryo (N)	
3 d soak (dormant seed)	1.8759±0.1129	a	0.4512±0.0219	a	0.1489±0.0130	b
75 d moist chilling (non-dormant)	1.7048±0.1177	ab	0.4179±0.0450	a	0.2045±0.0114	a
1 d germination conditions	1.5603±0.0989	b	0.3423±0.0250	b		
4 d germination conditions	1.4158±0.0956	b	0.2956±0.0321	c		

[a] The mechanical resistance of the different seed parts was measured using a Digital Force Gauge mounted with a stainless steel needle with tip diameter of 0.5 mm (closely approximating that of the embryo). The needle and seed piece were carefully aligned and the needle lowered through the seed piece at a rate of 5 mm sec^{-1}.

Table 4.3. Germination[a] percentage and rate after 20 days[b] of embryos isolated from dormant seed (3 day soak) or non-dormant seed (moist chilled) in different concentrations of PEG. Data are the average of 3 replications with 20 seeds in each (±SD). ANOVA and shortest range significances were used to determine significant differences at P=0.05 (lowercase letters) (Mo, 1984). Experiment performed and data collected by Chengwei Ren.

Osmotic Potential		Embryos of dormant seeds				Embryos of seeds after moist chilling			
% PEG	MPa	% Germination[a]		Germination rate		% Germination		Germination rate	
5	-0.023	100	a	66±3.5	b	-		-	
10	-0.127	100	a	67±2.1	b	100	a	79±0.9	a
15	-0.296	100	a	58±3.7	b	100	a	67±1.9	b
20	-0.684	57±3.33	c	15±1.4	d	77 ± 3.3	b	29±1.5	c

[a] Isolated embryos did not exhibit true germination, radicles did not elongate but shoots did grow.
[b] Seed was soaked 3 d in running water before dissection of dormant embryos or following a 12 d water soak and 75 d moist chilling for non-dormant embryos. Following dissection seeds were incubated at 25°C 8h light, 15°C 16 h dark.

4.3.3 Changes in ABA during sequential removal of structures surrounding the embryo

ABA and the major ABA metabolites were monitored in the embryos and megagametophytes over a 6 d incubation period in germination conditions (Figs. 4.2-4.4). After 6 d in germination conditions, the various treatments involving sequential removal of tissues surrounding the embryo, had elicited 2%, 17%, 67% and 100% germination (Intact seed, Hole in Top, Intact Mega and Embryo, respectively) (Fig. 4.1).

For the two treatments that elicited the greatest difference in germination capacity (i.e. Embryo vs. Intact seed), the ABA levels within the embryo were very closely associated (in a negative manner) with the ability of embryos/seeds to germinate (Fig. 4.2a). Isolation of embryos from seeds and their placement on 1% agar, a treatment that elicited 100% germination led to large and rapid decline in embryo ABA (by approx. 80%), within 12 h (Fig. 4.2a). In contrast, in embryos of intact seeds (2% germination at d 6 and 7% germination at d 18), the ABA level increased over the first 12 h; by d 6, the ABA level was similar to that present before incubation. However, for the two treatments that elicited 17% and 67% germination at d 6 (but a greater % germination by d 18; Hole in Top and Intact Mega treatments, respectively), the association between a decreased

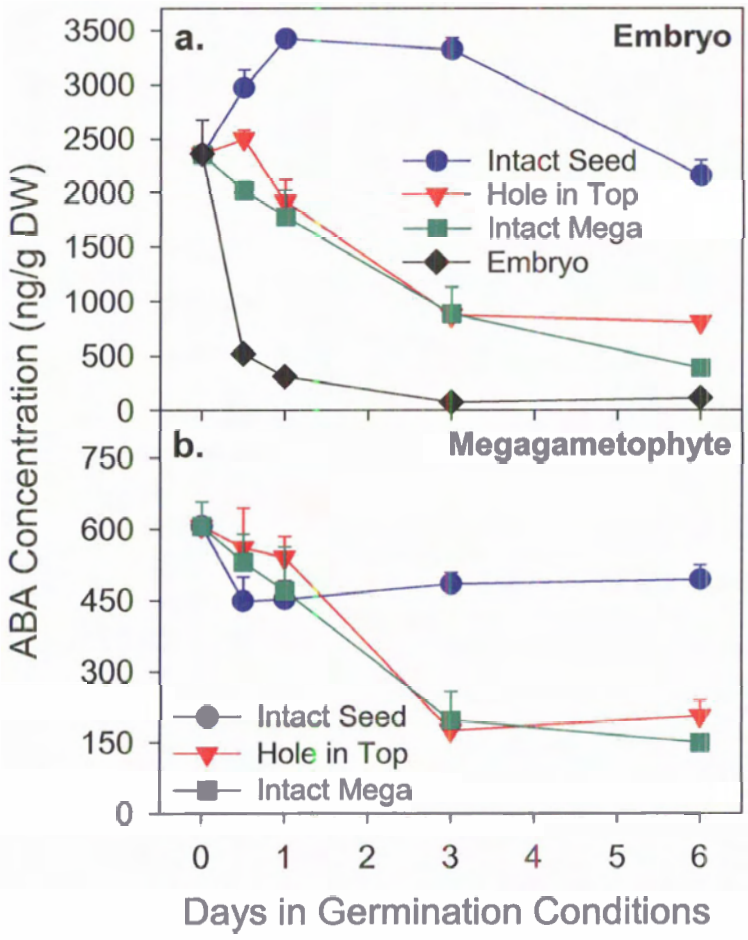


Fig. 4.2. Changes in ABA in embryos (**a**) and megagemtophytes (**b**) of western white pine following dissection treatment and incubation in germination conditions (23°C 16 h light). The metabolite neo-PA was not detected. Seeds were soaked 3d in water prior to dissection and incubation in germination conditions. Data are expressed on a ng per g DW basis and are based on 3 replicate extractions of 20 embryos or 15 megagametophytes each (± SE). See Fig. 4.1 for germination details and seed dissection illustrations.

ABA level and increased germination capacity was less clear. Both treatments led to a gradual decrease in ABA in the embryo and megagametophyte especially during the first 3 d (Fig. 4.2); although by d 6, the latter treatment (Intact Mega) resulted in lower amounts of ABA in the embryo (Fig. 4.2a).

In all treatments, levels of ABA in the megagametophyte tissues were approx. 20-40% lower than those of embryo samples (Fig. 4.2b).

4.3.4 Changes in ABA metabolites during removal of structures surrounding the embryo

4.3.4.1 8'-Hydroxylation of ABA

Isolation of embryos from seeds (a treatment resulting in 100% germination) led to a slight increase in PA followed by a decline (Fig. 4.3a) and DPA decreased throughout the 6 d (Fig. 4.3b). In marked contrast, the embryos of intact seeds, in which minimal germination was elicited, exhibited an increase in both products of 8' hydroxylation (PA and DPA) through the 6 d incubation (Fig. 4.3a, b). The Hole in Top treatment (which stimulated some germination – 17% at d 6 and 59% at d 18), elicited a slight increase of PA in the embryo during the 6 d incubation period, but this was not accompanied by any change in DPA. Embryos with only the megagametophyte surrounding them (Intact mega, which stimulated 67% germination by d 6), exhibited a transient yet considerable increase in 8'-hydroxylation-related metabolism of ABA (increased PA and DPA) over the first day; this was followed by a decline over the remaining 5 d period. The PA/DPA metabolites of the megagametophytes exhibited the same general trends in changes over the 6 d incubation period as those found in the embryo except that DPA showed a decreasing trend in all treatments (Figs. 4.3a, b; 4.4a, b).

Overall and in general, the largest transient increases (and decreases) in PA and DPA were observed in the Intact Mega samples, in both the embryo and megagametophyte tissues (Figs. 4.3a, b; 4.4a, b). Therefore, in terms of germination capacity, the treatments that were most effective in this regard, appeared to effect greater changes in the capacity of seeds to turnover (or transport) the products of 8' hydroxylation (PA and DPA). However, because ABA or metabolite flux and transport were not measured directly, this assumption remains speculative. For example, embryos of intact seeds, a treatment that

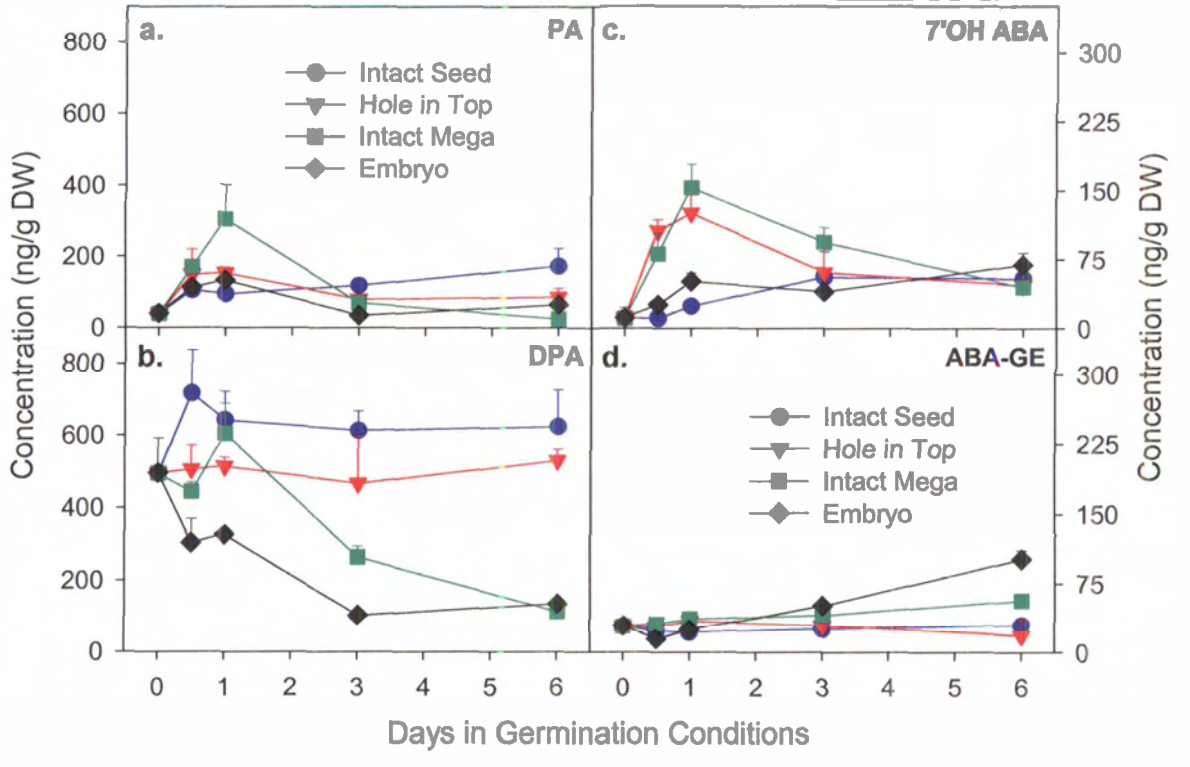


Fig. 4.3. Changes in PA (**a**), DPA (**b**), 7'OH ABA (**c**), and ABA-GE (**d**) in embryos of western white pine following dissection treatment and incubation in germination conditions (23°C 16 h light). The metabolite neo-PA was not detected. Seeds were soaked 3d in water prior to dissection and incubation in germination conditions. Data are expressed on a ng per g DW basis and are based on 3 replicate extractions of 20 embryos each (± SE). See Fig. 4.1 for germination details and seed dissection illustrations.

did not elicit germination, had increased DPA levels that were maintained throughout the 6 d incubation period (Fig. 4.3b) and it is unknown to what extent DPA was being metabolized in this tissue.

4.3.4.2 7'-Hydroxylation of ABA

There was generally no association between the capacity of a treatment to stimulate germination and changes in 7'-OH ABA in the embryo (Fig. 4.3c). For example, the isolated embryo- and intact seed-treatments (that elicited 100% and 2% germination, respectively) led to similar (slight and steady) increases in this metabolite over the 6 d period (Fig. 4.3c). The Hole in Top and Intact Mega treatments led to increases in 7'OH ABA in the embryo over the first day, followed by a decline (Fig. 4.3c).

Negligible levels of 7'OH ABA were observed in the megagametophyte and the treatments did not stimulate any changes in this metabolite (Fig. 4.4c). In addition, the metabolite neo-PA, a product of 9'-hydroxylation, was not detected in either the embryo or megagametophyte tissues (data not shown).

4.3.4.3 ABA Conjugation to Glucose

Following their isolation from the seed, embryos exhibited a large increase in ABA-GE over the 6 d incubation period, which increased approx. 3-fold (Fig. 4.3d). A similar but less pronounced increase in this metabolite was observed in the embryos of seeds surrounded by only the megagametophyte (Intact Mega treatment; Fig. 4.3d). No increase of ABA-GE occurred in embryos of seeds subjected to the other treatments (Intact Seed and Hole in Top treatments) (Fig. 4.3d).

In the megagametophyte, ABA-GE did not change appreciably during the 6 d incubation period regardless of the treatment (Fig. 4.4d) and remained at levels similar to that found in moist chilled seeds (Chapter 3) and in seeds during fluridone/GA treatments (Chapter 5).

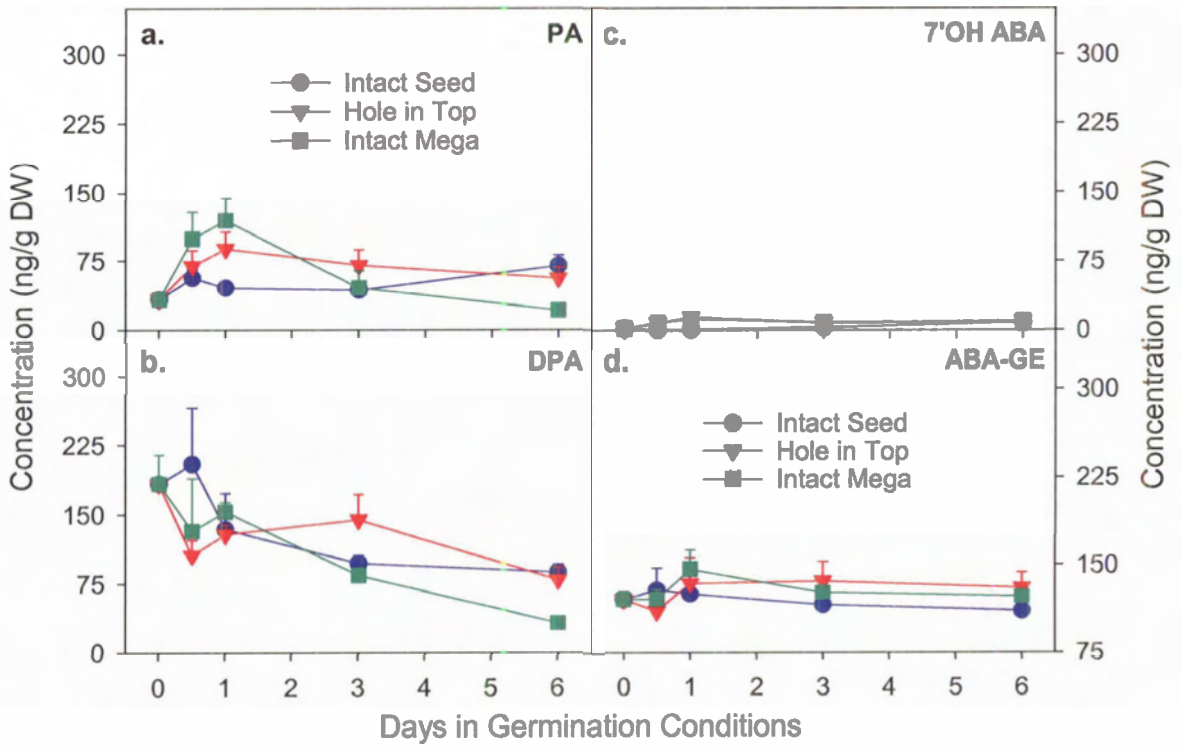


Fig. 4.4. Changes in PA (**a**), DPA (**b**), 7'OH ABA (**c**), and ABA-GE (**d**) in megagametophytes of western white pine following dissection treatment and incubation in germination conditions (23°C 16 h light). The metabolite neo-PA was not detected. Seeds were soaked 3d in water prior to dissection and incubation in germination conditions. Data are expressed on a ng per g DW basis and are based on 3 replicate extractions of 15 megagametophytes each (±SE). See Fig. 4.1 for germination details and seed dissection illustrations.

4.4 Discussion

The structures enclosing the embryo of western white pine seed include the seed coat, nucellar membrane/nucellar cap, and megagametophyte; these prevent germination via a 'coat-enhanced' dormancy mechanism (Hoff, 1987; Dumroese, 2000). How do these tissues function to maintain dormancy? As noted in the introduction, the mechanism may involve the following: (i) interference with water uptake, (ii) interference with gas exchange, (iii) prevention of the exit of inhibitors from the embryo, (iv) supply of inhibitors to the embryo, or encouraging inhibitor synthesis within the embryo, and (v) mechanical restraint (Bewley and Black 1994). The actions of several of these factors seem to contribute to the deep primary dormancy of western white pine seeds. Only interference with water uptake can be ruled out as a cause for dormancy (Terskikh et al., 2005). Seeds become saturated with water during a 12 d water soak at 23°C and no observable changes in hydration are evident during a subsequent 98 d moist-chilling period (Terskikh et al., 2005; Terskikh et al., unpublished data). However, supply of adequate moisture to the seed during moist chilling (i.e. maintenance of moisture content close to that of a fully imbibed seed) is an important determinant in dormancy breakage (Feurtado et al., 2003; Chapter 2, section 2.4).

4.4.1 Mechanical restraint is an important facet of the dormancy mechanism but the mechanism by which it is released is still uncertain

The hard outer seed coat and megagametophyte provide mechanical restraint as the puncture force required to penetrate these structures decreases after moist chilling and into germination (Table 4.2). A decrease in puncture force, prior to germination, has also been noted in white spruce (megagametophyte and nucellus), muskmelon (*Cucumis melo*) (perisperm), pepper (*Capsicum Annuum*) (endosperm), lettuce (*Lactuca sativa*) (endosperm), tomato (*Lycopersicon esculentum*) (endosperm), and *Datura ferox* (endosperm) (Tao and Khan, 1979; Watkins and Cantliffe, 1983; Groot and Karssen, 1987; Welbaum et al., 1995; Downie and Bewley, 1996; Sánchez et al., 1986). In agreement with Hoff (1987), the megagametophyte of white pine seems to restrict radicle elongation to a greater extent than the nuclellar cap/membrane and seed coat; however, all three tissues provide some 'restraint' on the radicle. Seed coat removal in the

micropylar end promoted 25% germination of seeds, while removal of the nucellar cap/membrane elicited a further 23% germination; in addition to removing these tissues, removal of the innermost tissue surrounding the embryo – the megagametophyte – in the micropylar end triggered a further 43% germination (i.e. 91% of seeds treated in this manner germinated) (Table 4.1).

There are several examples, most from seeds of angiosperms, which suggest that enzymatic degradation is the mechanism underlying a decrease in the mechanical restraint imposed by a 'living' tissue on the embryo. The classic model is that of tomato seeds. The endosperm of tomato seeds contains high amounts of galactomannans as a food reserve; these hemi-cellulose polysaccharides may also contribute to mechanical restraint and thus prevent embryo expansion (Groot et al., 1988). It is the enzyme endo-β-mannanase which increases in the micropylar region prior to radicle emergence that weakens galactomannans (Nomaguchi et al., 1995; Toorop et al., 1996). However, it has been questioned whether endo-β-mannanase is in itself sufficient for completion of germination since the enzyme can still accumulate in the absence of germination (Toorop et al., 1996; Bewley, 1997; Toorop et al., 2000). Indeed, several enzymes may contribute to weakening of the micropylar region and include: endo-β-mannanase, β-mannosidase, α-galactosidase, polygalacturonase, xyloglucan endotransglycosylase, peroxidase, β-1,3-glucanase, chitinase, and expansin. The primary evidence for their involvement is detection of the enzyme (or expression of the corresponding gene) in the micropylar region prior to tomato seed germination (Sitrit et al., 1999; Chen and Bradford, 2000; Nonogaki et al., 2000; Chen et al., 2002; Feurtado et al., 2001; Mo and Bewley, 2002; Morohashi, 2002). (Notably chitinase and β-1,3-glucanase may have alternative roles as fungal antagonists) (Wu and Bradford, 2003). Seeds of *Datura ferox* also produce endo-β-mannanase, β-mannosidase, and expansin in the micropylar endosperm prior to germination and in response to light perceived in the embryo via phytochrome and GA (Sánchez and de Miguel, 1997; Arana et al., 2005). In western white pine seeds several cell-wall hydrolytic/modifying enzymes have been characterized (endo-β-mannanase, α-galactosidase, polygalacturonase, and pectin methyl esterase); however, none shows a trend suggestive of its involvement in dormancy termination (Ren and Kermode, unpublished results). Indeed a mechanism to induce enzymatic-mediated weakening of

the megagametophyte may not be required to elicit germination of western white pine seeds. For example, the seeds have a cavity below the radicle, where remnants of the suspensor reside, and only the nucellar cap/membrane and seed coat surround the seed (ie. the radicle is not totally encapsulated by the megagametophyte) (Terskikh et al., 2005). Structural changes, revealed by scanning electron microscopy, show that the nucellar cap/membrane becomes more porous during dormancy termination (moist chilling) (Ma, Ren, Kermode, unpublished results). Tillman-Sutela and Kauppi (2000) suggest that opening of the seed coat, in various conifer species including western white pine, is mainly mechanical and primarily operates through enlargement of the imbibed endotesta cells lining the micropylar canal. The hypertrophied nucellar apex, swelling remnants and mucous substances in the micropyle further enhance seed coat splitting, which is reinforced by an unfolding of the nucellar cap as the radicle elongates (Tillman-Sutela and Kauppi, 2000).

The embryo itself also changes during moist chilling as it prepares for ensuing conditions ideal for germination. The mechanical strength of the cells of the embryo increases throughout moist chilling (Table 4.2) and this is likely indicative of increased turgor. In addition, with increased moist chilling of seeds, the isolated embryos become less sensitive to negative osmotic potentials (Table 4.3). This increased growth potential may allow germination in a wider range of environmental conditions. Increases in embryo growth potential (during germination) have been reported in seeds of several species such as *Datura ferox*, lettuce, and *Xanthium pensylvanicum,* and in many cases, growth potential is promoted by GA (Esashi and Leopold, 1968; Carpita et al., 1979a; Carpita et al., 1979b; de Migel and Sánchez, 1992; Arana et al., 2005).

Potentially acting as an antagonist of GA, classic experiments have demonstrated that applied ABA inhibits cell wall loosening and elasticity in *Brassica napus* embryos (Schopfer and Plachy, 1985; see Chapter 1, section 1.2 for further discussion). Further, in coffee (*Coffea arabica*) seeds, applied ABA inhibits the increase in embryo pressure potential and the second phase of endosperm cap weakening (de Silva et al., 2004). The authors suggest that an increase in endogenous ABA acts in a similar manner – i.e. it slows germination by restricting embryo growth potential and the second step of endosperm cap weakening (de Silva et al., 2004). Applied ABA also inhibits the second

phase of endosperm weakening in tomato seeds (Toorop et al., 2000). However, in *Arabidopsis* seeds, rupture of the seed coat and endosperm and mobilization of the cell walls surrounding the radicle proceed in the presence of 20 μM ABA. However, these processes (lipid mobilization and cell wall hydrolysis) still require the action of GA (Penfield et al., 2004). Whether GA or ABA participates in the regulation of embryo growth potential in western white pine is unknown; however inhibiting ABA biosynthesis and providing GA does stimulate germination (see Chapter 5).

4.4.2 Structures surrounding the embryo contribute to and maintain ABA homeostasis

Western white pine seeds need adequate air exchange for effective dormancy breakage (see Chapter 2; Feurtado et al., 2003). In addition, Dumroese (2000) found that germination increases if seeds, with a portion of the seed coat removed, are cultured with the opening exposed towards the air, rather than towards a moist substratum; moist chilling negates this effect. Further, the nucellar cap together with the nucellar membrane, probably provide little mechanical restraint but may act as a barrier to oxygen, and at the same time prevent the release and metabolism of inhibitors. Certainly the germination of seeds increases when these structures were removed in the present study (Table 4.1). To further investigate the mechanism involved, levels of ABA and of the major ABA metabolites were monitored during various germination-promoting treatments (Fig. 4.1a) that involved the sequential removal of structures surrounding the embryo. Indeed, ABA levels decreased with sequential removal of structures surrounding the embryo and megagametophyte (Fig. 4.2) and there was a close association between a rapid decline in ABA and the ability of embryos/seeds to germinate.

ABA in isolated embryos decreased approx. 95%, from 2400 to 100 ng/g DW, during a 6 d incubation in germination conditions (Fig. 4.2a). A significant decrease in ABA, from 876 to 55 ng/g FW, also occurs in isolated embryos of Douglas-fir (*Pseudotsuga menziesii*) seeds during a 7 d culture period (Bianco et al., 1997). The decrease in ABA levels in western white pine embryos, as in other treatments and tissues, is the result of a shift in ABA homeostasis towards catabolism or transport of ABA. The levels of the metabolites PA, DPA, and 7'OH ABA tended to be lowest for the treatments that

stimulated the greatest decline in ABA levels (i.e. Embryo, Intact Mega, and Hole in Top). Germination capacity seemed to correlate well with the ability of seeds (especially the embryos) to further metabolize (or transport) the products of 8' hydroxylation (PA and DPA), rather than with the ability of seed tissues to form these two metabolites. ABA decreased most markedly in isolated embryos as compared to the levels found within embryos that were enclosed by seed tissues, especially during the first day (i.e. in embryos of seeds of the Intact Mega and Hole in Top treatments). This may be because of a greater transport (or leaching) of ABA from the embryo into the surrounding medium; the presence of the megagametophyte, nucellar membrane, and seed coat likely inhibit leaching of ABA. However, in a separate experiment, when isolated embryos were imbibed on a wire screen in 100% RH, significant decreases in ABA were observed after 1 d (1820 to 204 ng/g FW); nevertheless, the decrease in ABA was greater in embryos placed on a moist substratum (1820 to 14 ng/g FW) (Ren and Kermode, unpublished results). This suggests that most of the ABA in isolated embryos is being catabolized rather than simply being leached (transported) into the surrounding medium. ABA analyses of the agar that embryos were incubated upon would test this assumption. In both lettuce and *Arabidopsis* Columbia, increased levels of PA and DPA during 2 mM GA_3 treatment or seed imbibition, respectfully, are only observed when the amounts present in seeds and the surrounding medium are summed together (Gonai et al. 2004; Kushiro et al. 2004). However, in barley seeds, more PA is found in the embryo and endosperm compared to in the imbibition medium (Jacobsen et al. 2002).

Two of the treatments (Hole in Top and Intact Mega) exhibited similar patterns of changes in ABA during the 6 d incubation period. This is somewhat surprising since the presence of the hard seed coat in the Hole in Top treatment is expected to create an additional barrier preventing the escape of ABA (via leaching or transport) and to reduce air exchange for catabolism of ABA (see below for further discussion). Moreover, the germination elicited by the two treatments was considerably different – especially at 6 d (67% versus 17% for Intact Mega and Hole in Top treatments, respectively). Thus, ABA alone is not the only factor functioning to enforce dormancy in western white pine seeds. Because the hard seed coat is mostly intact in the Hole in Top treatment, it likely provides an additional restraint that the embryo (radicle) is unable to overcome.

In intact seeds, ABA levels increased and remained high throughout the incubation period, although by 6 d they had declined slightly as compared to the pre-incubation levels (Fig. 4.2). In addition, an increase followed by a subsequent decline in ABA metabolites did not occur to the extent that was observed in the other treatments (Figs. 4.3, 4.4). This is consistent with previous findings. Dormant imbibed seeds, kept in warm moist conditions for 30 d (after an initial 13 d of soaking), maintain high ABA levels, while the amounts of PA, 7'OH ABA, and DPA decrease or remain at steady-state levels (Feurtado et al., 2004; see Chapter 3). Thus, in the absence of conditions required to break dormancy (e.g. removal of structures surrounding the embryo), the change towards enhanced ABA breakdown (reflected also by a continued catabolism/transport of PA/DPA) does not occur to the same extent. DPA is increased in embryos of the dormant intact seed, but it is unknown whether it is subjected to further catabolism under this condition (Fig. 4.3b).

In addition to the observed decreases in ABA during seed coat removal and embryo isolation, the changes in the catabolites suggest that ABA is being actively degraded. However, as with our previous study the levels of the catabolites do not account for the observed decreases in ABA (compare Figs. 4.2, 4.3, 4.4). The increase in ABA-GE in the isolated embryos after 6 d in germination conditions is also noteworthy (Fig. 4.3d). Since the isolated embryos 'germinated' faster than seeds subjected to the other treatments especially by d 6 (Fig. 4.1b), these embryos would have progressed into the seedling growth stage. It is becoming apparent that catabolism of ABA through glucosylation occurs during seedling growth. For instance, in *Arabidopsis* seedlings (ecotype Columbia), there is a significant increase in ABA-GE as seeds enter into the seedling growth stage (i.e. after germination). ABA-GE levels increase approx. 7-fold during the transition from germinating seed to seedling (Chiwocha et al. 2005). Chiwocha et al. (2005) suggest that ABA-GE may provide an inactive but accessible pool of hydrolysable ABA available for seedling growth and stress responses.

PA, DPA, and 7'OH ABA peaked after 1 d in germination conditions in embryos surrounded by the megagametophyte (Intact Mega treatment); this suggests that both the 8'- and 7'-hydroxylation pathways are being used to catabolize ABA (Fig. 4.3). However, in the megagametophytes of these seeds, only the PA metabolite increased. Thus, the

predominant pathway of ABA catabolism in the megagametophyte is the 8'-hydroxylation pathway. Consistent with this, very little 7'OH ABA was observed in the megagametophytes of seeds subjected to any of the treatments (Fig. 4.4). PA and 7'OH ABA also increased in the embryo (and PA increased in the megagametophyte) during the 6 d incubation of seeds subjected to the Hole in Top treatments (Figs. 4.3, 4.4). These results are consistent with that found during moist chilling of western white pine seed – i.e. ABA is catabolized through the 8'- and 7'-hydroxylation pathways in the embryo and via 8'-hydroxylation in the megagametophyte (Feurtado et al., 2004; Chapter 3).

In summary, the embryo-covering structures, such as the seed coat, nucellar cap/membrane, and megagametophyte, in western white pine seeds seem to inhibit ABA breakdown and production of the hydroxylated catabolites 8'OH ABA and 7'OH ABA. Thus, the structures surrounding the embryo operate in a dormancy mechanism through both mechanical restraint and by modulating the homeostasis of a germination inhibitor, namely ABA. However, the supposition that the 'covering-structures' may affect ABA homeostasis by inhibiting air exchange is not entirely supported by the present data. Oxygen, or lack thereof, can be a determinant in the dormancy status of seeds (Baskin and Baskin, 2001, references therein, Adkins et al., 2002), and has also been linked to the ability of seed tissues (e.g. the embryo and megagametophyte) to metabolize ABA. For instance, Douglas-fir seed germination is negatively affected by oxygen deprivation and dormant unchilled seeds are especially sensitive to oxygen deprivation than are their non-dormant chilled counterparts (Corbineau et al., 2002). In isolated embryos of beechnut, a decrease in the oxidative products of (+)-[^{3}H]-ABA (e.g. PA and DPA) occurs as a result of either lowering oxygen availability or by feeding the embryos tetcyclasis (a monooxygenase inhibitor). Interestingly, the oxidative products are reduced in the same manner in the presence of the seed coat and pericarp leading the authors to conclude that the covering structures limit the oxygen supply to the embryo (Barthe et al., 2000). Indeed, the ABA 8'-hydroxylase enzyme, which forms the unstable intermediate, 8'OH ABA, before PA is formed, is a cytochrome P450 monooxygenase (CYP707A subfamily, see Chapter 1, section 1.6, and Chapter 6) and is inhibited by oxygen deprivation and tetcyclasis (Krochko et al., 1998; Kushiro et al., 2004). The same may be true for the ABA 7'-hydroxylase enzyme.

How might the seed coat and the nucellar cap/membrane modulate oxygen diffusion into the megagametophyte and embryo to subsequently affect ABA catabolism, or catabolism of PA/DPA? The presence of phenolic compounds (or others) that consume oxygen may provide an explanation. For example, the ovary cap or operculum of sugar beet (*Beta vulgaris*) consumes gas (presumably oxygen) when seeds are imbibed, and this is linked to the oxidation of various phenolic compounds. When the non-living operculum is removed germination occurs and dormancy is released (Coumans et al., 1976; see Bewley and Black, 1994, for further review). The glumellae of barley (*Hordeum vulare*) also consumes oxygen through the oxidation of phenolic compounds, which gradually diminishes as dormancy is released during after-ripening (Lenoir et al., 1986). Interesting future experiments that deserve further investigation include: (1) whether higher oxygen environments stimulate germination and if this is accompanied by decreases in ABA and increases (followed by subsequent metabolism) of the catabolites PA, DPA, or 7'OH ABA, and (2) whether the oxygen consuming capacity of the seed coat changes during moist chilling.

Thus, if one were to model dormancy breakage in western white pine, two requisite events would be: (1) a shift in ABA homeostasis, in part modulated by the seed coat and covering structures, and (2) an increase in the growth capacity of the embryo coincident with a decrease in the force required to penetrate structures surrounding the radicle. GA and ABA may modulate these latter two events, especially an increase in the growth capacity of the embryo, although definitive evidence for this in white pine awaits further investigation. Of note, the GA requirement for germination in *Arabidopsis* is determined by both the mechanical restraint provided by the seed coat and also by embryonic ABA (Debeaujon and Koornneef, 2000).

CHAPTER 5

Disrupting abscisic acid homeostasis in western white pine (*Pinus monticola* Dougl. Ex D. Don) seeds induces dormancy termination and changes in abscisic acid catabolites

5.1 Introduction

Western white pine (*Pinus monticola* Dougl. Ex D. Don) seeds exhibit pronounced primary dormancy at maturity. The dispersed seed is acclimatized to undergo a lengthy over-wintering period of cold-moist conditions before germination and seedling establishment in the spring. Under laboratory or nursery conditions, dormancy-breaking procedures for western white pine rely on a moist-chilling treatment of approx. 90 d, which is generally preceded by a water soak of up to 14 d (Hoff 1987; Feurtado et al., 2003; see Chapter 2). Seed dormancy in this species is primarily 'coat-enhanced'; imposed by the seed coat, nucellar membrane, and megagametophyte (Hoff 1987; Dumroese 2000). However, recently, the dormancy mechanism in western white pine seed was linked to the sesquiterpenoid hormone abscisic acid (ABA) (Feurtado et al., 2004). ABA levels in embryos and megagametophytes decrease significantly during dormancy termination (through moist chilling) and are coincident with an increase in the germination capacity of seeds. In the absence of conditions required to break dormancy, seeds display no net changes in ABA biosynthesis and catabolism since ABA levels remain high (Feurtado et al.,, 2004, see Chapter 3).

Over the past decade, studies have shown that continued synthesis of ABA is necessary for dormancy maintenance in imbibed seeds or embryos of many species including *Arabidopsis thaliana* (Cape Verde Islands ecotype), barley (*Hordeum vulgare*), beechnut (*Fagus sylvatica*), Douglas-fir (*Pseudotsuga menziesii*), lettuce (*Lactuca sativa*), sunflower (*Helianthus annus*), and tobacco (*Nicotiana plumbaginifolia*) (Le Page-Degivry and Garello 1992; Wang et al., 1995; Bianco et al., 1997; Le Page-Degivry et al., 1997; Yoshioka et al., 1998; Grappin et al., 2000; Ali-Rachedi et al., 2004). To prove that ABA biosynthesis is required for dormancy maintenance these studies used the herbicide fluridone (1-methyl-3-phenyl-5-(3-tri-fluoromethyl-(phenyl))-4-(1*H*)-pyridinone), which blocks the phytoene desaturase enzyme during the synthesis of carotenoids and thus inhibits ABA biosynthesis (Bartels and Watson 1978; Finkelstein and Rock 2002). For instance, when dormant tobacco seeds are imbibed, there is an accumulation of ABA. Fluridone, effective in breaking dormancy, inhibits this ABA accumulation during imbibition and causes a decrease in ABA levels similar to those found in non-dormant afterripened seeds. In addition, exogenous application of

gibberellic acid (GA_3) also initiates a decline in ABA levels in the dormant seed, although the decline is not as pronounced as that induced by fluridone treatment (Grappin et al., 2000). In lettuce seeds, a combined treatment of fluridone and GA_3 is needed to overcome thermoinhibition of germination at 33°C. While fluridone acts to suppress ABA biosynthesis, GA seems to stimulate ABA catabolism and, thus, the two treatments act in concert to decrease ABA to levels sufficient for germination (Gonai et al., 2004).

The dynamic levels of ABA and ABA catabolites present in seeds are the result of the constant flux of biosynthesis versus catabolism (Cutler and Krochko 1999; Nambara and Marion-Poll 2005). Catabolism of ABA can occur through a multitude of pathways, often depending on the species, developmental timing or tissue. The major pathway is through 8'-hydroxylation to form 8'-hydroxy ABA, which reversibly cyclizes to phaseic acid (PA). The recent identification of the cytochrome P450 CYP707A family as ABA 8'-hydroxylases represents a major step forward in our knowledge of ABA catabolism (Kushiro et al., 2004; Saito et al., 2004). Further reduction of PA can take place to form dihydrophaseic acid (DPA). ABA and ABA metabolites (PA and DPA) can also become conjugated with glucose forming an ester (PA) or an ester or glucoside (ABA and DPA). Other minor pathways include formation of 7'-hydroxy ABA (7'OH ABA) and ABA 1',4' diols (Cutler and Krochko 1999; Zeevaart 1999). Recently, a new metabolite, 9' hydroxy-ABA and its cyclized form neo-PA were discovered in *Brassica napus* siliques (Zhou et al., 2004).

To continue our characterization of the dormancy mechanisms in western white pine seeds and, in particular, the role of ABA and its catabolism, we addressed the following questions: (1) Is ABA biosynthesis absolutely necessary for dormancy maintenance and (2) Do the levels of ABA and its major catabolites (PA, DPA, 7'OH ABA, neo-PA, and ABA-GE) change in seeds that have been subjected to fluridone and GA treatments, alone, and in combination?

5.2 Materials and Methods

5.2.1 Seed Material and Fluridone/GA Treatments

Details on seed materials and Pw seedlot 08006 are in section 3.2.1.

Dry seeds were allowed to warm to room temperature for ~4-6 h from storage at -20°C before soaking. Seeds were soaked for 8 d at 25°C in a 10 L water bath with 5 L water exchanged daily. Sterilization occurred with 3% H_2O_2 (v/v) for 10 min and seeds were rinsed several times with ddH_2O. Seeds were then soaked in 50 mL Falcon tubes (under gentle agitation) with 1 of 4 different treatments: water with 0.05% Tween 20 (Control), 50 μM GA_{4+7} with 0.05% Tween 20 (GA), 100 μM Fluridone with 0.05% Tween 20 (Fluridone), and 100 μM Fluridone and 50 μM GA_{4+7} with 0.05% Tween 20 (Fluridone/GA). GA_{4+7} was dissolved in EtOH and fluridone dissolved in DMSO. To avoid precipitation, fluidone solutions were added to rapidly-stirring solutions and 0.05% Tween 20 was added as a surfactant to avoid fluridone precipitation. Controls contained the appropriate amounts of EtOH and DMSO. After 3 d, seeds were surface-dried and placed upon Whatman 3MM paper supported by 15-ply K-22 germination paper (Seedburo Equipment Company) in a deep-dish 9 cm Petri plate that had been moistened with ~23 mL of the aforementioned treatment solutions. Seeds were then transferred to germination conditions (23°C, 16-h photoperiod, and light intensity ~100 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Germination was monitored for 30 d.

5.2.2 Extraction of ABA and ABA metabolites

Seeds were dissected into embryo and megagametophyte at various times during fluridone and/or GA treatments. Seedcoats were discarded since it was previously determined that the amounts of ABA and ABA metabolites in the seedcoat are low after prolonged water soaks (see section 3.3). After dissection, seed parts were frozen in dry ice before storage at -80°C. Following lyophilization for 24 h, embryo and megagametophyte samples were weighed to determine the dry weights (DW). Once samples had been lyophilized they were stored over Drierite desiccant (Anachemia) at room temperature. For embryo samples, 20 seed parts were used (~20 mg DW). For embryo samples that had germinated, DW were ~40-45 mg. Megagametophyte samples

were weighed out into 150 mg DW aliquots (20 megagametophytes ~200 mg DW). Further details of the extraction of ABA and ABA metabolites are described in section 3.2.3.

5.2.3 Quantification of ABA and ABA metabolites

Quantification of ABA and ABA metabolites follows sections 3.2.4 and 4.2.3.

5.3 Results and Discussion

5.3.1 Fluridone and GA are needed to successfully break seed dormancy in white pine

Germination of western white pine seeds was monitored after different treatments with fluridone and/or GA. An 11-d soak in water at 25°C yielded 17% germination (Fig. 5.1; Control). This lengthy pre-soak is needed for efficient dormancy breakage during subsequent moist chilling (Feurtado et al., 2003); thus, its use was continued during fluridone/GA treatments. GA_{4+7} and fluridone treatments alone were slightly promotive as dormancy-breaking treatments and yielded 31% and 50% germination, respectfully. Effective dormancy-termination was elicited by the combination of GA_{4+7} and fluridone, in which germination was 77% after 30 d (Fig. 5.1, Fluridone/GA). However, even this combined treatment was not as efficient as 98 d of moist chilling which elicts ~ 90% germination and seed germinate faster, and more synchronously (Feurtado et al., 2004).

The initial finding that GA did not appreciably stimulate germination was not surprising since exogenous GA_{4+7} application does not always lead to dormancy breakage (Bewley and Black, 1994). Similar to the findings presented here for western white pine, 100 μM GA_3 has a weak stimulatory effect on dormancy termination of seeds of the Cape Verde Islands ecotype of *Arabidopsis* (*Arabidopsis* Cvi) (Ali-Rachedi et al., 2004). As noted in the introduction, fluridone can break dormancy in a number of species. However, it is less common for both fluridone and GA to be required for dormancy termination. In many instances where *de novo* ABA biosynthesis has been shown to be important for dormancy maintenance, fluridone works sufficiently and GA_3 acts to only enhance the germination rate and synchronicity (Grappin et al., 2000; Ali-Rachedi et al., 2004). However, fluridone prevents thermodormancy in lettuce seeds at 28°C but when the temperature is increased to

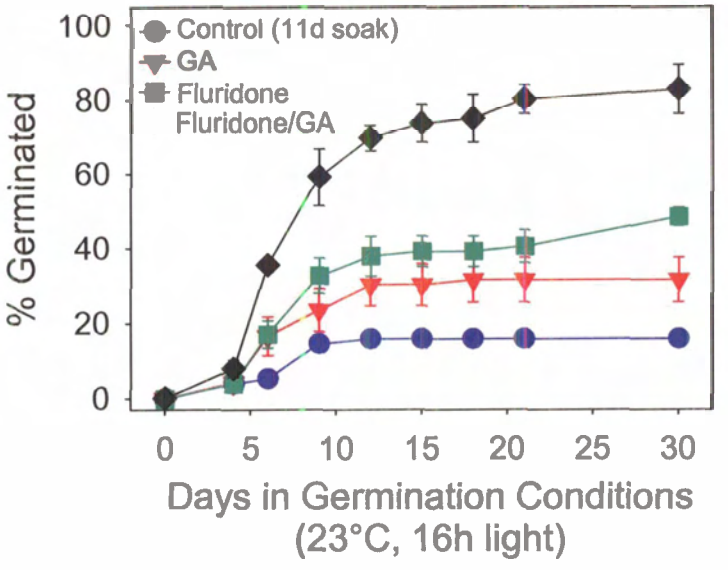


Fig. 5.1. Germination of western white pine seeds after fluridone and/or GA treatment. Seeds were soaked 8 d in water and 3 d in 100 μM fluridone and/or 50 μM GA prior to incubation in germination conditions on the aforementioned solutions. Germination was monitored for 30 d and was scored on the basis of protrusion of the radicle through the hard outer seedcoat. Data are based on 6 replicates of 25 seeds each (±SE).

33°C fluridone alone is no longer effective and the addition of 2 mM GA_3 is needed to restore germination (Gonai et al., 2004). Although it is unknown why such a high level of GA_3 (2 mM) is required, the authors suggest an increased sensitivity to ABA may account for the requirement for GA at 33°C (Gonai et al., 2004). In the context of a discussion on germination parameters, it is perhaps not surprising that both fluridone and GA are needed to break dormancy in western white pine, given the fact that normally these seeds require a lengthy moist chilling period (e.g. 90 d) to fully terminate dormancy (Feurtado et al., 2004). Similarly, in yellow cedar (*Chamaecyparis nootkatensis*) seeds, which normally require a 90-d dormancy-breaking treatment (30 d of warm, moist conditions followed by 60 d of moist chilling), GA_3 has a greatly reduced effect when used alone. However, when GA is combined with fluridone, germination increases with an increased time of exposure to the treatment (Schmitz et al., 2001).

5.3.2 Changes in ABA

During the treatment soak prior to incubation in germination conditions, ABA levels in the embryo decreased in the control, GA, and fluridone treatments to similar extents; however, ABA levels in the fluridone/GA treatment in the embryo were similar to the megagametophyte in that they remained constant throughout the initial treatment soak (Fig. 5.2a, b). ABA levels in megagametophytes were approx. 5-fold lower than in embryos. It is unknown why ABA did not drop in the embryo during the initial soak for the fluridone/GA treatments and a subsequent experiment yielded similar results (data not shown). When seeds were placed in germination conditions, ABA levels in embryos and megagametophytes in all treatments decreased further until just prior to the peak in germination (i.e. at 3 d in germination conditions) (Fig. 5.2a, b). Following the peak in germination, as seed populations returned to maintain a 'dormant' state, and seeds that were going to germinate had done so, ABA levels in embryo and megagametophyte tissues increased in all treatments except for those with fluridone (Fig. 5.2a, b).

The decline in ABA levels in embryos and megagametophytes of control seeds by approx. one-half by 3 d in germination conditions is noteworthy and has been found in other species. In dormant imbibed seeds of *Arabidopsis* Cvi, Douglas fir, and in thermo-dormant lettuce seeds imbibed at 33°C, there is an initial decrease in ABA levels before

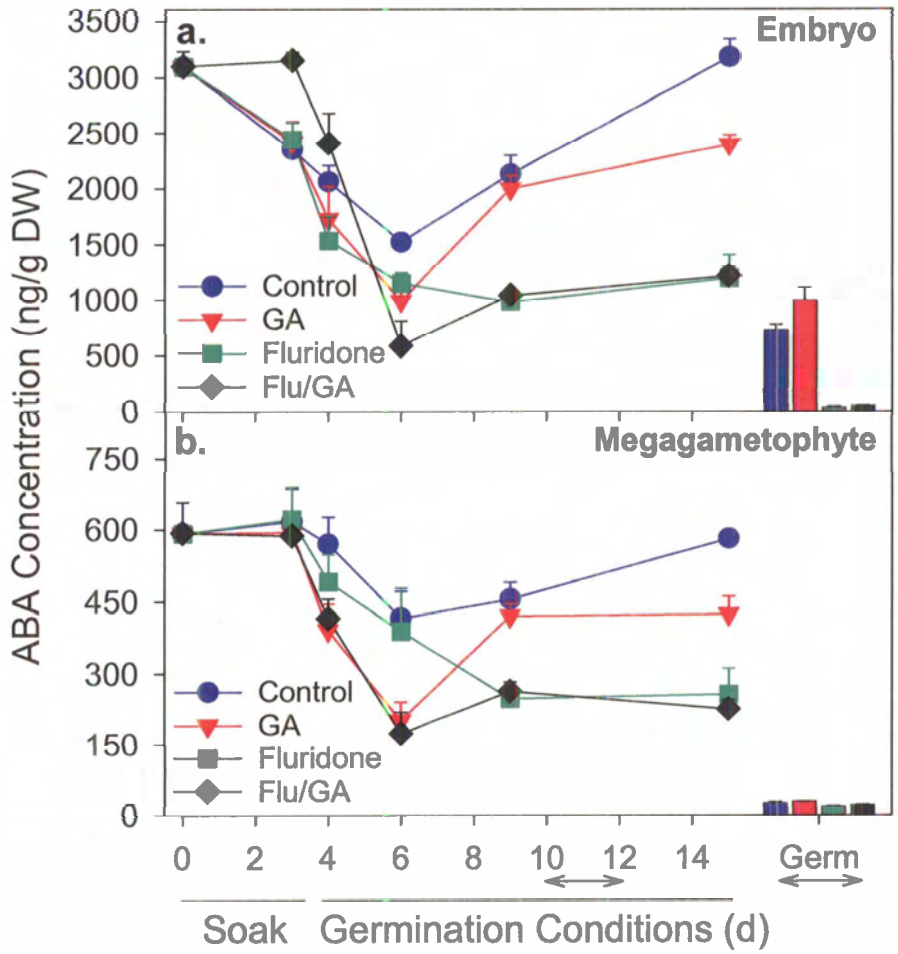


Fig. 5.2. ABA changes within the embryo (**a**) and megagametophyte (**b**) during fluridone/GA treatments. Ungerminated seeds were analysed before and after a 3 d soak in 100 μM fluridone and/or 50 μM GA and throughout 12 d in germination conditions (15 d total treatment time). Germinated seeds (Germ) were collected between 7-9 d in germination conditions and are denoted by the double-arrowed line. Data are expressed on a ng per g DW basis and are based on 3 replicate extractions of 20 embryos or 15 megagametophytes each (±SE). See Fig. 5.1 for germination details.

an increase. One could attribute the decrease in ABA to a portion of the seed population germinating, as seeds from *Arabidopsis* Cvi, Douglas fir, lettuce, and white pine all exhibit a decrease in ABA levels prior to germination (Bianco et al.,, 1997; Corbineau et al.,, 2002; Ali-Rachedi et al., 2004; Feurtado et al., 2004; Gonai et al., 2004). However, the decline in ABA levels is not accounted for by the proportion of seeds that germinate. Thus, at least for some species, this appears to be a characteristic of early imbibition / dormancy maintenance; ABA homeostasis initially favors catabolism (or transport) of ABA rather than biosynthesis. Once the seeds clear this 'transitory' period (where in some examples seed imbibition is occurring) there is a shift in ABA homeostasis to favour ABA biosynthesis and ABA levels increase as dormancy is maintained. Thus, once seeds are fully-imbibed, one could speculate that the seeds are sensing their environment, sensing that the requisite events required for dormancy termination have not taken place. A similar, but often more pronounced, 'catabolic-burst' or decrease in ABA levels is seen in many non-dormant (e.g. after-ripened or moist-chilled) seeds when placed in conditions conducive to germination (Corbineau et al., 2002; Gubler et al., 2005). In non-dormant *Arabidopsis* Columbia seeds there is a drop in ABA levels during early imbibition and this is associated with an increase in the expression of an ABA 8'-hydroxylase (*CYP707A2*) and combined levels of PA and DPA (Kushiro et al., 2004). Thus, one could question whether similar regulatory mechanisms controlling ABA flux occur during early imbibition / incubation of dormant and non-dormant seeds in conditions normally favorable for germination. This is an intriguing question that perhaps awaits a detailed analysis of ABA biosynthetic and catabolic genes/proteins during seed dormancy maintenance, termination, and germination.

GA_{4+7} treatment of western white pine seeds led to a more pronounced decrease in ABA levels than did the control treatment and its effect on ABA was similar to that elicited by the fluridone treatment (embryo) and to the fluridone/GA treatment (megagametophyte) until 3 d into germination conditions (Fig. 5.2a, b). After 3 d in germination conditions ABA of the embryo and megagametophyte increased in seed populations to a level that was similar to that of control (dormant imbibed) seeds, albeit lower. It is interesting that fluridone elicited somewhat greater germination of western white pine seeds, than did the GA treatment (50% versus 31%), but this was not reflected

in a greater decline of ABA level. However, the increase in ABA levels in GA treated seeds during d 3 to 6 in all probability prevented further seeds from germinating as 'dormancy' was re-initiated. Thus, because the GA effect on ABA metabolism (i.e. one that favored catabolism) was only transitory (until 3 d) the majority of seeds had not had sufficient chance to germinate before another shift in ABA homeostasis occurred, this time to favor ABA biosynthesis (from 3 to 6 d). This did not occur in fluridone-treated seeds since ABA biosynthesis was continuously blocked. A similar transient decrease in ABA levels, below that of dormant-imbibed seeds, is also seen in GA_3-treated *Arabidopsis* Cvi and thermo-dormant lettuce seeds (Ali-Rachedi et al., 2004; Gonai et al., 2004).

Fluridone treatments help to reveal the degree of ABA turnover that occurs during the initial or early period of dormancy maintenance (if one assumes that no ABA biosynthesis or formation of ABA is occurring with fluridone application). ABA levels during the initial soak periods were similar in control-, GA-, and fluridone-treatments suggesting that ABA biosynthesis was minimal in control and GA treatments and ABA was simply being catabolised or leached out of the seed into the water bath. When the seeds were put into germination conditions, the rate of catabolism in embryos increased in the fluridone samples and 900 ng/g DW was metabolised (transported) during the first d in germination conditions. During 1 to 3 d, 200 ng/g DW was metabolized per d; during d 3 to 6, the catabolism rate declined to only 50 ng/g DW per d, and did not decrease thereafter (Fig. 5.2a). Similar trends are revealed in the megagametophyte (Fig. 5.2b). Thus, it seems that ABA catabolism (or transport) ceases at 6 d in germination conditions. The fluridone data support the notion that under normal circumstances, i.e. in dormant imbibed (control) seeds, there is a shift in ABA homeostasis from a catabolic state towards one where ABA biosynthesis predominates as dormancy maintenance is continued.

The fluridone/GA treated seeds further reveal a nature of the dormancy mechanism in western white pine, and reveal a more precise action of GA. Since little or no ABA is synthesized during fluridone treatment, GA must have an effect on ABA catabolism or transport since ABA levels decline further when GA is added to fluridone-treated seeds. In addition, even if one does not account for changes in ABA sensitivity that may occur

during dormancy maintenance in white pine seeds, the decrease in ABA levels in fluridone- and GA-treated seeds suggests that one can invoke a threshold model to explain ABA levels and dormancy maintenance. The inhibition of ABA biosynthesis with fluridone alone or stimulation of ABA metabolism towards catabolism or transport with GA alone was not sufficient to decrease ABA levels below the threshold needed to terminate dormancy. It was only when the two treatments were combined that ABA levels dropped sufficiently for germination to proceed. The situation may be similar to that of thermo-dormant lettuce seed (Gonai et al., 2004).

In embryos of 7-9 d germinated seeds capable of biosynthesizing ABA (i.e. control and GA treatments), ABA was present at similar levels to those found in germinated seeds after a moist chilling treatment (Feurtado et al., 2004). However, in fluridone-treated seeds (with or without GA), ABA levels were very low in germinated seeds (Fig. 5.2a, b). In the megagametophytes, ABA was present at low levels in all treatments, again similar to that found for germinated seeds following a moist chilling treatment (Feurtado et al., 2004). In germinated seeds, ABA and ABA metabolite levels were higher in seeds capable of ABA biosynthesis (control and GA) and virtually non-existent in germinated seeds that had been treated with fluridone. These results suggest that there is *de novo* ABA synthesis taking place late during germination or during early seedling growth. The *de novo* synthesis of ABA is conceivably needed to sustain optimal seedling growth and stress responses (Fineklstein et al 2002; Feurtado et al., 2004). The rise in metabolites in the germinated seedling suggests that ABA levels are being dynamically maintained at this stage through the continued balance of biosynthesis and catabolism.

5.3.3 Catabolism of ABA through Hydroxylation

In the embryo, once seeds were transferred to germination conditions, a distinct peak in PA occurred in fluridone/GA treatments between 0 and 6 d (Fig. 5.3a). DPA, which decreased in all treatments over time, decreased slowest in the fluridone/GA treatment (Fig. 5.3b). In the megagametophyte, similar trends were observed. However, a peak in PA occurred in all treatments between 0 and 3 d in germination conditions and continued to peak until 6 d in the fluridone and fluridone/GA treatments (Fig. 5.4a, b). The data suggests that ABA was metabolized via the 8'-hydroxylation pathway to PA and DPA.

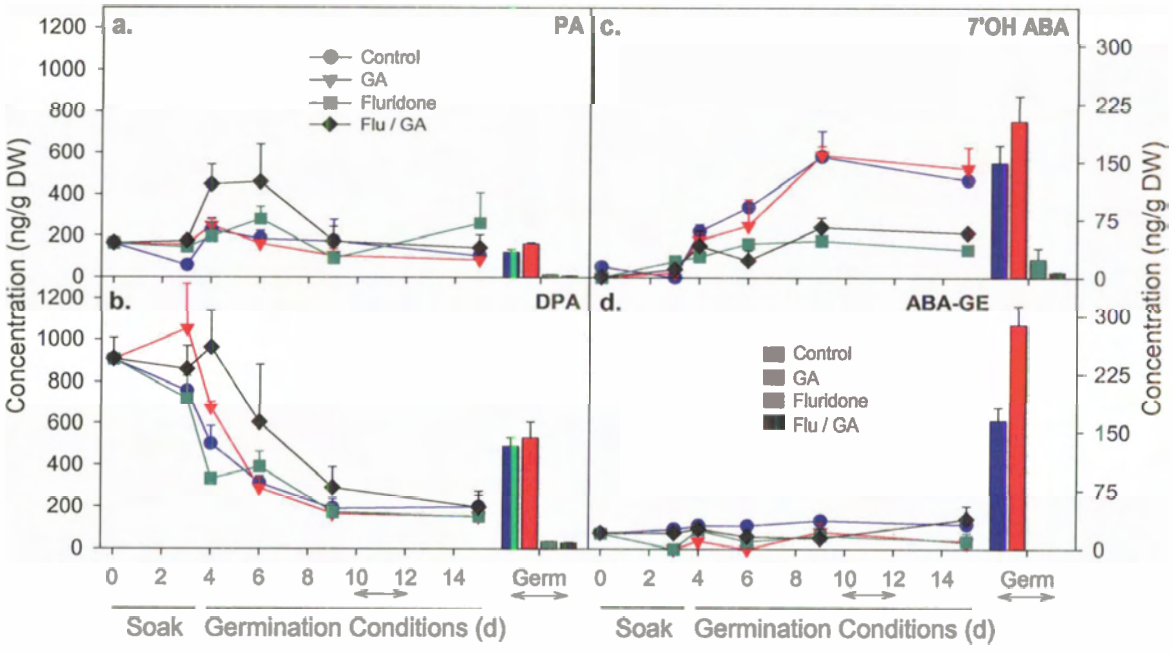


Fig. 5.3. PA (**a**), DPA (**b**), 7'OH ABA (**c**), and ABA-GE (**d**) changes within the embryo during fluridone/GA treatments. Ungerminated seeds were analysed before and after a 3 d soak in 100 µM fluridone and/or 50 µM GA and throughout 12 d in germination conditions (15 d total treatment time). Germinated seeds (Germ) were collected between 7-9 d in germination conditions and are denoted by the double-arrowed line. Data are expressed on a ng per g DW basis and are based on 3 replicate extractions of 20 embryos each (±SE). See Fig. 5.1 for germination details.

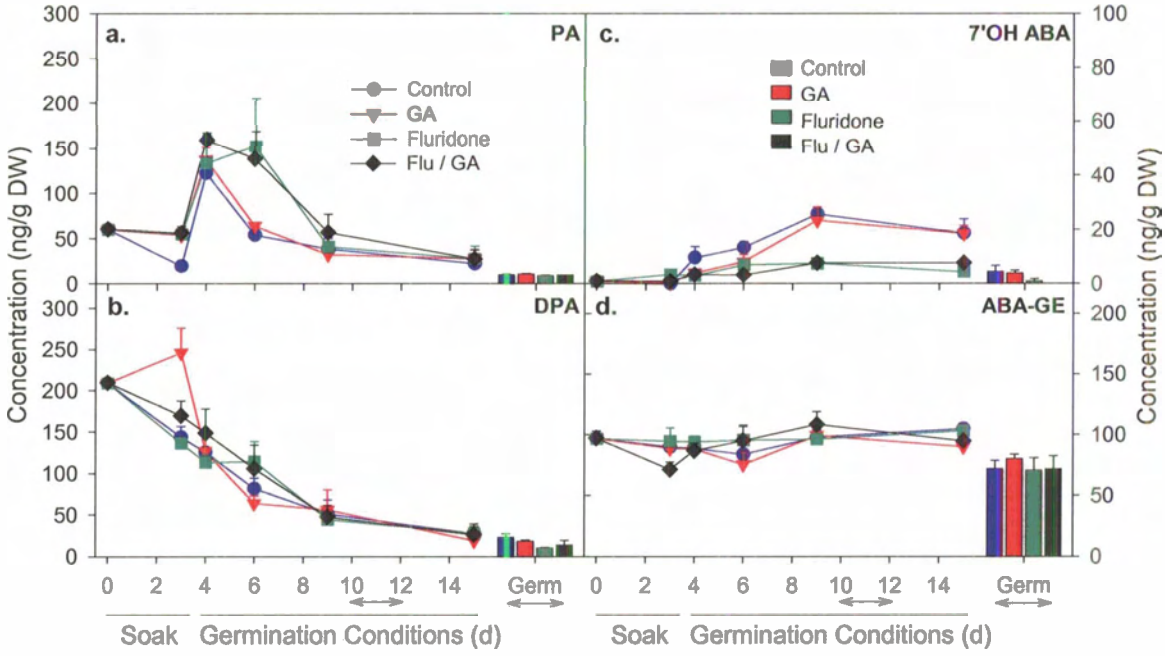


Fig. 5.4. PA (**a**), DPA (**b**), 7'OH ABA (**c**), and ABA-GE (**d**) changes within the megagametophyte during fluridone/GA treatments. Ungerminated seeds were analysed before and after a 3 d soak in 100 μM fluridone and/or 50 μM GA and throughout 12 d in germination conditions (15 d total treatment time). Germinated seeds (Germ) were collected between 7-9 d in germination conditions and are denoted by the double-arrowed line. Data are expressed on a ng per g DW basis and are based on 3 replicate extractions of 15 megagametophytes each (±SE). See Fig. 5.1 for germination details.

This is especially evident in the fluridone/GA treatment where the largest decrease in, or catabolism of, ABA was seen.

Hydroxylation of ABA at the 7'OH position was an important catabolic pathway in dormant imbibed seed. In the embryo, as ABA levels increased, when seed populations were maintaining dormancy during the latter stages in germination conditions (between 6 and 12 d in germination conditions), 7'OH-ABA also increased. These increases occurred to a greater extent in the control and GA-treated samples, whose ABA levels were higher, but slight increases were also seen in the fluridone and fluridone/GA samples (Fig. 5.3c). Similar trends were seen in the megagametophyte tissues except the levels of 7'OH-ABA were approx. 7-fold lower (Fig. 5.4c). During moist-chilling, 7'OH-ABA increases during the latter half of the treatment and peaks in embryos upon transfer of the seeds to germination conditions; this pathway is minimal in the megagametophyte of moist-chilled seed (Feurtado et al., 2004).

The increases of 7'OH-ABA in tissues actively biosynthesizing ABA and maintaining higher ABA levels (i.e. control-, GA-treated-, and germinated-seeds) suggest that ABA accumulation may activate its own catabolism, thereby providing a means to self-regulate ABA levels. Overexpression of the 9-*cis*-epoxycarotenoid dioxygenase (NCED) gene in tobacco, either constitutively or in an inducible manner, not only resulted in increased ABA levels but also elicited an increase in PA, but interestingly not the metabolite DPA (Qin and Zeevaart 2002). Also, the recently-identified ABA 8'-hydoxylase gene family in *Arabidopsis* (P450 CYP707A1 to 4), is induced by ABA in 2-week-old plants cultured on 1 μM (+)-ABA for 6 h (Saito et al., 2004). Several other genes involved in ABA biosynthesis and catabolism are also induced by ABA, often under certain developmental conditions or in selected species / ecotypes / mutants (reviewed in Xiong and Zhu 2003). Thus, the presumption that 7'-hydroxylation may be a pathway that is activated to help regulate ABA homeostasis in dormant imbibed seeds of western white pine is intriguing and awaits further investigation (i.e. cloning of the genes involved and measurement of their expression).

The 9'-hydroxylation pathway (Zhou et al., 2004) as measured by neo-PA formation was not an apparent pathway for metabolism in western white pine seeds and was present at very low or undetectable amounts at all timepoints (not shown).

5.3.4 Conjugation of ABA to glucose

Conjugation of ABA to glucose, through formation of the glucose ester, was not an apparent route of metabolism during dormancy termination. ABA-GE was present at very low levels in embryos of ungerminated seeds regardless of whether fluridone or GA was used (Fig. 5.3d). This contrasts what is observed in embryos during moist-chilled-induced dormancy termination of seeds, in which there is a slight accumulation of ABA-GE during moist-chilling before a decrease during germination (Feurtado et al., 2004). ABA-GE in the megagameophytes was present at constitutive levels and remained unchanged even in megagametophytes from germinated seeds (Fig. 5.4d). Identical findings were found in megagametophytes that had been subjected to moist-chilling as the dormancy-breaking treatment (Feurtado et al., 2004). ABA-GE did, however, increase significantly in embryos of germinated seeds in which ABA biosynthesis was not inhibited (Fig. 5.3d, Control and GA; Feurtado et al., 2004). Thus, it seems the increased ABA generated through *de novo* synthesis following germination is catabolized to ABA-GE (although ABA is also catabolized via 8'- and 7'-hydroxylation). It has been suggested that a proportion of the ABA-GE may provide an inactive but accessible pool of hydrolysable ABA available for seedling growth and stress response (Chiwocha et al 2005; see Chapter 4, section 4.4.2, for further discussion).

In conclusion, the dormancy mechanism of western white pine has been further investigated, in particular, the contribution of ABA. From previous work, we have shown that ABA homeostasis (the balance of biosynthesis to catabolism) undergoes at least three distinct changes during the transition from dormancy to germination and seedling growth in western white pine seeds (Feurtado et al., 2004). Here, disrupting ABA homeostasis through the use of fluridone and GA shows that there are distinct phase changes in ABA homeostasis even during incubation of dormant-imbibed seeds. Seeds initially display a catabolic state and more ABA is degraded than produced; as seeds progress ABA homeostasis slowly changes to favour ABA biosynthesis, presumably helping to maintain a dormant state. Future work should concentrate on: (i) determining the transport and leaching of ABA and metabolites (ii) determining how key control genes such as NCED and ABA 8'-hydroxylase (*CYP707A*) change in response to different dormancy breaking

procedures as well as in the dormant imbibed seed and (iii) how endogenous GA may contribute to dormancy termination.

CHAPTER 6

Expression of zeaxthanin epoxidase, 9-*cis* epoxycarotenoid dioxygenase, and abscisic acid 8'-hydroxylase genes suggests a fine balance is needed to maintain abscisic acid equilibrium during dormancy termination in western white pine (*Pinus monticola* Dougl. Ex D. Don) seeds

6.1 Introduction

The sesquiterpene phytohormone abscisic acid (ABA) plays a key role in plant responses to environmental stresses such as drought, cold, and salinity and in developmental processes such as embryo maturation, seed dormancy, and germination (Finkelstein and Rock, 2002; Nambara and Marion-Poll, 2005). As such, mutants deficient in ABA (ABA auxotrophs) from various plant species are altered in their response to abiotic stress and degree of seed dormancy (i.e. display wilty phenotypes or reduced dormancy) and, in part, helped reveal that ABA content is a key determinant of these physiological processes (Iuchi et al., 2001; Schwartz et al., 2003a; Nambara and Marion-Poll, 2005, references therein). In seeds, ABA levels usually peak during mid-development and regulate key events during seed formation such as the deposition of storage reserves, prevention of vivipary, acquisition of desiccation tolerance, and induction of primary dormancy (Phillips et al., 1997; Kermode, 2004; see Chapter 1, section 1.3). Moreover, in the mature imbibed seed, *de novo* ABA synthesis is often needed to maintain a dormant state (Grappin et al., 2000; Gubler et al., 2005; see Chapters 1, 5).

As a sesquiterpene, ABA is synthesized *indirectly* via the tetraterpene C_{40} carotenoids, in turn, carotenoids, and thus ABA itself, are formed from the methylerythritol 4-phosphate (MEP) pathway in plastids (reviewed in Finkelstein and Rock, 2002; Taylor et al., 2005). Although not solely committed to ABA biosynthesis, mutants impaired in the epoxidation of zeaxanthin were first discovered through their ABA-deficient phenotype (Marin et al., 1996). As such, zeaxanthin is usually considered a general starting point in the ABA biosynthetic pathway (Schwartz et al., 2003a). It is within plastids, that zeaxanthin proceeds to antheraxanthin, violaxanthin, neoxanthin, and finally xanthoxin. Although other minor synthesis routes have been suggested, xanthoxin is chiefly converted to ABA in the cytosol by two enzymatic steps via abscisic aldehyde (Cutler and Krochko, 1999; Nambara and Marion-Poll, 2005). Just as they helped in defining a role for ABA in stress and developmental processes, ABA-biosynthetic mutants have been invaluable tools for dissection of the biosynthetic pathway. In fact, most of the genes in the ABA biosynthetic pathway have been isolated through identification and characterization of ABA deficient mutants (Nambara and Marion-Poll,

2005). Further, molecular studies to elucidate the regulatory aspects of ABA biosynthesis are now possible and several have begun to reveal the complexity of the process (Seo and Koshiba, 2002; Xiong and Zhu, 2003).

Epoxidation of zeaxanthin to all-*trans*-violaxanthin is accomplished by the enzyme zeaxanthin epoxidase (ZEP; EC 1.14.13.90) (Bouvier et al., 1996). The *ABA2* gene, encoding ZEP, from *Nicotiana plumbaginifolia* marked the first ABA biosynthetic gene to be cloned (Marin et al., 1996). Epoxidation of zeaxanthin is not considered a rate-determining step for ABA synthesis in photosynthetic tissues but it is in non-chloroplast containing tissues such as seeds (Schwartz et al., 2003a). However, the first committed step to ABA biosynthesis, and thus deemed rate-determining, is the oxidative cleavage of 9-*cis*-violaxanthin and 9'-*cis*-neoxanthin, accomplished by the enzyme 9-*cis*-epoxycarotenoid dioxygenase (NCED; EC 1.13.11.51) (Schwartz et al., 2003b). The first *NCED* gene to be identified was from the *vp14* mutant of maize (*Zea mays*) (Tan et al., 1997). Subsequent identification of *NCED* in *Arabidopsis* has revealed a gene family of 9 members, 5 of which are presumed to be involved in ABA biosynthesis (Iuchi et al., 2001; Tan et al., 2003). Analyses of *NCED* family member expression and of *Atnced* mutants have revealed the plasticity present within plants (with respect to ABA synthesis) as genes show developmental and tissue specificity and overlap their functions to create functional redundancies (Tan et al., 2003; Lefebvre et al., 2006). For example, it is only when double *Atnced6 Atnced9* mutants are produced that reduced seed dormancy is observed (Lefebvre et al., 2006). The conversion of xanthoxin to abscisic aldehyde is accomplished by a short-chain dehydrogenase / reductase (SDR) family enzyme (AB-SDR, EC 1.1.1.288). AB-SDR was identified as the *Arabidopsis aba2* locus and represents the only species in which a gene encoding an enzyme with xanthoxin dehydrogenase activity has been identified (Cheng et al., 2002; González-Guzmán et al., 2002). *Ataba2* allelic mutants, such as *gin1* and *sre1*, showed that ABA biosynthesis is a prerequisite for both sugar- and salt-sensitivities during germination and seedling growth (Cheng et al., 2002; González-Guzmán et al., 2002; Chen et al., 2006). The last step in the ABA biosynthetic pathway, converting abscisic aldehyde to ABA, is accomplished by abscisic aldehyde oxidase (AB-AO, EC 1.2.3.14). Deficiency of AB-AO activity results from either mutations in the apoenzyme itself (tomato *sitiens* and *aao3* of *Arabidopsis*) or

in a molybdenum co-factor required for its activity (tomato *flacca* and *aba3* of *Arabidopsis*) (Schwartz et al., 2003a; Taylor et al., 2005). Similar to NCED, AB-AO are multi-gene families in *Arabidopsis* and tomato (*Lycopersicon esculentum*); however, are not strictly devoted to ABA biosynthesis and family members also participate in, for example, the synthesis of indole-3-acetic acid (e.g. *AAO1* in *Arabidopsis*) (Seo et al., 1998; Akaba et al., 1999; Min et al., 2000; Seo et al., 2000a). Characterization of an *aao3* mutant revealed an ABA-deficient wilty phenotype in leaves and subsequent identification of the salt-resistant *sre2* locus as allelic mutants of *aao3* revealed a role for ABA biosynthesis in seeds (Seo *et al.*, 2000b; González-Guzmán et al., 2004).

ABA can be catabolised or inactivated either through oxidation or conjugation at various positions in the molecule and the preference for one pathway versus another is dependent on the plant species, tissue, and developmental timing (Cutler and Krochko, 1999; Zeevaart, 1999). However, the major pathway by which ABA is catabolized is through hydroxylation at the 8' position to form 8'-hydroxy ABA, which reversibly cyclizes to phaseic acid (PA). Further reduction of PA can take place at the 4' position to form dihydrophaseic acid (DPA). ABA and ABA metabolites (PA and DPA) can also become conjugated with glucose forming an ester (PA) or an ester or glucoside (ABA and DPA). Other minor pathways include formation of 7'-hydroxy ABA (7'OH ABA) and ABA 1',4' diols (Cutler and Krochko 1999; Zeevaart 1999). A new metabolite, 9'-hydroxy ABA and its cyclized form neo-PA were discovered in *Brassica napus* siliques (Zhou et al. 2004).

The ABA 8'-hydroxylase enzyme, which converts ABA to 8'OH ABA, has been characterized as an integral membrane protein with properties of a cytochrome (CYP) P450 monooxygenase (Krochko et al., 1998). Recently, the *CYP707A* subfamily in *Arabidopsis* was identified as the ABA 8'-hydroxylase gene family (Kushiro et al., 2004; Saito et al., 2004). Saito et al. (2004) and Kushiro et al. (2004) showed that the CYP707A subfamily (CYP707A1 to A4; EC 1.14.13.93) converts ABA to PA *in vitro*, but not to other hydroxylated catabolites such as 7'OH ABA or 9'OH ABA. Analogous to NCED, analysis of *CYP707A* expression and mutants have revealed the subfamily can act in concert to create functional redundancy (e.g. under rehydration after drought treatment) and also in isolation (e.g. during release of seed dormancy) to help maintain ABA

homeostasis. For example, while *cyp707a3* mutants germinate to a greater extent than Columbia wild-type (wt), *cyp707a2* mutants display a significant degree of dormancy (which may be linked to the higher ABA levels observed in mature seeds of *cyp707a2* compared to wt) (Kushiro et al., 2004). Two ABA glycosyltransferases (ABA GT) have been identified in *Arabidopsis* and Azuki bean (*Vigna angularis*); both form the glucose ester of ABA in *in vitro* assays, although the latter glycosylates *trans*-ABA better than the natural enantiomer (+)-ABA (Xu et al. 2002; Lim et al. 2005). The ABA GT from Azuki bean gene is ABA-inducible and also increases in response to drought and wounding in hypocotyls but not in leaves (Xu et al., 2002).

As evidence continues to build, it is becoming evident that concentrations of 'bioactive ABA' within a tissue or cell are dynamically maintained through fine-tuning of the rates of biosynthesis, catabolism and transport. Further, ABA itself seems to actively control its own 'pool-size' through feed-back and feed-forward mechanisms of regulation on synthesis and catabolism genes, respectively (Nambara and Marion-Poll, 2005). The ABA biosynthetic pathway has been dissected to a large extent. However, the same level of detail has not been obtained with regard to the degradation of ABA. In addition, the movement and transport of ABA is even less well understood (see Davies et al., 2005, for review). However, the recent identification of the ABA 8'-hydroxylase gene family in *Arabidopsis* and two glycosyltransferases that are able to glucosylate ABA has furthered our knowledge of ABA catabolism tremendously and has opened up avenues to study the key relationship between ABA synthesis and catabolism. One of the more important aspects that deserves further attention is how ABA levels are regulated and further information on ABA homeostasis may be gained through concurrent analysis of both ABA biosynthetic and catabolic gene expression.

Recently, the dormancy mechanism in western white pine (*Pinus monticola*) seed was linked to abscisic acid (ABA) and the ability of seed tissues to catabolise ABA (Chapter 3; Feurtado et al., 2004). Thus in order to further understand the role of ABA biosynthesis and catabolism during seed dormancy release in western white pine, we (1) cloned the genes zeaxthanin epoxidase (*ZEP*), 9-*cis* epoxycarotenoid dioxygenase (*NCED*), and abscisic acid 8'-hydroxylase (*CYP707A*) during various stages of dormancy maintenance and germination to screen for different paralogous genes expressed and (2)

monitored expression of these genes during dormancy termination (moist chilling) and germination.

6.2 Materials and Methods

6.2.1 Seed material and dormancy-breaking treatments

Mature seeds of western white pine seed lot 08006 were obtained from the British Columbia Ministry of Forests, Tree Seed Center in Surrey, BC, Canada. Seed lot 08006 was collected in 1981 from an elevation of 909 m at 51°31' latitude and a longitude of 119°12'. Seeds were stored at -20°C before use.

For RNA extraction timepoints, both for cloning work and expression analysis, dry seeds were subjected to a running water soak for 3 d (at approx. 20°C) before transfer to a 25°C 10 L ddH_2O bath (5 L of water was exchanged daily) for 10 d. Before transfer to moist chilling conditions, seeds were sterilized in 3% H_2O_2 for 25 min and rinsed with sterile water several times. After sterilization, seeds were surface dried until seed surface-moisture disappeared. Seeds were then placed on Whatman 3MM paper supported by 15-ply K-22 germination paper (Seedburo Equipment Company, Chicago, Illinois, USA) that had been moistened with approx. 25 mL ddH_2O in a clear plastic seed box (11.2 x 11.2 x 3.6 cm, Hoffman Manufacturing Co., Albany, Oregon, USA). Following 98 d of moist chilling at 3 ± 2°C in the dark, seeds were transferred to germination conditions (23°C, 16 h photoperiod, and light intensity approx. 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$). After a brief rinse with water, seeds were maintained in the same plastic seed box except that 50 mL de-ionized distilled water was present. For some extractions, seeds were subjected to only the initial water soak (e.g. a 3 d soak or a 3 d + 10 d soak), or to a partial dormancy-breaking treatment (13 d soak, plus 30-60 d of moist chilling), prior to their transfer to germination conditions. Other treatments included a 3 d water soak 25°C and 3 d imbibition under germination conditions, a 12 d water soak 25°C and 12 d imbibition under germination conditions, a 3 d soak 20°C, followed by removal of the hard seed coat, and transfer and incubation on 1% agar at germination conditions for 18 h (see Section 4.2.4 for details on the sterilization protocol used).

A drought stress was also imposed on 7 d-old seedlings. Seedlings were allowed to loose 15% fresh weight before transfer to a sealed plastic bag for approx. 6 h. Following

the 6 h drought stress, a rehydration treatment was also performed where drought-stressed seedlings were allowed to recover water in a germination box (see above) for approx. 6 h.

Following treatments, seeds were either dissected into separate embryo and megagametophyte samples (e.g. for qPCR expression analysis), had the hard outer seed coat removed (e.g. most timepoints used for cloning work), or were used intact (e.g. drought and rehydration seedling treatments, and dry seeds) before being frozen in dry ice or liquid N_2. Tissues were stored at -80°C before use in RNA extractions.

6.2.2 RNA extraction

6.2.2.1 Whole seeds and seed parts

Extraction from whole dry seeds used approx. 50 seeds (0.9 g FW); while, for seeds with the hard seed coat removed, approx. 40-50 seeds were used for RNA extraction. For extraction from isolated megagametophytes approx. 325 mg or 25 megagametophytes were used. Tissues were ground to a fine powder in liquid nitrogen and then transferred to a 15 mL Falcon tube containing 0.5 g polyvinylpolypyrrolidone (PVPP, Sigma) and 4 mL RLT buffer from the RNeasy Midi Kit (Qiagen) and 80 µL β-mercaptoethanol (20 µL/mL; BioRad). PVPP stock was made by hydrating with DEPC-treated water and autoclaving; 4 mL volume was added to each Falcon tube and excess water was removed by centrifugation; the final volume of PVPP was approx. 2 mL after removal of excess water. After addition of powdered tissue, the tube was vortexed vigorously for 5 min. Further extraction occurred with addition of 2 mL $CHCl_3$: isoamyl alcohol (24:1) and vortexing for 2 min. The samples were then centrifuged at 5000*g* for 20 min. The aqueous supernatant was then transferred to a new 15 mL Falcon tube with 4 mL (1 volume) of phenol: $CHCl_3$: isoamyl alcohol (25:24:1, pH 8.0) (Sigma) already added. The tube was inverted gently several times until a milky consistency was observed. The solution was then centrifuged in a 15 mL Corex tube at approx. 11000*g* for 15 min. The aqueous phase was transferred to a 15 mL Falcon tube and 1/10 volume of 2 M sodium acetate (pH 4.8) and 1 volume of isopropanol were added. After inverting the tube to mix, the solution was precipitated at -80°C for 1 h, and then thawed at room temperature until liquid. The tube was then centrifuged at 5700*g* for 15 min at 4°C and the supernatant

discarded. The pellet was washed with 2 mL of 75% ethanol and centrifuged at 6500*g* for 10 min at 4°C. Once the pellet was dissolved in 0.5 mL DEPC-treated water, 1 volume of 4 M lithium acetate (pH 4.8, Sigma) was added. After an overnight precipitation at 4°C, the pellets were collected by centrifugation at 6500*g* for 15 min at 4°C. The pellets were washed briefly with 1 mL 75% ethanol and centrifuged at 6500*g* for 10 min at 4°C. Pellets were dissolved in 0.5 mL DEPC-treated water before addition of 2 mL RLT Buffer and 1.4 mL 100% ethanol.

RNA was then purified through RNeasy midi columns following the manufacturer's instructions (Qiagen). The RNA solution was poured into a midi column and the flow-through discarded after centrifugation at 1200*g* for 3 min. Two mL RW1 solution was added and then centrifuged at 1200 *g* for 2 min, then at 3700*g* for 3 min, and the flow-through discarded. An on-column DNase treatment was performed to eliminate contaminating genomic DNA; 20 µL of DNase and 140 µL RDD Buffer (RNase-Free DNase Set, Qiagen) was pipetted onto the column membrane. After 15 min at room temperature, 2 mL RW1 was added for 5 min and the flow-through discarded after a 5 min centrifugation at 1200*g*. To wash further, 2.5 mL of RPE solution was added and centrifuged at 1200*g* for 2 min, and the flow-through discarded. An additional 2.5 mL RPE wash followed; after centrifugation at 1200*g* for 2 min, and then at 3700*g* for 5 min to totally remove any traces of ethanol, the flow-through was discarded. To elute the RNA, the column was transferred to a 15 mL collection tube (from RNeasy Kit) and 150 µL of RNase-free water was added to the column membrane and stood for 10 min at room temperature. The eluate was collected by centrifuging at 1200*g* for 3 min. The elution was repeated with 100 µL of RNase-free water and a final centrifugation at 3700*g* for 3 min was performed. For some samples with lower amounts of RNA, noted by the size of the pellet before column purification, smaller elution volumes were used. RNA was quantified by measuring absorbance at 260 nm and quality was checked using A_{260}/A_{280} ratios and formaldehyde agarose gel electrophoresis.

6.2.2.2 Isolated embryos

Twenty-Five embryos (approx. 50 mg FW) were ground in liquid nitrogen to a fine powder. For 6 d germinated embryos, approx. half the material was used (i.e. 12 embryos

or approx. 100 mg FW). The procedure for whole seeds and seed parts, using RNeasy midi columns (section 6.2.2.1 above), was modified to accommodate smaller tissue amounts and purification using the RNeasy Mini Kit (Qiagen). The powder was transferred to a 2 mL microcentrifuge tube containing approx. 50 mg PVPP (0.5mL volume in solution, hydrated with DEPC-treated water, 0.2 mL final volume of PVPP after excess water removal), 700 µL RLT buffer from the RNeasy Mini kit (Qiagen) and 14 µL β-mercaptoethanol. The tube was then periodically vortexed vigorously for 5 min. After addition of 350 µL of $CHCl_3$: isoamyl alcohol (24:1), the tubes were vortexed for 2 min and centrifuged at 16000*g* for 10 min. The supernatants were transferred to new 2 mL micocentrifuge tubes with 700 µL (1 volume) of phenol : $CHCl_3$: isoamyl alcohol (25:24:1, pH 8.0) already added. The tubes were inverted several times to mix (gentle mixing here or layers will not separate after centrifugation) and centrifuged at 16000*g* for 10 min. The aqueous phase supernatant was transferred into a new 2 mL tube and 1/10 volume of 2 M sodium acetate (pH 4.8) and 1 volume of isopropanol were added before flicking the tube to mix. The solution was precipitated at -80°C for 1 h and thawed at room temperature until liquid. Following centrifugation at 16000*g* for 10 min at 4°C, the pellet was washed with 0.7 mL 75% ethanol by vortexing briefly. The solution was then spun at 16000*g* for 10 min at 4°C. The pellet was resuspended in 100 µL of DEPC-treated water to dissolve and 1 volume of 4 M lithium acetate (pH 4.8) was added to precipitate the RNA. After an overnight incubation at 4°C, the RNA was collected by centrifugation at 16000*g* for 10 min at 4°C and the supernatant discarded. The pellet was again washed with 0.5 mL 75% ethanol and centrifuged at 16000*g* for 7 min at 4°C. To dissolve the RNA, 100 µL of DEPC-treated water was added and the tube flicked gently. Three-hundred and fifty µL RLT buffer (from RNeasy kit) and 250 µL of 100% ethanol were added and the contents mixed thoroughly by flicking.

RNA was then further purified using RNeasy mini columns (Qiagen) according to the manufacturer's instructions. The solutions were loaded into RNeasy mini columns with 2 mL collection tubes, centrifuged at 16000*g* for 15 sec, and the flow-through discarded. The columns were washed with 350 µL RW1 solution and centrifuged for 15 sec at 16000*g* (discard flow-through). On-column DNase digestion was preformed to remove unwanted genomic DNA; 10 µL of DNase and 70 µL RDD Buffer was pipetted onto the

column membrane. After 15 min at room temperature, 350 μL RW1 was added to the column, the tubes centrifuged for 15 sec at 16000*g*, and the flow-through and 2 mL collection tube discarded. An ethanol wash, 500 μL of RPE solution, was applied, the tubes centrifuged at 16000*g* for 15 sec, and the flow-through discarded. A second 500 μL RPE wash was performed before centrifugation at 16000*g* for 2 min. To complete remove all traces of ethanol, a second centrifugation step was performed for 1 min in a new collection tube. RNA was eluted by transferring the column to a new 2 mL collection tube (from RNeasy Kit), adding 50 μL of RNase-free water to the column membrane, letting it stand for 10 min at room temperature, and centrifuging at 16000*g* for 1 min to collect flow-through. The elution was repeated with 50 μL of RNase-free water but only letting it stand for 5 min at room temperature. For some samples with lower amounts of RNA, noted by the size of the pellet before column purification, smaller elution volumes were used. RNA was quantified by measuring absorbance at 260 nm and quality was checked using A_{260}/A_{280} ratios and formaldehyde agarose gel electrophoresis.

6.2.3 cDNA synthesis for RT-PCR

First strand cDNA was synthesized using the Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. Each component was mixed and centrifuged briefly before use. Two μg of total RNA was reverse transcribed using oligo$(dT)_{20}$ as a primer. The RNA (+ DEPC-water totaling 8 μL), 1 μL 50 μM primer, and 1 μL 10 mM dNTPs were incubated at 65 °C for 5 min to denature and then placed on ice for at least 1 min prior to adding the RT enzyme mix (10 μL + 10 μL = 20 μL total reaction volume). RT enzyme mix consisted of 1 μL (200 U) Superscript™ III RT enzyme, 1 μL (40 U) RNaseOUT™, 2 μL 0.1 M DTT, 4 μL 25 mM $MgCl_2$, and 2 μL 10X RT buffer. The cDNA synthesis reactions were incubated at 50°C for 50 min and the reaction terminated at 85°C for 5 min before chilling on ice. To remove unwanted RNA templates, 1 μL (2U) RNase H treatment was performed for 20 min at 37°C. Reactions were stored at –20°C until use. cDNA reactions were diluted 1/5 for use in PCR amplification; of this 1/5 dilution 1.5-6 μL was used in PCR reactions (sections 6.2.4 and 6.2.6).

6.2.4 RT-PCR to produce amplicons with sequence homology to *CYP707A*, *NCED*, and *ZEP*

A degenerate primer approach was used to clone cDNA fragments with homology to *CYP707A*, *NCED*, and *ZEP* from western white pine seeds during various stages of dormancy termination and germination. Degenerate primer design for *ZEP* and *NCED* was based on (Chernys and Zeevaart, 2000; Römer et al., 2002) and amino acid alignment of 15 ZEP and 19 NCED sequences in GenBank. Degenerate primer design for cloning of *CYP707A* was based on amino acid alignment of 9 sequences including spruce and loblolly pine ESTs (CO217842 and CF388492). Since *ZEP* is not generally a gene family, only cDNA produced from one tissue, dormant imbibed seeds, was used for cloning. However, since *NCED* and *CYP707A* are present as multi-gene families (Tan et al., 2003; Kushiro et al., 2004; Saito et al., 2004) multiple tissues were used for cloning (Table 6.1).

PCR reactions were optimized for quantity and specificity by adjusting the annealing temperature and concentration of primers. Table 6.2 lists the degenerate primers used. For *CYP707A* cloning, a proof-reading enzyme, ProofStart® DNA Polymerase (Qiagen), was used. The standard parameters used for PCR cycling for ProofStart® were: 95°C for 5 min, 40 cycles of 94°C for 30 sec, 52°C for 1 min, and 72°C for 1.33 min, and 72°C for 10 min. For cDNA timepoints that had very low expression of *CYP707A*-like sequences (e.g. dry seed), 50 PCR cycles were used sometimes in combination with a second round of PCR. Following the first round of PCR, PCR products corresponding to the correct size were agarose gel purified using the MinElute Kit (Qiagen) prior to the second PCR reaction (see section 6.2.5). The dNTP concentration used was 300 μM for each dNTP and the degenerate primer concentration was 2 μM for each primer in a 50 μL reaction with 2.5 U of ProofStart®.

For *NCED* and *ZEP* cloning, *Taq* Polymerase (Qiagen) was used since substrate specificity and product quantity were improved. The PCR parameters for *Taq* were: 94°C for 4 min, 40 cycles of 94°C for 30 sec, 52°C (ZEP) or 55°C (NCED) for 1 min, and 72°C for 1 min, and 72°C for 10 min. ZEP amplification used a PCR additive, Q-Solution (Qiagen), to increase product yield and specificity. Similar to *CYP707A*, *NCED* cloning of cDNA timepoints with low expression was accomplished by increasing the PCR cycle

Table 6.1. Western white pine timepoints for cDNA production and screening for cDNA clones with homology to *CYP707A*, *NCED*, and *ZEP* sequences. Dash (-) indicates tissue not checked, sequence sizes in base pairs (bp) are shown along with tentative sequence names.

Stage	Timepoint	CYP707A		NCED		ZEP	
		# Clones Sequenced	Products Observed	# Clones Sequenced	Products Observed	# Clones Sequenced	Products Observed
Dormant	Dry Seed	3	994 bp, *PmCYP707A1p*	3	742 bp, *PmNCED1*	-	-
	3d soak + 3d Imbibe	-	-	6	*PmNCED1*	3	671 bp *PmZEP*
	12d soak + 12d Imbibe	-	-	6	*PmNCED1*	3	*PmZEP*
Dormancy Breakage	41d Moist Chilling	2	*PmCYP707A1p*	-	-	-	-
	50 d Moist Chilling	2	*PmCYP707A1p*	3	*PmNCED1*	-	-
Germination	1d Germination Conditions	3	*PmCYP707A1p*	3	*PmNCED1*	-	-
	18 h Intact Mega (-seed coat)	7	*PmCYP707A1p*	-	-	-	-
Stress and Recovery	6h Drought 7d old seedling	-	-	3	733 bp, *PmNCED2*	-	-
	6h Drought 6h Rehydration 7d old seedling	7	*PmCYP707A1p*; 997 bp, *PmCYP707A2p*	-	-	-	-

number to 50 and/or a second round of PCR. The dNTP concentration used was 200 μM for each dNTP and the degenerate primer concentration was 1 μM for each primer in a 50 μL reaction with 2.5-3.5 U of *Taq*. The cDNA timepoints that were screened, including the number of clones sequenced and products obtained are shown in Table 6.1.

Table 6.2. Degenerate primers used to clone CYP707A-, NCED-, and ZEP-like sequences from western white pine. CYP_5'_4 and CYP_5'_5 were used to reduce overall degeneracy of the 5' primer but only CYP_5'_5 yielded PCR products of the correct size.

Sequence	Name	Amino acid sequence deduced from
5' TAGGNTGYCCNTGYGTRATG 3'	CYP_5'_4	LGCPCVM
5' TAGGNTGYCCNTGYGTYATG 3'	CYP_5'_5	LGCPCVM
5' GGCATGAANGTRTTNGGYTT 3'	CYP_3'_6	KPNTFMP
5' TYGAYGGNGAYGGNATGGT 3'	NCED_1_5'	FDGDGMV
5' GCGTTCCAGAGATGRAARCARAARCA 3'	NCED_3_3'	CFCFHLWNA
5' GGNAARATGCARTGGTAYG 3'	ZEP_1_5'	GKMQWYA
5' GGCATGAANGTRTTNGGYTT 3'	ZEP_2_3'	WFEDDDA

6.2.5 Cloning PCR products with homology to CYP707A, NCED, and ZEP

PCR products were purified using agarose gel electrophoresis (1.2% gels for *CYP707A* and 1.7% for *NCED* and *ZEP*) and the MinElute Gel Extraction kit (Qiagen) according to the manufacturer's instructions. All centrifugation steps were carried out at 16000*g* in a microcentrifuge. Approx. 100-150 mg agarose gel slices containing the desired PCR product were excised and placed into 1.5 mL microcentrifuge tubes. Three volumes of Buffer QG were added and the gel slices melted by incubation at 50°C for 10 min (tubes were vortexed every 2-3 minutes during incubation). One volume of isopropanol was added and the tubes mixed by inverting several times. Samples were applied to MinElute columns, centrifuged for 1 min, and the flow-through discarded. Five hundred μL of Buffer QG was applied as a wash and the columns were centrifuged for 1 min before discarding the flow-through. A wash with 750 μL of Buffer PE and incubation for 4 min occurred before centrifuging for 1 min and discarding the flow-

through. Residual ethanol was removed by an additional centrifuge of 1 min. DNA was eluted with 10 μL of Buffer EB (10 mM Tris-Cl, pH 8.5). Buffer stood on the column for at least 1 min before centrifugation for 1 min. Purified PCR products were either used directly in a TOPO® cloning reaction or stored at -20°C.

Cloning reactions used the TOPO-TA Cloning® kit (Invitrogen) using the pCR®2.1-TOPO® vector with DH5α™-T1^R competent cells according to the manufacturer's recommendations. If a proof-reading polymerase was used then the 3'-end A-base overhang was added to the end of purified blunt-ended PCR products with the A-Addition Kit (Qiagen). Briefly, 8 μL (0.5-1 μg) of purified PCR product was mixed with 2 μL of 5x Qiagen A-Addition Master Mix prior to a 30 min incubation at 37°C.

The TOPO® cloning reaction was accomplished using 1-4 μL of purified PCR product from the MinElute Kit (Qiagen) or after the A-Addition reaction. Salt solution, sterile water, and TOPO® vector were added to the PCR product for a final volume of 6 μL. The TOPO® cloning reaction was mixed gently and incubated at room temperature for 5 to 20 minutes depending on the concentration of purified PCR product obtained. The TOPO® reaction was either used directly for transformation or stored at -20°C for 1-3 d.

For plasmid transformation, 2 μL of the TOPO® cloning reaction was added to 50 μL DH5α™-T1^R *Escherichia coli* competent cells that had been thawed on ice. The cells with added plasmid were gently mixed before incubation in ice for 30 min. After the cells were heat-shocked for 30 seconds in a 42°C water bath without shaking, they were immediately transferred to ice. After 3-4 minutes, 250 μL of SOC medium was added to the cells and the tube was shaken horizontally at 200 rpm at 37°C for 1 h. Ten to 50 μL of each transformation was spread onto prewarmed selective plates. To ensure even spreading of smaller volumes (20 μL and under), 20 μL SOC medium was added. Selective plates contained 50 μg/mL Kanamycin and 40 μL of 40 mg/mL of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) in dimethylformamide (DMF) for blue/white colony screening. Plates were allowed to dry before being inverted and grown at 37°C overnight (15-17 h).

Twelve to 24 white colonies were picked with a toothpick into 20 μL sterile water; 14 μL were pipetted into 2.5-3 mL LB medium (with 50 μg/mL Kanamycin) and 3 μL was used in a PCR reaction to check the insert of interest. PCR cycling parameters followed those described above (section 6.2.4). Plasmids were purified using the QIAprep® Spin Miniprep kit (Qiagen). The required proprietary buffers (e.g. Buffer P1) were used according to the kit instructions. All centrifugation steps were carried out at 16000*g* in a microcentrifuge. Bacterial cultures were centrifuged for 45 sec. The bacterial cells were resuspended in 250 μL of Buffer P1 containing RNase A. Two-hundred and fifty μL of Buffer P2 was added and the tubes gently inverted 4-6 times to mix. Next, 350 μL of Buffer N3 was added and the tubes were again gently inverted 4-6 times. The samples were centrifuged for 10 min. During the centrifugation, a QIAprep spin column was placed in a 2 mL collection tube and the supernatants obtained by centrifugation were applied to the QIAprep spin column by pipetting. The samples were centrifuged for 1 min and the flow-through discarded. The QIAprep spin column was washed by adding 500 μL of Buffer PB, centrifuging for 1 min, and discarding the flow-through. The column was again washed using 750 μL of Buffer PE and centrifuged for 1 min. The flow-through was again discarded and the QIAprep spin column was centrifuged for an additional 1 min to remove residual ethanol. The columns were then placed in clean 1.5 mL microfuge tubes. The DNA was eluted by adding 50 μL of Buffer EB (10 mM Tris-Cl, pH 8.5), letting stand for 1 min, and centrifuging for 1 min. Purified plasmids were stored at -20°C.

Selected plasmid clones containing inserts (Table 6.1) were sequenced in both the forward (T7 Forward Primer) and reverse (M13 Reverse Primer) directions to check for homology to *CYP707A*, *NCED*, or *ZEP* sequences in GenBank.

6.2.6 RACE-PCR to obtain the full length mRNA sequences for *CYP707As*

To facilitate further work with CYP707A, full-length cDNA sequences were obtained using RNA-Ligase Mediated RACE PCR using the GeneRacer™ Kit (Invitrogen). RNA from 7 d-old white pine seedlings that had undergone a 6 h dehydration / 6 h rehydration treatment (see section 6.2.1) were used to obtain the full length sequence of both *PmCYP707A1* and *PmCYP707A2*. RACE-ready cDNA was prepared as follows. The

5'end phosphate group on truncated and non-mRNA was removed from 4 μg of total RNA by dephosphorylating the RNA with calf-intestinal phosphatase (CIP) in a 10 μL reaction at 50°C for 1 h. The RNA was phenol:chloroform purified and precipitated with 10 mg/mL mussel glycogen, 3 M sodium acetate, and 95% ethanol. After 10 min on dry ice, the precipitated RNA was collected by centrifugation at 16000*g* for 20 minutes. The pellet was rinsed with 70% ethanol and centrifuged at 4°C for 2 min at 16000*g*. After air drying the pellet, the RNA was dissolved in 7 μL of DEPC-treated water. The mRNA was decapped at the 5'end by using tobacco acid pyrophosphatase (TAP) in a 1 h reaction at 37°C. The RNA was purified and precipitated as described previously. The GeneRacer™ RNA Oligo was ligated to the decapped mRNA using T4 RNA Ligase. Prior to addition of T4 RNA Ligase and incubation for 1 h at 37°C, RNA secondary structure was relaxed by incubation at 65°C for 5 min and chilling on ice for 2 min. The RNA was purified and precipitated a third time as previously except that the final dissolving volume was 10 μL. The ligated mRNA was reverse transcribed as described in section 6.2.3 except that the GeneRacer™ oligo dT primer was used and the reaction was carried out for 1 h at 50°C.

The proof-reading enzyme Platinum® Pfx DNA Polymerase (Invitrogen) was used to generate 5' and 3' RACE PCR products for *PmCYP707A1* and *PmCYP707A2*. PCR reactions contained 300 μM dNTPs, 1 mM $MgSO_4$, and 0.3-0.9 μM primers in a 50 μL volume with 1.25 U of Pfx enzyme. In some reactions the concentration of 10X Platinum® Pfx amplification buffer was doubled (i.e. 20X) to increase product yield. Primers used for RACE are detailed in Table 6.3.

i) To amplify the 5'end of *PmCYP707A1* touchdown PCR was used in combination with semi-nested PCR. PCR conditions for the first round of PCR were: 94°C for 2 min, 5 cycles of 94°C for 30 sec and 70°C for 1.5 min, 5 cycles of 94°C for 30 sec and 68°C for 1.5 min, and 25 cycles of 94°C for 30 sec, 65°C for 30 sec, and 68°C for 1.25 sec, before 10 min at 68°C. The first round of PCR used PCRx Enhancer Solution (Invitrogen) at 1X concentration for improved yield. One μL from the first round of PCR was used for the subsequent second

round of PCR. PCR cycling was: 94°C for 2 min, 22 cycles of 94°C for 30 sec, 65°C for 30 sec, and 68°C for 1.17 min, and 10 min at 68°C.

ii) For the 3'end of *PmCYP707A1*, one round of touchdown PCR was used and the reaction conditions were as follows: 94°C for 2 min, 5 cycles of 94°C for 30 sec and 70°C for 1:30 second, 5 cycles of 94°C for 30 sec, 65°C for 30 sec, and 68°C for 1.5 min, 25 cycles of 94°C for 30 sec, 60°C for 30 sec, and 68°C for 1.5 min, and 10 min at 68°C.

iii) For the 5'end of *PmCYP707A2*, touchdown PCR was used in combination with semi-nested PCR. The first round PCR cycling conditions were: 94°C for 2 min, 5 cycles of 94°C for 30 sec and 70°C for 1.5 min, 5 cycles of 94°C for 30 sec and 68°C for 1.5 min, 26 cycles of 94°C for 30 sec, 65°C for 30 sec, and 68°C for 45 sec, and 68°C for 10 min. The second round of PCR conditions were: 94°C for 2 min, 24 cycles of 94°C for 30 sec, 62°C for 30 sec, and 68°C for 40 sec, before 10 min at 68°C.

iv) The 3'end of *PmCYP707A2* was amplified using both touchdown PCR and nested PCR. PCR conditions for the first round were: 94°C for 2 min, 5 cycles of 94°C for 30 sec and 68°C for 1.75 min, 5 cycles of 94°C for 30 sec, 65°C for 30 sec, and 68°C for 1.5 min, 26 cycles of 94°C for 30 sec, 60°C for 30 sec, and 68°C for 1.5 min, followed by 10 min at 68°C. PCR cycling for the second round was: 94°C for 2 min, 23 cycles of 94°C for 30 sec, 60°C for 30 sec, 68°C for 1 min, and 68°C for 10 min.

6.2.7 Cloning *CYP707A* RACE fragments

Cloning of *CYP707A* RACE fragments was similar to cloning the degenerate primer PCR products (section 6.2.5). However, the Zero Blunt® TOPO® PCR Cloning Kit for Sequencing (Invitrogen), with the pCR®4Blunt-TOPO® vector and TOP10 Chemically Competent *E. coli*, was used according to the manufacturer's suggestions. In addition, since the pCR®4Blunt-TOPO® vector contains the ccdB gene, blue/white colony screening (i.e. addition of X-Gal to the selective plates) was not necessary.

Table 6.3. **(a)** RACE PCR primers used to amplify the 5' and 3' ends of PmCYP707A1 (CYP) and PmCYP707A2 (CYP2). GR2 and CYP_GR4 were not used in cloning reactions. **(b)** PCR primer pairs used to generate RACE products and the sizes of products that were generated.

(a) Sequence	Primer Name
5' CGACTGGAGCACGAGGACACTGA 3'	GeneRacer™ 5' (GR1)
5' GGACACTGACATGGACTGAAGGAGTA 3'	GeneRacer™ 5' Nested (GR2)
5' GCTGTCAACGATACGCTACGTAACG 3'	GeneRacer™ 3' (GR3)
5' CGCTACGTAACGGCATGACAGTG 3'	GeneRacer™ 3' Nested (GR4)
5' CTGTGAGCTTCCGTCCGTTGATTG 3'	CYP_GR1
5' GAGAACAGTGCCACTTGGAATGAAAGC 3'	CYP_GR2
5' GGAGGGCCAATAATGTGGTAAACAAT 3'	CYP_GR3
5' CACAGCTGAACAAGAATCCATTAGACAA 3'	CYP_GR4
5' AGCAGCCCCACCTCAAAAGCAT 3'	CYP2_GR1
5' CACAATTGACCGAATCTTGGCG 3'	CYP2_GR2
5' TTGCGGAAAGGAGATCTACCACTGAG 3'	CYP2_GR3
5' AGGCTATTCATGAGGCCAAAGGTG 3'	CYP2_GR4

(b) Primer pair		End	Product Size (bp)	Overlap with previous cloning (bp)
GR1	CYP_GR1	5'	907	557
GR1	CYP_GR2	5'	666	316
GR3	CYP_GR3	3'	1119	508
GR1	CYP2_GR1	5'	699	313
GR1	CYP2_GR2	5'	595	209
GR3	CYP2_GR3	3'	1239	251
GR4	CYP2_GR4	3'	1001	26

6.2.8 Sequence analysis and primer design

PCR primers, including degenerate, standard, and qPCR primers, were designed with the help of PrimerSelect (DNAStar Inc., Madison, WI, USA), Primer3 (Rozen and Skaletsky, 2000) (http://biotools.umassmed.edu/bioapps/primer3_www.cgi), and OligoPerfect™ Designer (Invitrogen) (http://www.invitrogen.com/content.cfm?pageid=9716).

Sequence analysis was carried out using SeqMan and Editseq (DNAStar Inc., Madison, WI, USA) and World Wide Web resources including BLAST searches of the National Center for Biotechnology Information (NCBI) database (Altschul et al., 1990) and Baylor College of Medicine (BCM) sequence analysis tools (Smith et al., 1996). Sequence alignments were performed using the BCM Multiple Alignment Program (MAP) (Huang, 1994) or CLUSTALW 1.83 (Higgins et al., 1994)

(http://www.ebi.ac.uk/clustalw/). Pretty printing and shading of multiple alignment files was accomplished using Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html). Comparisons of sequences for percent identity used ALIGN (Myers and Miller, 1989) (http://www.ch.embnet.org/software/LALIGN_form.html).

6.2.9 Quantitative real-time RT-PCR of *CYP707A*, *NCED*, and *ZEP* genes

Quantitative real-time RT-PCR (qPCR) was performed using the Superscript™ III Platinum® Two-Step qRT-PCR kit and Platinum® SYBR® Green qPCR SuperMix UDG (Invitrogen). Two μg of RNA was used for production of cDNA samples with 10 μL 2X RT reaction mix (2.5 μM oligo$(dT)_{20}$, 2.5 ng/μL random hexamers, 10 mM MgCl2, and dNTPs) and 2 μL RT Enzyme mix (Superscipt™ III and RNaseOUT™) in a 20 μL synthesis reaction. After gentle mixing, the tubes were incubated for 10 min at 25°C and then for 50 min at 42°C. The reactions were terminated by 5 min incubation at 85°C. Following chilling on ice, 1 μL of *E. coli* RNase H (2 U/μL) was added to remove unwanted RNA templates. cDNA reactions were stored at -20°C until use.

cDNA reactions were diluted 0.4x and 0.04x so that 2.5 μL would yield 1:1 and 1:10 dilutions respectfully. Further serial dilutions were created to yield 1:100, 1:1000, and 1:10000. For samples used to create standard curves to measure amplification efficiency, further dilutions of 1:20, 1:200, 1:2000, 1:20000, and 1:100000 were created. Gene specific qPCR primers were designed to produce fragments of approx. 100-300 bp (Table 6.4). qPCR primers were based on sequences obtained from cloning (section 6.2.5 and 6.2.7) for *PmCYP707A1*, *PmCYP707A2*, *PmNCED1*, *PmNCED2*, and *PmZEP*. *18S* rRNA primers were designed based on a consensus sequence obtained by aligning *Pinus monticola 18S* (AY527222) with *P. elliottii* (D38245), *P. luchuensis* (D38246), and *P. wallichiana* (X75080) *18S* sequences. Primers were tested in standard PCR reactions prior to qPCR analysis.

PCR amplification efficiency was tested using a serial dilution series of a cDNA timepoint to generate a standard curve. Standard curves for each gene varied from 1:1 to 1:10000 dilutions except *18S* which varied from 1:1000 to 1:100000 (see Appendix, Fig. A10). The slope of the line determined by plotting Ct versus relative dilution indicated

PCR efficiency (i.e. Efficiency = $[10^{(-1/slope)}]-1$; an efficiency of 100% would mean doubling of the PCR product every cycle). Only primers that produced between 90% and 110% efficiency were used. Primer dimer formation and non-specific amplification was checked by generating dissociation curves at the end of every qPCR reaction (see Appendix, Fig. A9). No template controls (NTC) were also included on every PCR plate to check for the formation of primer dimer artifacts.

Table 6.4. qPCR primers used to monitor expression of *PmCYP707A1* (CYP_q1, CYP_q2), *PmCYP707A2* (CYP_q5, CYP_q6), *PmNCED1* (NCED_q12, NCED_q13), *PmNCED1* (NCED_q8, NCED_q9) and *PmZEP* genes.

Primer Name	Sequence	Product Size (bp)	Annealing Temp (°C)
CYP_q1	5' CAGCGAAATACTGAACCAAATA 3'	235	57
CYP_q2	5' TGTGACAGCCTCAAGAAATG 3'		
CYP_q5	5' CTCACCTGTTCAAGCCCACA 3'	250	58
CYP_q6	5' AGCAGCCCCACCTCAAAAG 3'		
NCED_q12	5' TTCACGGGTCCTCAACGGTCATC 3'	113	57
NCED_q13	5' GAAGTGGAAGTAGCGTAGGTGTGG 3'		
NCED_q8	5' TCGCTGAAAGAACCTACAATGAC 3'	184	57
NCED_q9	5' GCTCACTCTCGTCTGAAGCA 3'		
ZEP_q3	5' ATCTGGGTGTTGGACTTGGAC 3'	191	58
ZEP_q4	5' CTGGTCGCTTGCTTTCTCAC 3'		
18S_q1	5' AAACGGCTACCACATCCAAG 3'	258	58
18S_q2	5' ACCGACCTTTACGACCCAAG 3'		
18S_q3	5' CATAAACGATGCCGACCAG 3'	252	57
18S_q4	5' CACCACCACCCATAGAATCA 3'		

PCR reactions were performed on a Stratagene MX3000P instrument and Ct values were obtained using the proprietary MX3000P software. SYBR® Green signal intensity was normalized to ROX reference dye. Twenty-five µL reactions consisted of 12.5 µL Platinum® SYBR® Green qPCR SuperMix-UDG, 0.1 µL 25 µM ROX, 0.2 µM of each primer (0.15 µM for *PmNCED1* and *PmNCED2*), 8.9 µL sterile water (9.15 µL for *PmNCED1* and *PmNCED2*), and 2.5 µL diluted cDNA. cDNA amounts for genes of interest (GOI) were equivalent to 1 µL from the cDNA synthesis reaction; while cDNA amounts for *18S* were diluted 1:10000 fold or equivalent to 10^{-4} µL from each reaction. Each PCR plate was mixed and spun down at 2100*g* for 1 min prior to running. PCR cycling conditions were: 2 min at 50°C, 4 min at 95°C, 40 cycles of 95°C for 30 sec, 1 min annealing (30 sec annealing time was used for *PmNCED1*), and 72°C for 30 sec,

followed by a dissociation curve cycle of 95°C for 30 sec, 55°C for 30 sec, and 95°C for 30 sec.

The mean Ct values of 2 biological replicates (with 3 technical replicates per biological replicate) were normalized against 1/10000 diluted *18S* rRNA as an internal control (see Appendix, Fig. A7, for example amplification plot). Relative expression ratios were calculated based on:

$$\text{Ratio} = \frac{(E^{\text{target}})^{\Delta Ct_{GOI}\,(\text{control-sample})}}{(E^{18S})^{\Delta Ct_{18S}\,(\text{control-sample})}}$$

where, E = the efficiency of the GOI or 18S internal control (e.g. 95% efficiency = 1.95); ΔCt = difference in Ct values between control (i.e. timepoint expression is based on or relative to) and sample (Pfaffl, 2001). Mean relative fold change relative to the 3 d soak embryo sample is presented ± standard error (SE).

6.3 Results

6.3.1 Recovery of *ZEP*- and *NCED*-like cDNA sequences from western white pine seeds

Conserved aa regions present in ZEP and NCED proteins (Chernys and Zeevaart, 2000; Römer et al., 2002) were used to design degenerate primers for PCR amplification of *ZEP*- and *NCED*-like sequences (Table 6.2). A 671 bp cDNA fragment was amplified from western white pine dormant-imbibed seeds (Table 6.1). When the 671 bp fragment and its 223 deduced aa fragment, hereafter named *PmZEP* and *Pm*ZEP, respectively, were used for a BLAST search of the NCBI Genbank the top hits were ZEP sequences from various species [data not shown; top hit nucleotide = apricot (*Prunus americana*) *ZEP* (AF159948, Bit Score = 91.7, E-value = 3^{-15}), protein = grape (*Vitis vinifera*) ZEP (AAR11195, Bit Score = 368, E-value = 8^{-101})]. At the amino acid level, when *Pm*ZEP was aligned with *Arabidopsis* ABA1, rice ZEP, and *N. plumbaginifolia* ABA2, identities of 72.2, 76.2, and 75.8% were observed, repectively (Fig. 6.1).

For *NCED*, a 742 bp fragment, designated *PmNCED1*, was amplified from dormant-imbibed seeds and from seeds during moist-chilling and germination (Table 6.1). A 733 bp fragment, designated *PmNCED2*, was amplified from 7 d germinated seeds that had

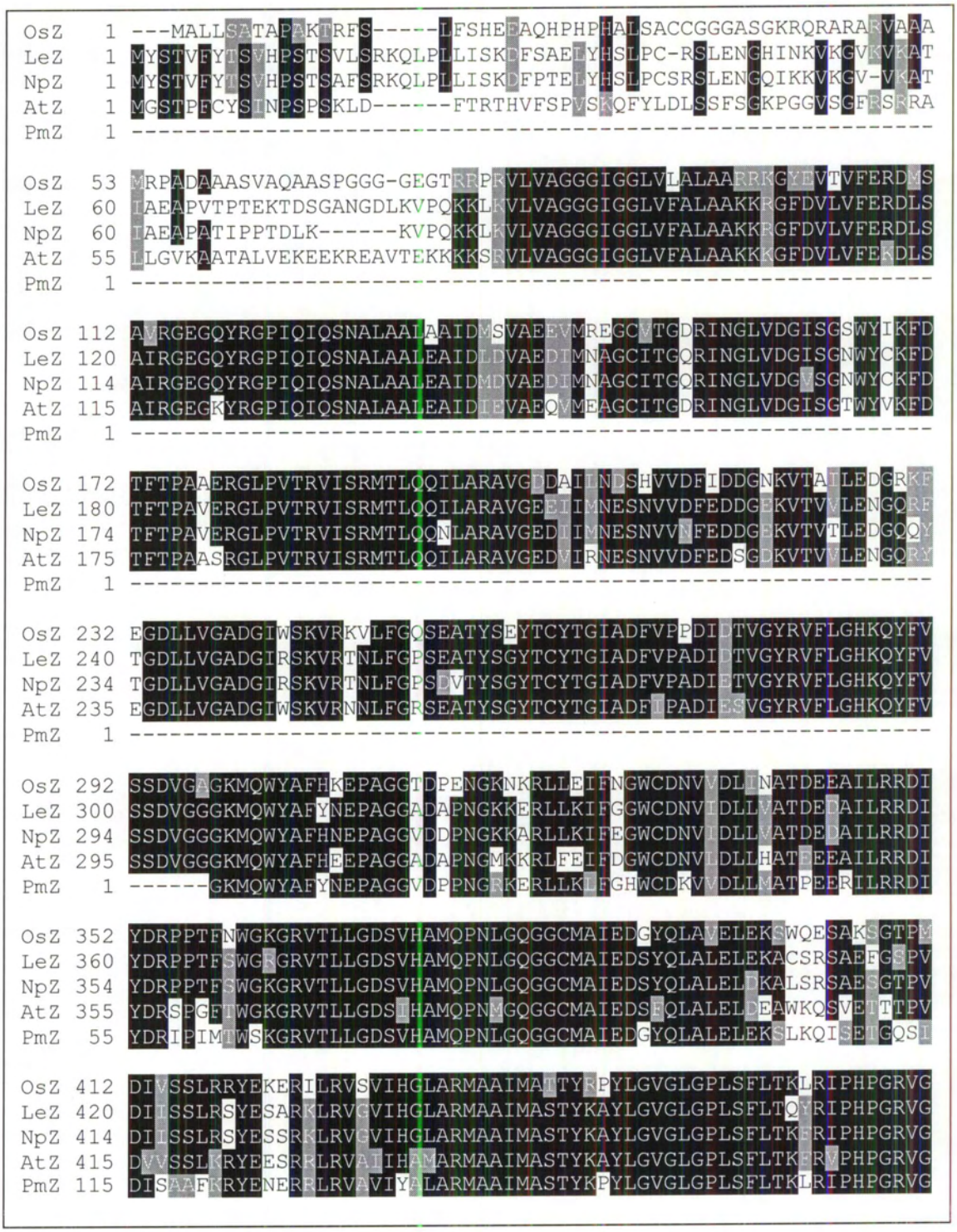


Fig. 6.1. Amino acid alignment of zeaxanthin expoxidase (ZEP) enzymes from representative species. *Arabidopsis thaliana At*ABA1 (AtZ) (At5g67030; NP_851285) (Xiong et al., 2002), rice *Os*ABA2 (*Oryza sativa*) (OsZ) (BAB39765) (Agrawal et al., 2001), tomato (*Lycopersicon esculentum*) *Le*ZEP (LeZ) (CAB06084) (Burbidge et al., 1997b), *Nicotiana plumbaginifolia Np*ABA2 (CAA65048) (NpZ) (Marin et al., 1996) are aligned with western white pine (*Pinus monticola*) *Pm*ZEP (PmZ).

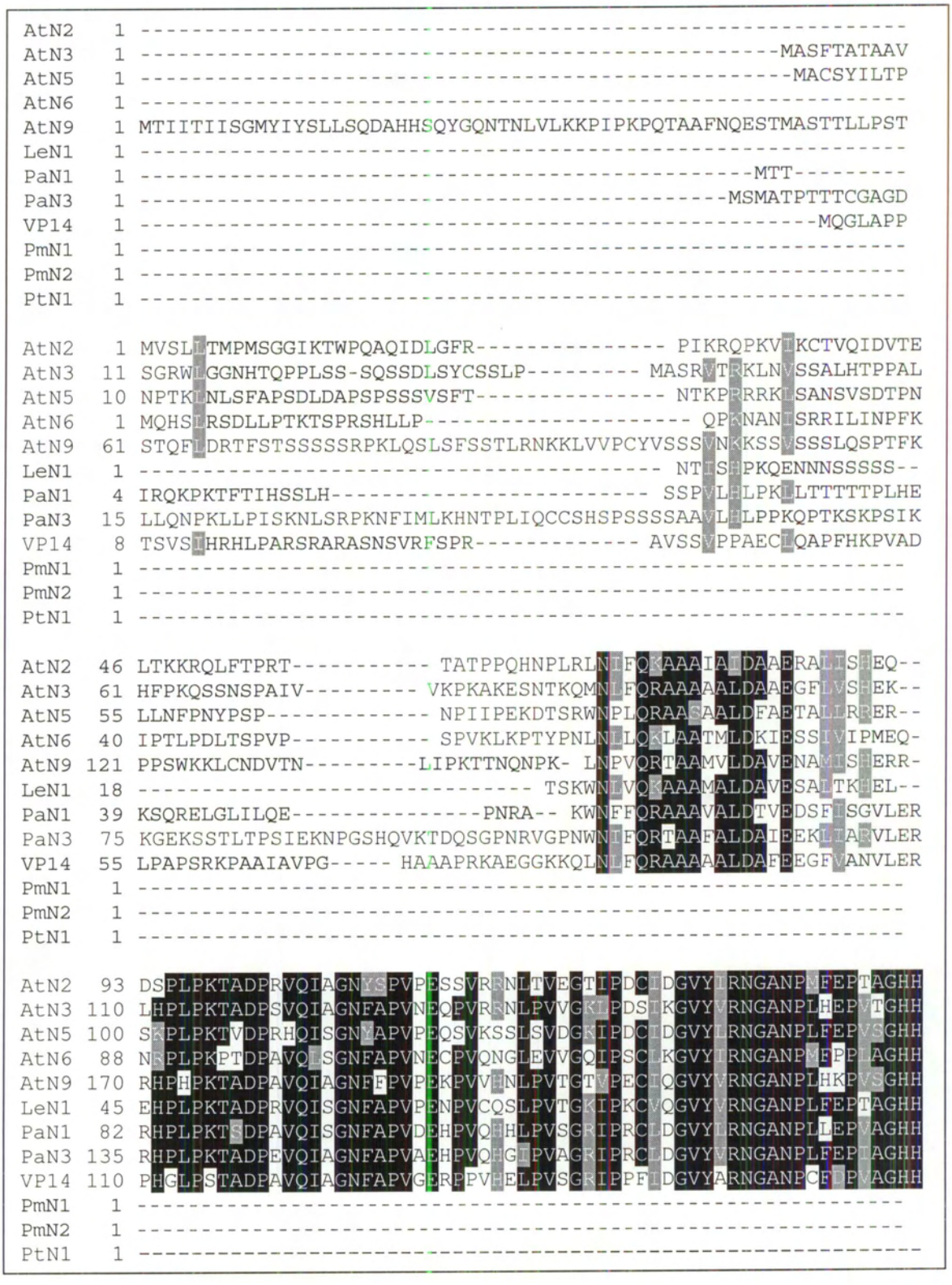
Fig. 6.1. Continued. Amino acid alignment of zeaxanthin expoxidase (ZEP) enzymes from representative species.

been subjected to a drought treatment (Table 6.1). When *PmNCED1* and *PmNCED2* and their respective proteins were used for a BLAST search of the GenBank the top hits were NCED sequences from various species [data not shown; *PmNCED1* top hit nucleotide = maize *VP14* (U95953, Bit Score = 63.9, E-value = 7^{-7}), protein = peanut (*Arachis hypogaea*) NCED (CAE00459, Bit Score = 300, E-value = 4^{-80}); *PmNCED2* top hit nucleotide = rice *NCED2* (AL731885, Bit Score = 68, E-value = 4^{-8}), protein = orchid (*Oncidium* Gower Ramsey) NCED (AAX85470, Bit Score = 392, E-value = 7^{-108})]. At the amino acid level, both *Pm*NCED1 and *Pm*NCED2 fragments exhibit greatest homology to NCED sequences as opposed to other carotenoid cleavage dioxygenases (CCDs) (Table 6.5; Fig. 6.2). *Pm*NCED1 and *Pm*NCED2 share 61.8% identity to each other.

Table 6.5. Comparison of the amino acid identities between *Pm*NCED1 and *Pm*NCED2 and those of the *Arabidopsis* 9-*cis* epoxycarotenoid dioxygenase/carotenoid cleavage dioxygenase (NCED/CCD) gene family and VP14 of maize (*Zea mays*) (Tan et al., 1997; Tan et al., 2003).

Sequence	*Pm*NCED1 % Identity	*Pm*NCED2 % Identity
*At*NCED2 (At4g18350)	56.9	67.1
*At*NCED3 (At3g14440)	58.5	70.4
*At*NCED5 (At1g30100)	61.8	67.9
*At*NCED6 (At3g24220)	57.1	61.1
*At*NCED9 (At1g78390)	59.6	70.4
*Zm*VP14 (AAB62181)	59.1	71.3
*At*CCD1 (At3g63520)	40.1	42.7
*At*CCD4 (At4g19170)	42.4	47.2
*At*CCD7 (At2g44990)	19.7	23.1
*At*CCD8 (At4g32810)	20.6	20.1

Fig. 6.2. Amino acid alignment of *9-cis* epoxycarotenoid dioxygenase (NCED) enzymes from representative species. *Arabidopsis At*NCED2 (AtN2) (At4g18350; NP_193569), *At*NCED3 (AtN3) (At3g14440; NP_188062), *At*NCED5 (AtN5) (At1g30100; NP_174302), *At*NCED6 (AtN6) (At3g24220; NP_189064), *At*NCED9 (AtN9) (At1g78390; NP_177960) (Tan et al., 2003), tomato (*Lycopersicon esculentum*) *Le*NCED1 (LeN1) (CAD30202) (Burbidge et al., 1999), maize (*Zea mays*) VP14 (VP14) (AAB62181) (Tan et al., 1997), avocado (*Persea americana*) *Pa*NCED1 (PaN1) (AAK00632), *Pa*NCED3 (PaN3) (AAK00623) (Chernys and Zeevaart, 2000), and loblolly pine (*Pinus taeda*) (PtN1) (BG039692) are aligned with western white pine (*Pinus monticola*) *Pm*NCED1 (PmN1) and *Pm*NCED2 (PmN2).


AtN2 1 ----------
AtN3 1 ----------MASFTATAAV
AtN5 1 ----------MACSYILTP
AtN6 1 ----------
AtN9 1 MTIITIISGMYIYSLLSQDAHHSQYGQNTNLVLKKPIPKPQTAAFNQESTMASTTLLPST
LeN1 1 ----------
PaN1 1 ----------MTT---------
PaN3 1 ----------MSMATPTTTCGAGD
VP14 1 ----------MQGLAPP
PmN1 1 ----------
PmN2 1 ----------
PtN1 1 ----------
AtN2 1 MVSLLTMPMSGGIKTWPQAQIDLGFR---------- PIKRQPKVIKCTVQIDVTE
AtN3 11 SGRWLGGNHTQPPLSS-SQSSDLSYCSSLP--------- MASRVTRKLNVSSALHTPPAL
AtN5 10 NPTKLNLSFAPSDLDAPSPSSSVSFT---------- NTKPRRRKLSANSVSDTPN
AtN6 1 MQHSLRSDLLPTKTSPRSHLLP---------- QPKNANISRRILINPFK
AtN9 61 STQFLDRTFSTSSSSSRPKLQSLSFSSTLRNKKLVVPCYVSSSVNKKSSVSSSLQSPTFK
LeN1 1 ---------- NTISHPKQENNNSSSSS--
PaN1 4 IRQKPKTFTIHSSLH---------- SSPVLHLPKLLTTTTTPLHE
PaN3 15 LLQNPKLLPISKNLSRPKNFIMLKHNTPLIQCCSHSPSSSSAAVLHLPPKQPTKSKPSIK
VP14 8 TSVSIHRHLPARSRARASNSVRFSPR---------- AVSSVPPAECLQAPFHKPVAD
PmN1 1 ----------
PmN2 1 ----------
PtN1 1 ----------
AtN2 46 LTKKRQLFTPRT---------- TATPPQHNPLRLNIFQKAAAIAIDAAERALISHEQ--
AtN3 61 HFPKQSSNSPAIV--------- VKPKAKESNTKQMNLFQRAAAAALDAAEGFLVSHEK--
AtN5 55 LLNFPNYPSP---------- NPIIPEKDTSRWNPLQRAASAALDFAETALLRRER--
AtN6 40 IPTLPDLTSPVP---------- SPVKLKPTYPNLNLLQKLAATMLDKIESSIVIPMEQ-
AtN9 121 PPSWKKLCNDVTN---------LIPKTTNQNPK- LNPVQRTAAMVLDAVENAMISHERR-
LeN1 18 ---------- TSKWNLVQKAAAMALDAVESALTKHEL--
PaN1 39 KSQRELGLILQE----------PNRA-- KWNFFQRAAAVALDTVEDSFLSGVLER
PaN3 75 KGEKSSTLTPSIEKNPGSHQVKTDQSGPNRVGPNWNIFQRTAAFALDAIEEKLIARVLER
VP14 55 LPAPSRKPAAIAVPG----- HAAAPRKAEGGKKQLNLFQRAAAAALDAFEEGFVANVLER
PmN1 1 ----------
PmN2 1 ----------
PtN1 1 ----------
AtN2 93 DSPLPKTADPRVQIAGNYSPVPESSVRRNLTVEGTIPDCIDGVYIRNGANPMFEPTAGHH
AtN3 110 LHPLPKTADPSVQIAGNFAPVNEQPVRRNLPVVGKLPDSIKGVYVRNGANPLHEPVTGHH
AtN5 100 SKPLPKTVDPRHQISGNYAPVPEQSVKSSLSVDGKIPDCIDGVYLRNGANPLFEPVSGHH
AtN6 88 NRPLPKPTDPAVQLSGNFAPVNECPVQNGLEVVGQIPSCLKGVYIRNGANPMFPPLAGHH
AtN9 170 RHPHPKTADPAVQIAGNFFPVPEKPVVHNLPVTGTVPECIQGVYVRNGANPLHKPVSGHH
LeN1 45 EHPLPKTADPRVQISGNFAPVPENPVCQSLPVTGKIPKCVQGVYVRNGANPLFEPTAGHH
PaN1 82 RHPLPKTSDPAVQISGNFAPVDEHPVQHHLPVSGRIPRCLDGVYLRNGANPLLEPVAGHH
PaN3 135 RHPLPKTADPEVQIAGNFAPVAEHPVQHGIPVAGRIPRCLDGVYVRNGANPLFEPIAGHH
VP14 110 PHGLPSTADPAVQIAGNFAPVGERPPVHELPVSGRIPPFIDGVYARNGANPCFIPVAGHH
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PmN2 1 ----------
PtN1 1 ----------

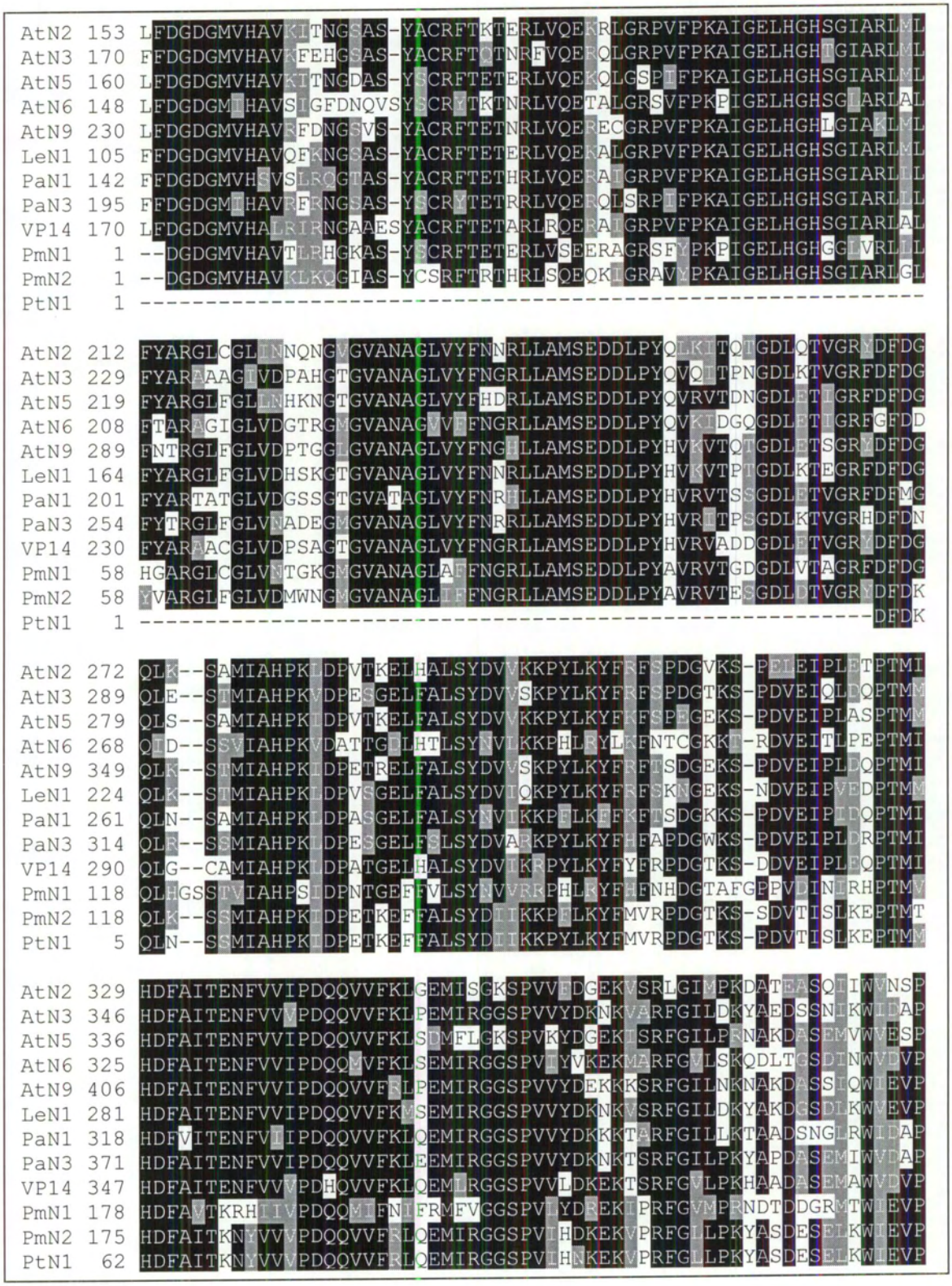


Fig. 6.2. Continued. Amino acid alignment of 9-*cis* epoxycarotenoid dioxygenase (NCED) enzymes from representative species.

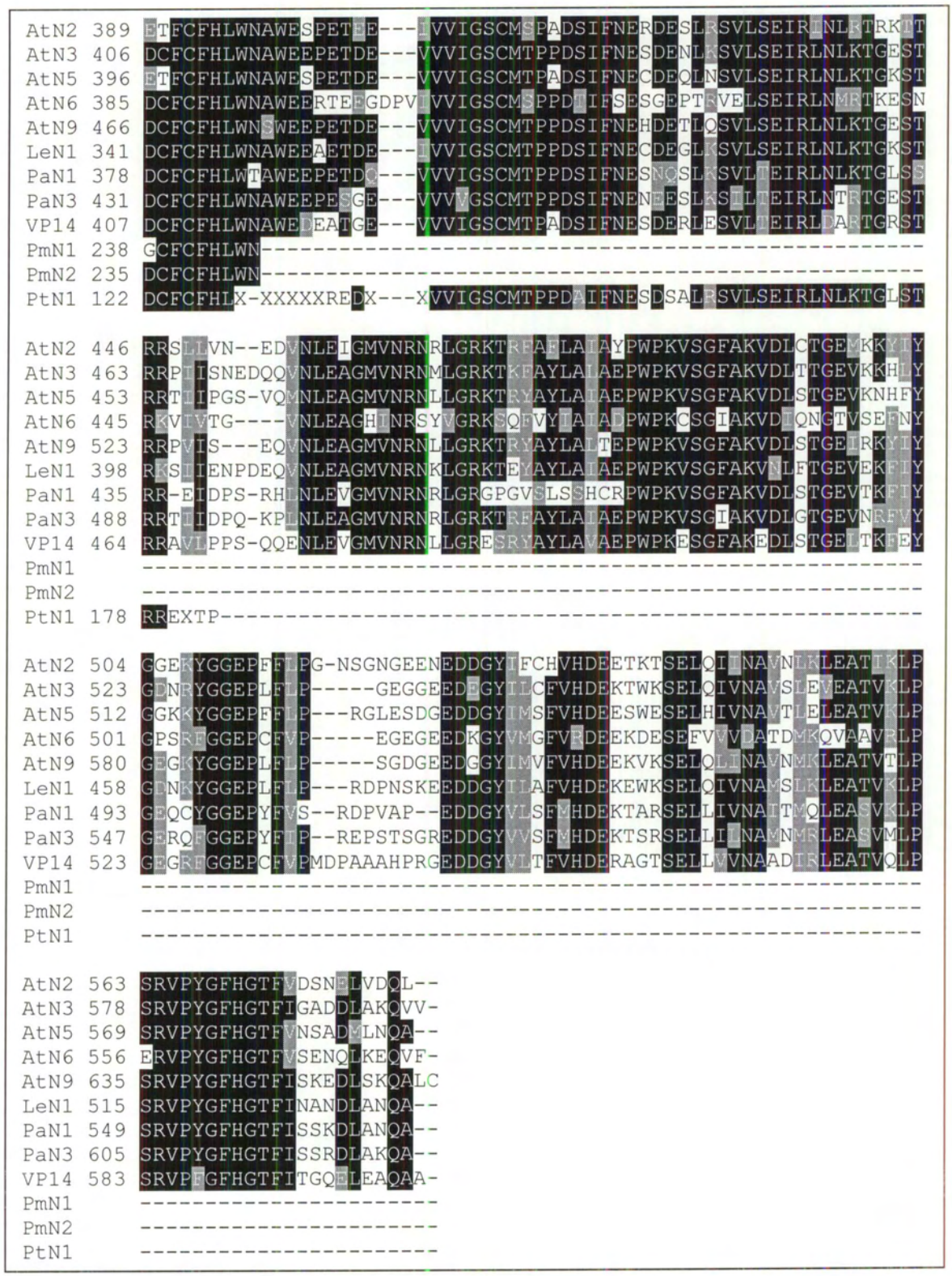


Fig. 6.2. Continued. Amino acid alignment of 9-*cis* epoxycarotenoid dioxygenase (NCED) enzymes from representative species.

6.3.2 Cloning full-length *CYP707A*-like cDNA sequences from western white pine seeds

Alignment of *Arabidopsis*, rice, and tomato CYP707A (ABA 8'-hydroxylase) sequences (Kushiro et al., 2004; Saito et al., 2004; Nelson et al., 2005a) with two EST sequences from loblolly pine (*Pinus taeda*) (CF388492, CF474400, CF667437) and sitka spruce (*Picea sitchensis*) (CO217842) were used to create degenerate primers for PCR amplification of *CYP707A*-like sequences from dormant-imbibed seeds and from seeds during moist-chilling and germination (Table 6.1). A 994 bp cDNA fragment, *PmCYP707A1p*, was cloned from various stages of seed dormancy-breaking and germination (Table 6.1), while a 997 bp fragment, *PmCYP707A2p*, was cloned from 7 d germinating seeds that had been subjected to a drought and rehydration treatment (Table 6.1). The full-length cDNA for *PmCYP707A1*, obtained using 5' and 3' RACE-PCR, contains an open reading frame of 1410 bp, and 5' and 3' untranslated regions of 86 and 379 bp, respectively (total length = 1875 bp). The full-length cDNA for *PmCYP707A2* contains an open reading frame of 1443 bp, and 5' and 3' untranslated regions of 113 and 495 bp, respectively (total length = 2051 bp).

The predicted molecular masses of the of *Pm*CYP707A1 and *Pm*CYP707A2 proteins are 53 and 54.4 kD, respectively, with isoelectric points of 9.33 and 8.93. Both proteins share hydrophobic amino terminuses (i.e. approx. the first 30 aa) which are characteristic of membrane targeting hydrophobic segments present in P450 enzymes (data not shown; Werck-Reichhart et al., 2002). In addition, other characteristics of P450 enzymes present in *Pm*CYP707A1 and *Pm*CYP707A2 include: a cluster of prolines (e.g. PPGS) following the hydrophobic region at the amino terminus, an E-R-R triad (E-X-X-R.....R motif) present at aa 337 and 341, respectively, and a cysteine in a potential heme-binding domain (at aa 413 and 417, respectively) (Fig. 6.3) (Werck-Reichhart et al., 2002).

When *PmCYP707A1* and *PmCYP707A2* and their respective proteins were used for a BLAST search of the NCBI GenBank the top hits were CYP707A sequences from various species [data not shown; *PmCYP707A1* top hit nucleotide = potato (*Solanum tuberosum*) *StCYP707A2* (DQ206631, Bit Score = 113, E-value = 2^{-21}), protein = *Arabidopsis* CYP707A1 (At4g19230; NP_567581, Bit Score = 648, E-value = 0); *PmCYP707A2* top hit nucleotide = potato (*Solanum tuberosum*) *StCYP707A2*

(DQ206631, Bit Score = 65.9, E-value = 5^{-7}), protein = *Arabidopsis* CYP707A1 (At4g19230; NP_567581, Bit Score = 641, E-value = 0)]. At the amino acid level, both *Pm*CYP707A1 and *Pm*CYP707A2 proteins share approx. 60% homology to other CYP707A sequences (Table 6.6; Fig. 6.3). *Pm*CYP707A1 and *Pm*CYP707A2 share 68.3% identity to each other.

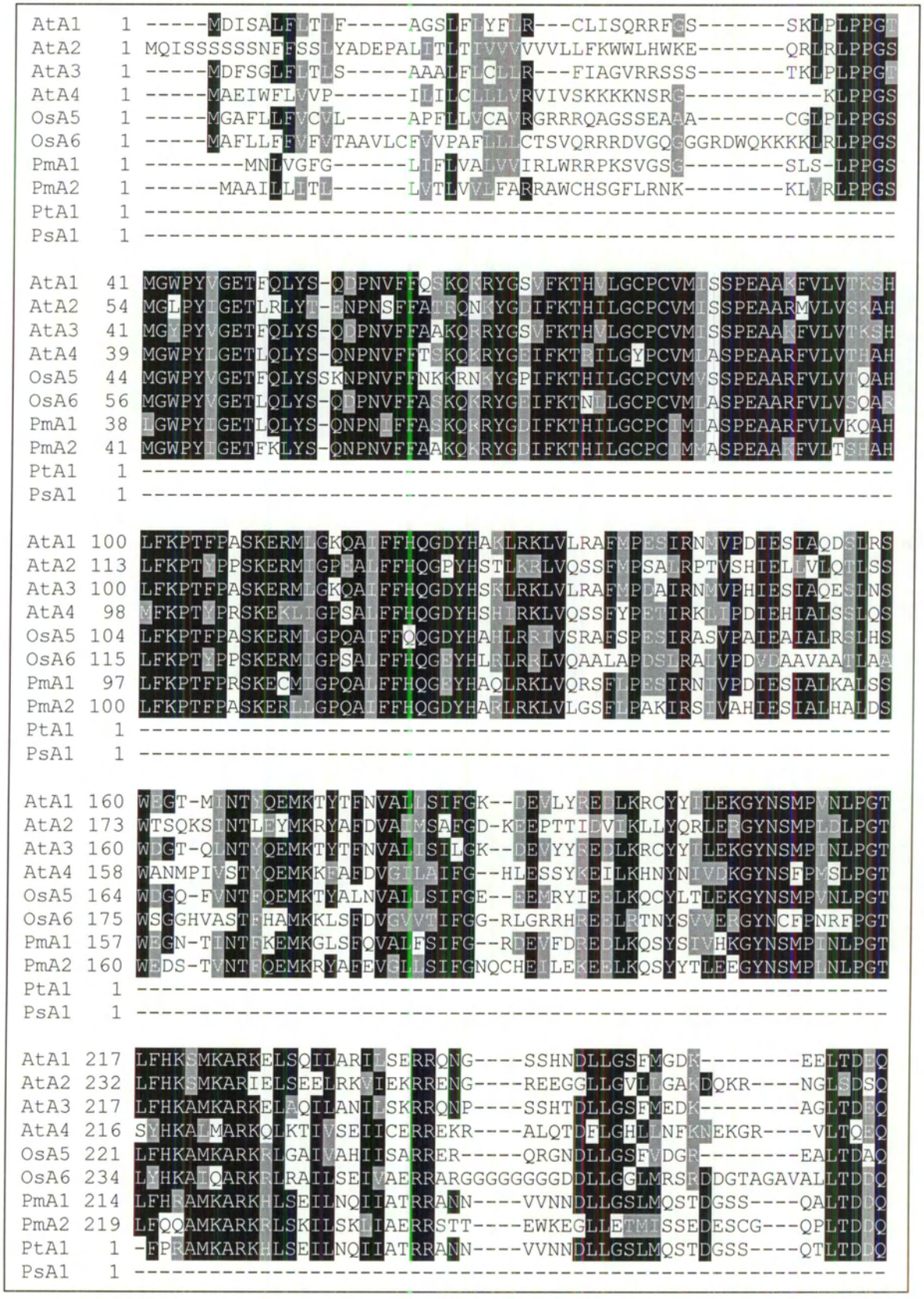
Table 6.6. Comparison of the amino acid identities between *Pm*CYP707A1 and *Pm*CYP707A2 and other CYP707A (ABA 8'-hydroxylase) genes from *Arabidopsis* and rice (*Oryza sativa*) (Kushiro et al., 2004; Nelson, 2005a).

Sequence	*Pm*CYP707A1 % Identity	*Pm*CYP707A2 % Identity
*At*CYP707A1 (At4g19230)	66.3	63.7
*At*CYP707A2 (At2g29090)	59.3	57.1
*At*CYP707A3 (At5g45340)	66.1	63.1
*At*CYP707A4 (At3g19270)	59.5	56.3
*Os*CYP707A5 (XP_467350)	61.8	60.7
*Os*CYP707A6 (XP_482909)	56.1	52.8

6.3.3 Expression of *PmCYP707A1*, *PmCYP707A2*, *PmNCED1*, *PmNCED2*, and *PmZEP* during moist-chilling-induced dormancy termination in western white pine

To begin to determine the potential roles of the putative white pine *CYP707A*, *NCED*, and *ZEP* gene products, the expression of these genes was analyzed using quantitative real-time PCR (qPCR) during dormancy termination (Figs. 6.4, 6.5). Dormancy termination was accomplished through a pre-treatment water soak of 13 d, followed by 98 d of moist chilling at 3°C, and subsequent placement in germination conditions (23°C, 16 h photoperiod). Timepoints for qPCR expression analysis were similar to germination and ABA and ABA metabolite analysis presented in Chapter 3 (Feurtado et al., 2004, see 6.4 below for discussion). However, a 60 d timepoint was done in place of 45 d moist chilling and a 3 d germination timepoint was omitted (compare Figs. 6.4, 6.5 to 3.1). Expression data is shown relative to the embryo tissues of the 3 d soak timepoint (Figs. 6.4, 6.5).

Fig. 6.3. Amino acid alignment of abscisic acid 8'-hydroxylase (CYP707A) enzymes from representative species. *Arabidopsis thaliana* CYP707A1 (AtA1) (At4g19230; NP_974574), CYP707A2 (AtA2) (At2g29090; NP_180473), CYP707A3 (AtA3) (At5g45340; NP_199347), CYP707A4 (AtA4) (At3g19270; NP_566628) (Kushiro et al., 2004; Saito et al., 2004), rice (*Oryze sativa*) CYP707A5 (OsA5) (similar to XP_467350), CYP707A6 (OsA6) (similar to XP_482909) (Nelson, 2005a), loblolly pine (*Pinus taeda*) ESTs (CF388492, CF474400, CF667437) denoted as PtA1, and sitka spruce (*Picea sitchensis*) EST (CO217842) denoted as PsA1 are aligned with western white pine (*Pinus monticola*) *Pm*CYP707A1 (PmA1) and *Pm*CYP707A2 (PmA2).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AtA2 1 MQISSSSSSNFFSSLYADEPALITLTTVVVVVVLLFKWWLHWKE-------QRLRLPPGS
AtA3 1 -----MDFSGLFLTLS-----AAALFLCLLR---FIAGVRRSSS-------TKLPLPPGT
AtA4 1 -----MAEIWFLVVP------ILILCLLLVRVIVSKKKKNSRG-----------KLPPGS
OsA5 1 -----MGAFLLFVCVL-----APFLLVCAVRGRRRQAGSSEAAA-------CGLPLPPGS
OsA6 1 -----MAFLLFFVFVTAAVLCFVVPAFLLLCTSVQRRRDVGQGGGRDWQKKKKLRLPPGS
PmA1 1 --------MNLVGFG------LIFLVALVVIRLWRRPKSVGSG--------SLS-LPPGS
PmA2 1 ------MAAILLITL------LVTLVVLFARRAWCHSGFLRNK--------KLVRLPPGS
PtA1 1 ----------
PsA1 1 ----------
AtA1 41 MGWPYVGETFQLYS-QDPNVFFQSKQKRYGSVFKTHVLGCPCVMISSPEAAKFVLVTKSH
AtA2 54 MGLPYIGETLRLYT-ENPNSFFATRQNKYGDIFKTHILGCPCVMISSPEAARMVLVSKAH
AtA3 41 MGYPYVGETFQLYS-QDPNVFFAAKQKRYGSVFKTHVLGCPCVMISSPEAAKFVLVTKSH
AtA4 39 MGWPYIGETLQLYS-QNPNVFFTSKQKRYGEIFKTRILGYPCVMLASPEAARFVLVTHAH
OsA5 44 MGWPYVGETFQLYSSKNPNVFFNKKRNKYGPIFKTHILGCPCVMVSSPEAARFVLVTQAH
OsA6 56 MGWPYVGETLQLYS-QDPNVFFASKQKRYGEIFKTNLLGCPCVMLASPEAARFVLVSQAR
PmA1 38 LGWPYIGETLQLYS-QNPNIFFASKQKRYGDIFKTHILGCPCIMIASPEAARFVLVKQAH
PmA2 41 MGWPYIGETFKLYS-QNPNVFFAAKQKRYGDIFKTHILGCPCIMMASPEAAKFVLTSHAH
PtA1 1 ----------
PsA1 1 ----------
AtA1 100 LFKPTFPASKERMLGKQAIFFHQGDYHAKLRKLVLRAFMPESIRNMVPDIESIAQDSLRS
AtA2 113 LFKPTYPPSKERMIGPEALFFHQGPYHSTLKRLVQSSFMPSALRPTVSHIELLVLQTLSS
AtA3 100 LFKPTFPASKERMLGKQAIFFHQGDYHSKLRKLVLRAFMPLAIRNMVPHIESIAQESLNS
AtA4 98 MFKPTYPRSKEKLIGPSALFFHQGDYHSHIRKLVQSSFYPETIRKLIPDIEHIALSSLQS
OsA5 104 LFKPTFPASKERMLGPQAIFFQQGDYHAHLRRIVSRAFSPESIRASVPAIEAIALRSLHS
OsA6 115 LFKPTYPPSKERMIGPSALFFHQGEYHLRLRRLVQAALAPDSLRALVPDVDAAVAATLAA
PmA1 97 LFKPTFPRSKECMIGPQALFFHQGEYHAQLRKLVQRSFLPESIRNIVPDIESIALKALSS
PmA2 100 LFKPTFPASKERLLGPQAIFFHQGDYHARLRKLVLGSFLPAKIRSIVAHIESIALHALDS
PtA1 1 ----------
PsA1 1 ----------
AtA1 160 WEGT-MINTYQEMKTYTFNVALLSIFGK--DEVLYREDLKRCYYILEKGYNSMPVNLPGT
AtA2 173 WTSQKSINTLFYMKRYAFDVATMSAFGD-KEEPTTIDVIKLLYQRLERGYNSMPLDLPGT
AtA3 160 WDGT-QLNTYQEMKTYTFNVALISILGK--DEVYYREDLKRCYYTLEKGYNSMPINLPGT
AtA4 158 WANMPIVSTYQEMKKFAFDVGILAIFG--HLESSYKEILKHNYNIVDKGYNSFPMSLPGT
OsA5 164 WDGQ-FVNTFQEMKTYALNVALLSIFGE--EEMRYIEELKQCYLTLEKGYNSMPVNLPGT
OsA6 175 WSGGHVASTFHAMKKLSFDVGVVTIFGG-RLGRRHREELRTNYSVVERGYNCFPNRFPGT
PmA1 157 WEGN-TINTFKEMKGLSFQVALFSIFG--RDEVFDREDLKQSYSIVHKGYNSMPINLPGT
PmA2 160 WEDS-TVNTFQEMKRYAFEVGLLSIFGNQCHEILEKEELKQSYYTLEEGYNSMPLNLPGT
PtA1 1 ----------
PsA1 1 ----------
AtA1 217 LFHKSMKARKELSQILARILSERRQNG----SSHNDLLGSFMGDK--------EELTDEQ
AtA2 232 LFHKSMKARIELSEELRKVIEKRRENG----REEGGLLGVLLGAKDQKR----NGLSDSQ
AtA3 217 LFHKAMKARKELAQILANILSKRRQNP----SSHTDLLGSFMEDK--------AGLTDEQ
AtA4 216 SYHKALMARKQLKTIVSEIICERREKR----ALQTDFLGHLLNFKNEKGR----VLTQEQ
OsA5 221 LFHKAMKARKRLGAIVAHIISARRER-----QRGNDLLGSFVDGR--------EALTDAQ
OsA6 234 LYHKAIQARKRLRAILSEIVAERRARGGGGGGGGGDDLLGGLMRSRDDGTAGAVALLTDDQ
PmA1 214 LFHRAMKARKHLSEILNQIIATRRANN----VVNNDLLGSLMQSTDGSS----QALTDDQ
PmA2 219 LFQQAMKARKRLSKILSKLIAERRSTT----EWKEGLLETMISSEDESCG---QPLTDDQ
PtA1 1 -FPRAMKARKHLSEILNQIIATRRANN----VVNNDLLGSLMQSTDGSS----QTLTDDQ
PsA1 1 ----------

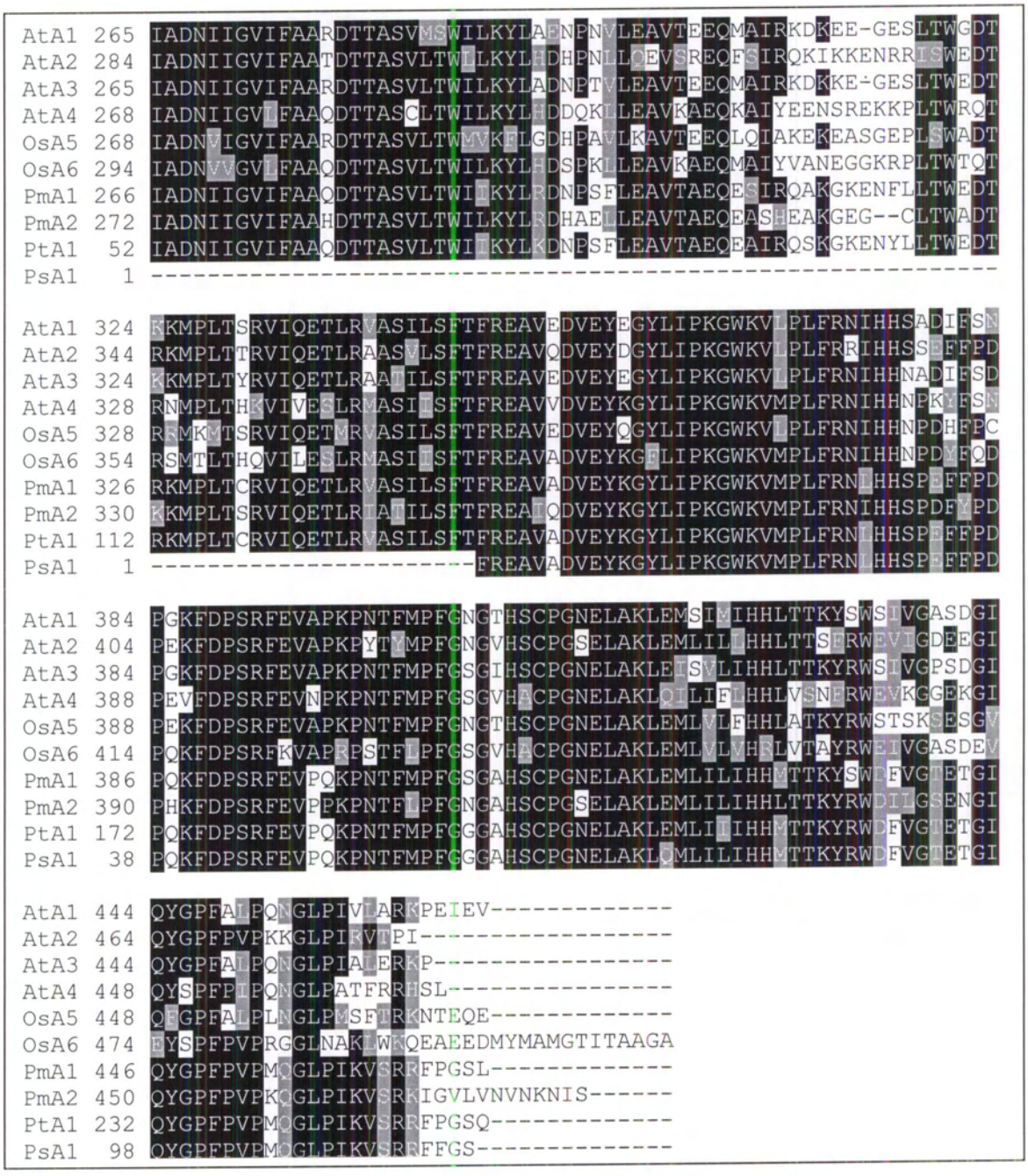

Fig. 6.3. Continued. Amino acid alignment of abscisic acid 8'-hydroxylase (CYP707A) enzymes from representative species.

6.3.3.1 *PmZEP*, *PmNCED1*, *PmNCED2* expression during dormancy termination

PmZEP expression in the embryo and megagametophyte increased slightly during the initial water soak, decreased through moist chilling and early germination, and increased in the germinated seedling. Expression in the megagametophyte was approx. 8-9 fold lower than that of the embryo (Fig. 6.4a). Overall, changes in *PmZEP* expression in both tissues were minimal and fold changes were less than 3 fold (Fig. 6.4a).

PmNCED1 expression in the embryo and megagametophyte increased slightly by 30 d moist chilling before decreasing significantly during the remainder of moist chilling and into germination and seedling growth, by approx. 1000 fold (Fig. 6.4b). Similar to *PmZEP*, expression of *PmNCED1* in the megagametophyte was approx. 10 fold lower than that of the embryo; however, by the germinated seedling stage expression was similar in both tissues (Fig. 6.4b). In contrast to *PmNCED1*, expression of *PmNCED2* was low throughout the water soak, moist chilling, and early germination (Fig. 6.4c; data not shown). However, *PmNCED2* increased in the germinated seedling by approx. 1500 and 100 fold in the embryo and megagametophyte, respectively (Fig. 6.4c). *PmNCED1* was the major *NCED*-homologous sequence expressed during seed dormancy breakage, while *PmNCED2* was the major *NCED*-homologous sequence expressed in the germinated seed (Fig. 6.4b, c; data not shown).

6.3.3.2 *PmCYP707A1* and *PmCYP707A2* expression during dormancy termination

PmCYP707A1 expression increased towards the end of moist chilling between 60 and 98 d in the embryo and between 30 and 60 d in the megagametophyte (Fig. 6.5a). Overall, expression of *PmCYP707A1* was lower in the megagametophyte compared to the embryo through 60 d moist chilling but became similar thereafter (Fig. 6.5a). In contrast to *PmCYP707A1*, *PmCYP707A2* expression decreased significantly throughout moist chilling and germination in both the embryo and megagametophyte tissues (by 20 and 50 fold, respectively, in the germinated seedling) (Fig. 6.5b). Expression between the embryo and megagametophyte was similar (Fig. 6.5b). Thus, *PmCYP707A1* was the major *CYP707A*-homologous sequence expressed during seed dormancy breakage, even though expression levels of *PmCYP707A1* and *PmCYP707A2* were similar during the initial water soak (Fig. 6.5; data not shown).

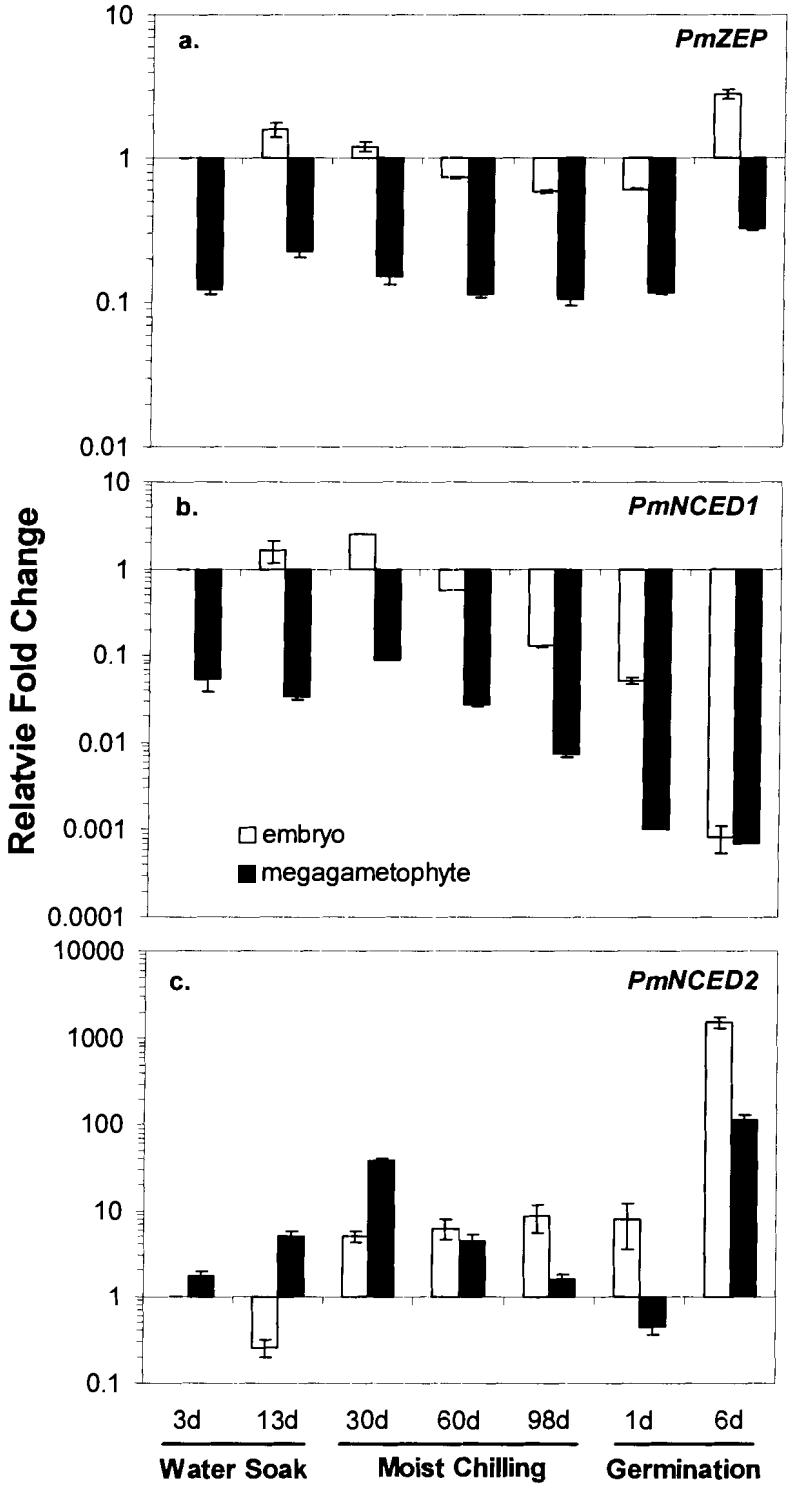

Fig. 6.4. Expression of western white pine cDNA sequences with homology to zeaxanthin expoxidase (*ZEP*) and 9-*cis* epoxycarotenoid dioxygenase (*NCED*) as monitored by quantitative real-time PCR analysis. *PmZEP* (a),*PmNCED1* (b), and *PmNCED2* (c) expression is expressed relative to the embryo 3d timepoint and all data was normalized using 18S rRNA as a internal control. An average from duplicate experiments (±SE) is shown.

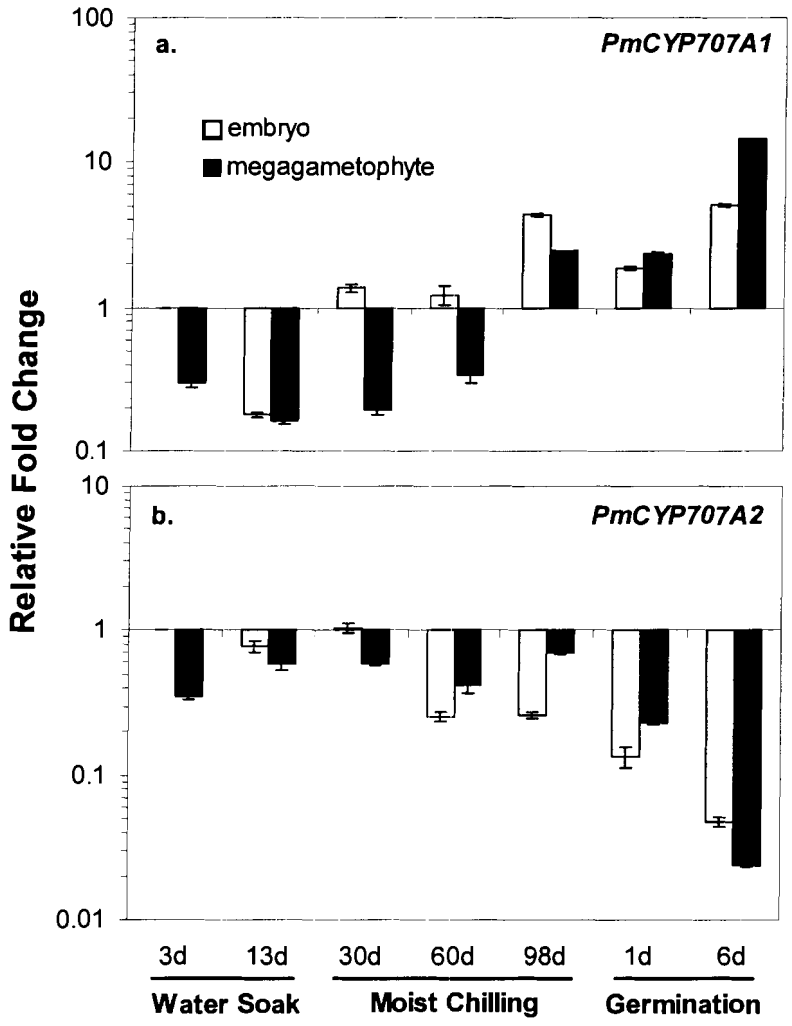

Fig. 6.5. Expression of western white pine cDNA sequences with homology to abscisic acid 8'-hydroxylase (*CYP707A*) genes as monitored by quantitative real-time PCR analysis. *PmCYP707A1* (a) and *PmCYP707A2* (b) expression is expressed relative to the embryo 3d timepoint and all data was normalized using 18S rRNA as an internal control. An average from duplicate experiments (±SE) is shown.

6.4 Discussion

ABA homeostasis within plant cells and tissues are under constant flux as a result of the opposing forces of biosynthesis versus degradation (Nambara and Marion-Poll, 2005). In seeds, changes in ABA equilibrium within embryos and surrounding seed tissues can contribute to dormancy inception, maintenance, and termination (Cutler and Krochko 1999; Schmitz et al. 2000, 2002; Finkelstein and Rock, 2002; Lefebvre et al., 2006). In the conifer western white pine, decreases in ABA and increases in ABA catabolite levels coincide with dormancy termination and increases in seed germinability (through a moist-chilling treatment) (Chapter 3; Feurtado et al., 2004). In this study, we sought to further investigate how ABA levels are dynamically maintained during dormancy termination in western white pine by monitoring changes in the expression of ABA biosynthetic and catabolic genes. To accomplish this goal, cDNA sequences with homology to zeaxthanin epoxidase (ZEP), 9-*cis* epoxycarotenoid dioxygenase (NCED), and abscisic acid 8'-hydroxylase (CYP707A) were cloned from western white pine seed during dormancy maintenance, termination, and germination.

6.4.1 Cloned sequences from western white pine seeds are probable *ZEP*, *NCED*, and *CYP707A* members

The recovery of sequences from western white pine seed tissues with high homology to existing *ZEP* and *NCED* at both the nucleotide and protein levels suggests *PmZEP*, *PmNCED1*, and *PmNCED2* do indeed encode ZEP and NCED enzymes. The deduced protein fragment, *Pm*ZEP, displayed approx. 75% identity to other known ZEP enzymes such as ABA1 from *Arabidopsis* and ABA2 from *N. plumbaginifolia* (Marin et al., 1996). The deduced protein fragments, *Pm*NCED1 and *Pm*NCED2, also displayed significant homology to NCED of approx. 59% and 68% identity, respectively (Table 6.5). However, NCED enzymes belong to a family of carotenoid cleavage dioxygenases (CCD) enzymes that can cleave an epoxycarotenoid at various positions in the carotenoid backbone (Tan et al., 2003). For example, the *Arabidopsis* NCED2, NCED3, NCED6, and NCED9 cleavage reaction takes place between positions C11 and C12 (C11',C12') in a *cis*-xanthophyll molecule, while the *Arabidopsis* CCD1 reaction takes place between positions C9 and C10 (C9',C10') (Iuchi et al., 2001; Schwartz et al., 2001). Thus, it is

possible that the *PmNCED1* and *PmNCED2* encode other CCD enzymes and not NCED. Nonetheless, the higher homology to *Arabidopsis* NCED sequences compared to other CCD sequences would suggest that the white pine sequences encode NCED enzymes (Table 6.5). Validation of the substrate preferences for would require full-length cloning and expression in a heterologous system such as yeast or *Escherichia coli* to test xanthophyll cleavage (Iuchi et al., 2001; Schwartz et al., 2001) or over-expression in *Arabidopsis* to test if the gene(s) produced an ABA over-accumulation phenotype (Lefebvre et al., 2006).

The *PmCYP707A1* and *PmCYP707A2* sequences are probable candidates encoding ABA 8'-hydroxylases based on their deduced protein identities of approx. 63% and 60% to the only confirmed 8'-hydroxylases, the CYP707A sub-family of *Arabidopsis* (Kushiro et al., 2004; Saito et al., 2004). When comparing *Pm*CYP707A1 and *Pm*CYP707A2 with the next highest cytochrome P450 family homologue CYP88A3, encoding *ent*-kaurenoic acid hydroxylase involved in GA biosynthesis (NP_172008; Helliwell et al., 2001), 33.3% and 31.0% identities are obtained, respectively.

The *Arabidopsis* recombinant CYP707A proteins convert ABA to PA *in vitro*, however other hydroxylated catabolites such as 7'-hydroxy or 9'-hydroxy ABA were not detected under the assay conditions used. Further, CYP707As do not appear to be involved in cyclization of 8'-hydroxy ABA to PA since ABA is primarily converted to 8'-hydroxy ABA in short incubation periods and then 8'-hydroxy ABA is autoisomerized to PA (Kushiro et al., 2004; Saito et al., 2004). It is interesting that neither 7'- or 9'-hydroxy ABA were detected in *Arabidopsis* under the assay conditions used; however, it is possible different assay conditions would favour the production of 7'- or 9'-hydroxy catabolites (e.g. changes to pH, temperature, oxygen concentration). Further, it is also possible for the same CYP sub-family in different species to hydroxylate a substrate at different positions. For example, CYP71D13 and CYP71D15 from peppermint (*Mentha piperita*) hydroxylate limonene at the C3 position while CYP71D18 from spearmint (*Mentha spicata*) modifies limonene at the C6 position (Karp et al., 1990; Lupien et al., 1999; Haudenschild et al., 2000). Thus, in species that show significant amounts of either 7'- or 9'-hydroxy ABA, CYP707As present may be 7'- or 9'-hydroxylases. This may be the case for western white pine, as significant amounts of 7'OH ABA are present during

dormancy termination (Figs. 3.5, 4.3, 5.3). Definitive experiments, gained through expression of the *Pm*CYP707As in a system such as yeast, await to test this assumption.

6.4.2 Expression of putative *PmZEP*, *PmNCED*, and *PmCYP707A* genes reveal coordinate regulation of ABA biosynthetic and catabolic genes

There are few reports of the expression of ABA biosynthetic and catabolic genes in seeds. Further, assorted studies that exist in the literature mostly focus on expression during seed development (Nambara and Marion-Poll, 2005). This is the first report of the expression of *ZEP*, *NCED*, and *CYP707A* genes during seed dormancy release in a deeply-dormant species such as conifers. The ability to relate ABA levels and the metabolite levels of PA and DPA (the products of 8'-hydroxylation) is particularly relevant when comparing the expression of white pine *ZEP*, *NCED*, *CYP707A* genes and will be focused on this discussion.

The marginally decreased expression of *PmZEP* in western white pine seeds during moist chilling and dormancy termination, as well as an increase in *PmZEP* expression during seedling growth, correlated with the observed decrease in ABA level during moist chilling and increase in ABA level in the germinated seedling (Figs. 3.3, 6.4a). The increased *PmZEP* in the embryo correlates with an increase in *de novo* synthesized ABA (see Chapter 5) in the embryo, however as the seedling is green at this stage the observed increase could also be associated with the photoprotective xanthophyll cycle (Havaux et al., 2005). The marginal changes and rather 'steady-state' expression observed suggest that *PmZEP* is not a rate-determining step during dormancy release in white pine.

However, it has been suggested that in non-photosynthetic tissues such as seeds and roots, where the concentration of epoxycarotenoids is lower, expression of *ZEP* may have regulatory role (Schwartz et al., 2003a). Supporting the contention that ZEP may be rate-determining in seeds, over- or antisense expression of *Np*ZEP generated plants with heightened or reduced seed dormancy, respectively (Frey et al., 1999). Expression studies in seeds have also correlated increases in ABA to expression of *ZEP*. During seed development in *N. plumbaginifolia*, *NpZEP* mRNA abundance has been shown to peak just before mid-development, at about the time when ABA concentrations are maximal (Audran et al., 1998). One possible explanation for the lack of change in *PmZEP* during

dormancy release and the observed changes in *NpZEP* during seed development is that during seed development when ABA levels are high, ZEP becomes limiting, as ABA levels fall to that present in the mature seed, ZEP is not longer limiting and expression remains somewhat steady especially as the seed/seedling prepares to become photosynthetic and has need of the xanthophyll cycle.

PmNCED1 was the major *NCED*-homologous sequence cloned from white pine during dormancy maintenance and termination (Table 6.1). *PmNCED2* was only cloned during a drought stress treatment on seedlings (Table 6.1). This suggests stage specific and development regulation of the various *NCEDs* in western white pine and is similar to that observed in *Arabidopsis* (see below). *PmNCED1* may be responsible for ABA synthesis during seed during dormancy maintenance and through moist chilling (Fig. 6.4b). It is also a much more likely candidate than *PmZEP* to be rate-determining for ABA biosynthesis in white pine seeds during dormancy maintenance and termination (Fig. 6.4a, b). Expression of *PmNCED1* correlates well with the observed decrease in ABA levels, in both embryo and megagametophyte tissues, during moist chilling and germination (i.e. as ABA levels decline so to does expression of *PmNCED1*) (Fig. 3.3). However, while ABA levels declined during the first 30 d of moist chilling, *PmNCED1* increased slightly (Figs. 3.3, 6.4b). *PmNCED2* expression also increased during this time, although it is far less abundant than *PmNCED1* during moist chilling (Fig. 6.4c, data not shown). Thus, the decline in ABA could be due to increased catabolism (e.g. expression of *CYP707As*) or as a result of changes in the protein levels of *Pm*NCED1 (and perhaps *Pm*NCED2). Alternatively, ABA may have been transported out of the embryo and megagametophyte following transfer to moist chilling.

PmNCED2 was present at relatively low levels during dormancy termination compared to the amount of transcript present at the germinated seedling stage (Fig. 6.4c). As such *PmNCED2* may be responsible for ABA synthesis in the growing seedling (Fig. 6.4c). It should be noted that the higher levels of ABA seen in the germinated seedling could be the result of seedling stress as parts of the germinated seeds were not in contact with the moist substratum (data not shown). However, as pointed out by Finkelstein and Rock (2002) ABA, at a certain endogenous concentration, is necessary for growth. For example, the ABA-deficient *Arabidopsis* mutants *aba1-1*, *aba1-3*, and *aba1-4* are stunted

in their growth due to a reduced capacity to transpire and establish turgor. When these mutants are sprayed with exogenous ABA, cell expansion and normal growth are restored (Finkelstein and Rock, 2002). Thus, the high level of expression of *PmNCED2* could be due to a requirement for ABA needed for optimal seedling growth or the result of stress-related ABA synthesis.

In *Arabidopsis*, *AtNCED3* is the major stress-induced form, although smaller relatively minor increases in *AtNCED2*, *AtNCED5*, and *AtNCED9* also occur during stress (Tan et al., 2003). *AtNCED3*, *AtNCED5*, *AtNCED6*, and *AtNCED9* are expressed during seed development in *Arabidopsis* siliques. *AtNCED3* is expressed predominantly in maternal tissues such as the funiculus (Tan et al., 2003). In addition, *AtNCED6* and *AtNCED9* have been further characterized and shown to be involved in dormancy induction (Lefebvre et al., 2006). Through qPCR analysis from seed tissues collected 10 d after pollination, *AtNCED3* was detected in both empty silique and seed tissues while *AtNCED5*, *AtNCED6*, and *AtNCED9* were only detected in seed tissues. Lefebvre et al. (2006) show that *AtNCED6* and *AtNCED9* are the most abundant *NCEDs* in seed tissues. Further localization of *AtNCED6* and *AtNCED9* through promoter GUS/GFP fusions and *in situ* hydridization showed differences in spatial and temporal expression. *AtNCED6* was specifically expressed in endosperm tissues from before fertilization and throughout endosperm development. *AtNCED9*, in contrast, was expressed in both embryo and endosperm tissues. In accordance with expected gene redundancy in the *NCED* family, seeds from single mutants of *Atnced6* or *Atnced9* displayed similar degrees of dormancy compared to wild-type. *Atnced6 / Atnced9* double mutants showed increased germination rates compared to wild-type; but still showed an intermediary rate of germination, between that of wild-type and the *aba3-1* mutant. Resistance to paclobutrazol during germination was enhanced for both single and double mutants but was greater for double mutants suggesting less GAs were required for *Atnced6*, *Atnced9*, and *Atnced6 / Atnced9* germination (Lefebvre et al., 2006). *AtNCED3* also seems to play a stress-regulatory role in seeds. *Arabidopsis sto1*, a salt-stress tolerant mutant that is allelic to *Atnced3*, can germinate when seeds are imbibed in a high osmotic solution (145 mM NaCl) (Ruggiero et al., 2004). Recently, during microarray analysis of *Arabidopsis Cvi* seeds, *AtNCED6*

and *AtNCED9* mRNAs were found to be more than 10-fold higher in dormant seeds compared to non-dormant seeds (Taylor et al., 2005, references therein).

PmCYP707A1 expression in the embryo and megagametophyte also correlated with the decline in ABA content during moist chilling in western white pine – to an extent. However, during early to mid-moist chilling, the expression of *PmCYP707A1* in both embryo and megagametophyte tissues is not reflective of the decreases in ABA and increases in PA and DPA observed (Figs. 3.3, 3.4, 6.5a). Only relatively small increases in expression of *PmCYP707A1* were observed during the first 60 d of moist chilling; the largest increase in *PmCYP707A1* occurred during 60 to 98 d and into germination (Fig. 6.5a). Yet, the modest increases in *PmCYP707A* expression may account for the increased catabolism through the 8'-hydroxylation pathway; especially since we cannot rule out changes at the protein level (i.e. perhaps the *Pm*CYP707A1 protein became more stable as moist chilling proceeded).

PmCYP707A2 expression did not correlate with the decrease in ABA levels and increase in PA and DPA observed through moist chilling (Figs. 3.3, 6.5b). Its expression decreased significantly during moist chilling and germination in the embryo and during germination in the megagametophyte (Fig. 6.5b). Thus, *PmCYP707A2* does not seem to play major role in the catabolism of ABA during dormancy termination in western white pine – but still contributes nonetheless. Other possibilities exist for the role *PmCYP707A2* may play in seeds: (1) it may have a greater function during seed development and/or in the dormant-imbibed seed since its expression was higher during the water soaks, and (2) it may be present to help counteract the expression of the biosynthetic gene *PmNCED1* (the expression pattern of *PmCYP707A2* was very similar to that of *PmNCED1*). Further expression analyses during seed development and in the dormant imbibed seed would help confirm these assertions.

Changes in the expression of four *CYP707A*s have been studied in *Arabidopsis* Columbia seeds. During mid-seed development, when ABA levels are higher, *CYP707A1* and *CYP707A3* are expressed abundantly; as ABA levels fall during late embryo development *CYP707A1* and *CYP707A3* are downregulated (Kushiro et al., 2004). CYP707A2 plays a major role in the decrease in ABA levels observed during early seed imbibition in non-dormant seeds, when corresponding increases in PA and DPA are seen

(Kushiro et al., 2004). *CYP707A2* transcripts are high in the dry seed and increase to peak within six hours after the start of imbibition; in comparison, only small increases in the CYP707A1 and A3 are seen and occur after 18-24 h imbibition (Kushiro et al., 2004). Further, when comparing *cyp707a2* and *cyp707a3* mutant seeds, only *cyp707a2* seeds show a hyper-dormant phenotype; this correlates with increased ABA levels in the mature seeds of *cyp707a2* plants (Kushiro et al., 2004).

In conclusion, the present work has identified potential ABA biosynthetic and catabolic genes in western white pine from the zeaxthanin epoxidase, 9-*cis* epoxycarotenoid dioxygenase, and abscisic acid 8'-hydroxylase (CYP707A) gene families. It is likely that the synergism between the expression of these genes (and their subsequent proteins) ultimately controls ABA levels during seed dormancy termination. Indeed, *PmNCED1* and *PmCYP707A1* seem likely candidates for the modulation of ABA levels during dormancy termination. As ABA levels decrease through dormancy termination, *PmNCED1* is down-regulated, while expression of *PmCYP707A1* is up-regulated. *PmNCED2* expression in the seedling and it's cloning from drought-stressed tissue suggest this NCED member may be involved in ABA synthesis in vegetative tissues and/or during stress-related ABA synthesis. However, additional information would be gained through a more thorough gene expression analysis of these genes. For example, we do not know the role these genes may play during seed dormancy maintenance or in the rest of the plant. Further, whether these genes are induced by various hormones is unknown (e.g. are they induced by ABA or GA?). Finally, over-expression of these genes in the relevant model system would help define their substrate specificities and cement their roles in seed dormancy termination in western white pine.

CHAPTER 7

Summary discussion

Primary dormancy, considered a 'developmental arrest' that blocks the transition to germination, requires specific environmental cues such as light and moist chilling to overcome constraints and permit germination. Dormancy is still an enigmatic process whose complexity is continually compounded through discovery of new factors involved in dormancy maintenance, release, and germination. Yet these discoveries have strong ramifications for they allow us to further develop and build on 'our current model' of seed dormancy. Strong evidence implicates a pivotal role for ABA in both dormancy induction and dormancy maintenance. However, it is becoming clear that a multitude of hormones participate to modulate dormancy induction, maintenance, termination and germination. In addition to ABA, these include GA, ethylene, cytokinin, auxin, and brassinosteroids (for review see Kucera et al., 2005).

Many of the discoveries made in recent years, in relation to factors controlling dormancy and germination (but also in general), have been from the model plant *Arabidopsis*, chiefly as a consequence of sequence availability and mutant analysis. However, there is also a need to focus our attention on other species for two important reasons. Clearly, the mechanistic and physiological aspects of dormancy are not the same for every species. Thus, our understanding and modeling of seed dormancy is greatly enhanced if new or orthologous factors are characterized from various species. Secondly, there is a need to focus our attention on agronomic and silviculturally important species and to understand the mechanisms in these species so as to make trait improvement and develop markers for traits such as dormancy. This study focused on the dormancy mechanism of the deeply-dormant-coniferous species western white pine, a potentially important timber species for reforestation. Specifically, we sought to improve the propagation of white pine and more thoroughly understand the complexities of the dormancy mechanism in this species by: (1) devising protocols that were more efficient and effective in breaking white pine seed dormancy than current protocols, and (2) elucidating the role of ABA and its metabolism in white pine seed dormancy.

During attempts to improve the dormancy-breaking protocol of western white pine, a number of conclusions became evident regarding the optimal conditions for effective and efficient dormancy release:

a) A critical factor in eliciting adequate dormancy-breakage of western white pine seeds is the maintenance of high seed moisture content during moist chilling (similar to that of the fully imbibed seed). However, high moisture content is not sufficient in itself to promote effective dormancy release and adequate gas exchange, between the seed and its environment, during moist chilling also seems required.

b) Prior to the moist chilling period, a prolonged water soak stimulates higher germination percentages. This water soak is more effective at higher (i.e. 25-27°C) rather than colder temperatures (i.e. ≤21°C).

Conclusions from studies focused on elucidating the role of ABA and its metabolism in western white pine seed dormancy provide evidence that ABA plays a key role in the maintenance of dormancy and can be summarized as follows:

a) Germination capacity of western white pine seeds is correlated to declines in ABA levels. A decline in ABA alone, below a threshold amount, may be sufficient to release dormancy of western white pine seeds; however, changes to ABA sensitivity (data not shown) and other hormonal signaling paths (e.g. GA) may also be important prerequisites.

b) During dormancy termination (e.g. moist chilling), the relative rate of ABA catabolism (or transport) exceeds ABA biosynthesis. Moreover, effective dormancy breaking treatments seem to increase the *capacity* for ABA catabolism and/or *reduce* synthesis of ABA when seeds are subsequently placed in germination conditions; thus the net effect is a shift to favor catabolism (or transport).

c) In addition to providing mechanical restraint, the seed coat and/or nucellar membrane also seem to modulate ABA homeostasis; however, the precise mechanisms of this control remains to be determined.

d) Catabolism of ABA in western white pine seeds occurs through 8'- and 7'-hydroxylation pathways and conjugation to glucose but does not seem to be metabolized through the 9'-hydroxylation pathway during dormancy termination. Generally, increases in ABA catabolite levels do not account for the observed declines in ABA during dormancy termination. Thus, other unmeasured pathways and end-points in catabolism exist in western white pine seeds. Transport of ABA or metabolites out of the seed is another viable option to explain this discrepancy.

e) Expression analyses of putative *ZEP*, *NCED*, and *CYP707A* genes suggest these genes are regulated at the level of transcription during dormancy termination. Down-regulation of *PmZEP* and *PmNCED1* expression through dormancy termination (moist chilling) partially agrees with observed levels of ABA. Up-regulation of *PmCYP707A1* during dormancy termination also correlates, to an extent, with declining ABA levels as well as increases in PA and DPA.

f) Similar to *Arabidopsis*, analyses of *NCED* and *CYP707A* putative genes from western white pine suggests overlapping but distinct developmental roles for these gene families in ABA biosynthesis and catabolism, respectively. For example, one *NCED* gene (*PmNCED1*) is expressed predominantly in the seed during dormancy while the other (*PmNCED2*) is expressed in the seedling.

Concluding Statement

The dormancy mechanism of western white pine is multi-faceted. One of the main factors implicated in the mechanism is the seed coat (Hoff et al., 1987), which exerts mechanical resistance to radicle protrusion (germination) and may act as a barrier to gas exchange. As a barrier to gas exchange it can feasibly interfere with the oxidative

catabolism of ABA. ABA is another chief component of the dormancy mechanism in western white pine seeds. The physiological manifestation of dormancy in western white pine, as with other physiological and developmental processes controlled by ABA, is ultimately dictated by the amount of endogenous ABA present in a given cell or tissue. These 'bioactive ABA pools' are controlled by the fine-tuning of the rates of *de novo* biosynthesis and catabolism (or transport).

While most of the ABA biosynthetic pathway has now been dissected and genes encoding the enzymes in the pathway cloned (chiefly in *Arabidopsis*), many questions remain as to the exact molecular mechanisms and regulatory points controlling the synthesis of ABA precursors. The gene(s) responsible for formation of neoxanthin (NSY) and the *trans*-to-*cis* isomerization reactions of neoxanthin and violaxanthin remain unidentified. In addition to NCED (the 'first committed step' to ABA synthesis) these genes could be key rate-determining control points leading to formation of ABA. Another ambiguity is whether 9-*cis*-violaxanthin or 9'-*cis*-neoxanthin acts as the predominant precursor; however, recent evidence suggests this may be tissue- or developmental-stage dependent (Rodrigo et al., 2006).

With regard to catabolism of ABA, recent identification, in *Arabidopsis*, of ABA 8'-hydroxylase genes as the cytochrome P450 *CYP707A* subfamily and a glycosyltransferase that specifically adds glucose to the 1' carbon group of (+)-ABA has opened up new avenues to explore the molecular mechanisms controlling ABA levels (Kushiro et al., 2004; Lim et al., 2005). We are now in a position to simultaneously monitor various developmental phases of the plant's lifecycle including during seed development (dormancy induction), dormancy maintenance, termination, and germination for expression of both synthesis and catabolism genes. This thesis study is but one of a handful of studies (perhaps together with Kushiro et al., 2004) which have presented such analyses and is the first of its kind in a deeply dormant coniferous seed. Our results presented here begin to reveal how co-ordinate simultaneous expression of ABA biosynthetic and catabolism genes helps regulate ABA levels (at the level of transcription). However, very little is known about the regulation of synthesis and catabolism genes at post-transcriptional levels and whether there is a potential for post-translational regulation – this area deserves more

attention. The identification of factors which regulate the expression of ABA biosynthesis and catabolism genes both at the level of transcription (e.g. characterization of important *cis* regulatory regions within promoters and also transcription factors which bind) and at the level of translation (e.g are proteins degraded through a ubiquitin-mediated 26S proteosome pathway?) will be important. Also, do some of the same factors that regulate biosynthesis genes also regulate catabolism genes? For example, ABA-responsive elements (ABREs) are present in the promoters of both *Arabidopsis NCED* and *CYP707A* genes (data not shown) and may bind common factors (e.g. basic region leucine zipper proteins such as ABI5 or the like); this may be a mechanism for the feed-back and feed-forward regulation of synthesis and catabolism genes by ABA. Further upstream in the regulatory cascade, it will be important to determine how light and cold specifically trigger changes to the seed's ability to synthesize and catabolise ABA. For example, do some of the same factors which have recently been shown to modulate GA synthesis genes (e.g. the basic helix-loop-helix proteins SPATUALA and PIL5; Penfield et al., 2005) also cross-talk with the ABA pathway and modulate ABA synthesis and catabolism?

There are also many more aspects specific to the dormancy mechanism of western white pine, especially in relation to ABA, that deserve follow up studies. Transport of ABA and its metabolites within the seed and between the seed and its surrounding environment is unknown; this is true for all plants where movement and transport of ABA is still a poorly understood subject. Further, whether the oxygen consuming/diffusing capacity of the seed coat/nucellar membrane change through moist chilling (dormancy termination) in western white pine is unknown; availability of oxygen to the embryo and megagametophyte tissues may subsequently affect ABA homeostasis (through the oxidative catabolism of ABA). Finally, the identification of candidate *CYP707A* genes in western white pine seeds should be followed with confirmation of these genes as ABA 8'-hydroxylase (or perhaps 7'- or 9'-hydroxylase) enzymes.

In conclusion, this study has cemented ABA as a causal factor for dormancy maintenance in western white pine. Changes to ABA homeostasis towards a state that favours ABA catabolism over biosynthesis ultimately contribute to dormancy

termination in this deeply dormant seed. Further experiments are greatly anticipated and will further our knowledge of this conifer model for seed dormancy and ABA metabolism in general.

APPENDIX

Supplemental figures

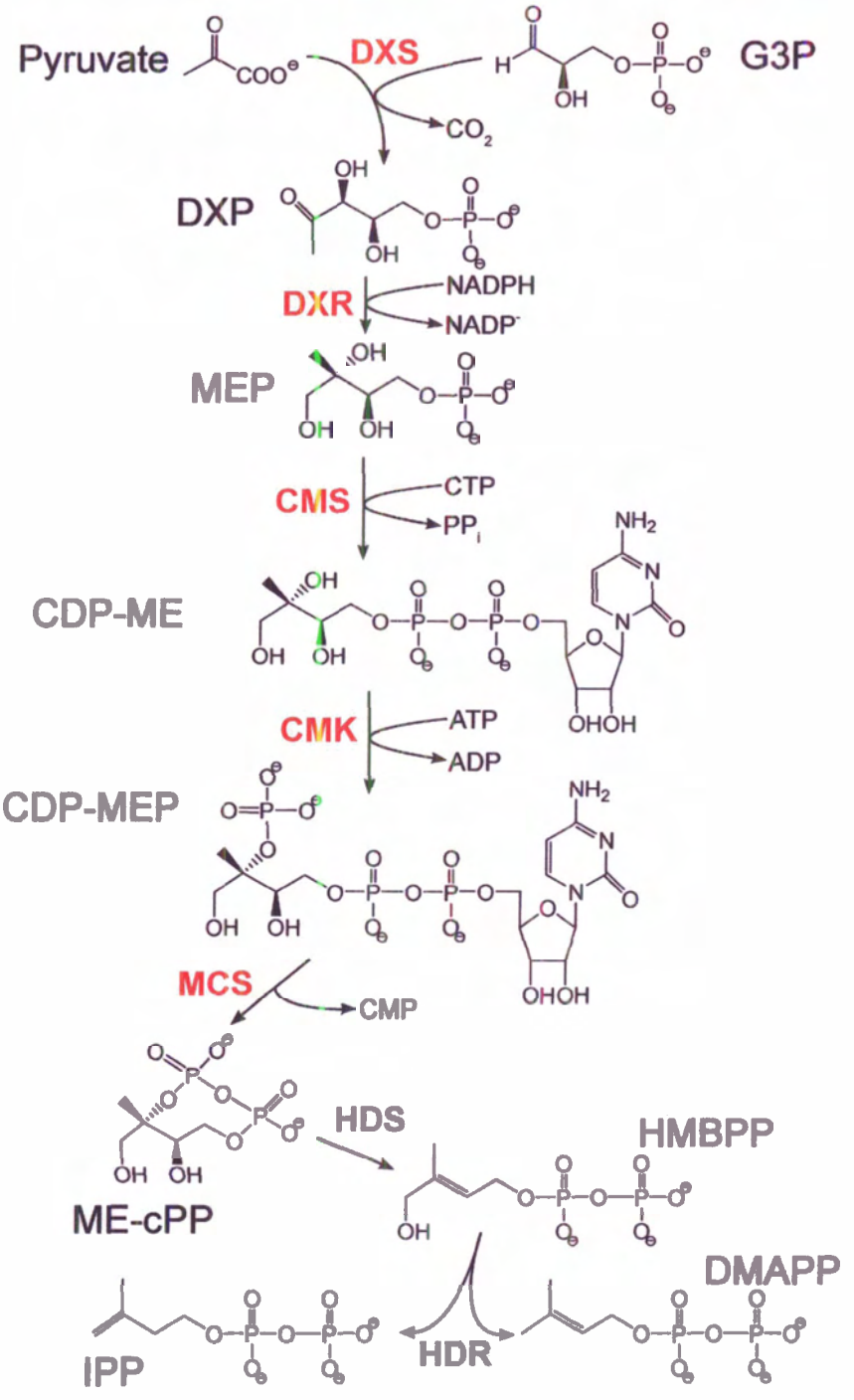

Fig. A1. Synthesis of IPP and DMAPP via the plastidial MEP pathway. G3P, D-Glyceraldehyde-3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, methylerythritol 4-phosphate; CDP-ME, 4-diphosphocytidyl methylerythritol; CDP-MEP, CDP-ME 2-phosphate ; ME-cPP, ME 2,4-cyclodiphosphate; HMBPP, hydroxymethylbutenyl diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate. DXS, DXP synthase; DXR, DXP reductoisomerase; CMS, CDP-ME synthase; CMK, CDP-ME kinase; MCS, ME-cPP synthase; HDS, HMBPP synthase; HDR, HMBPP reductase (or IDS, IPP / DMAPP synthase). IPP and DMAPP can be inter-converted by the enzyme IDI (IPP isomerase).

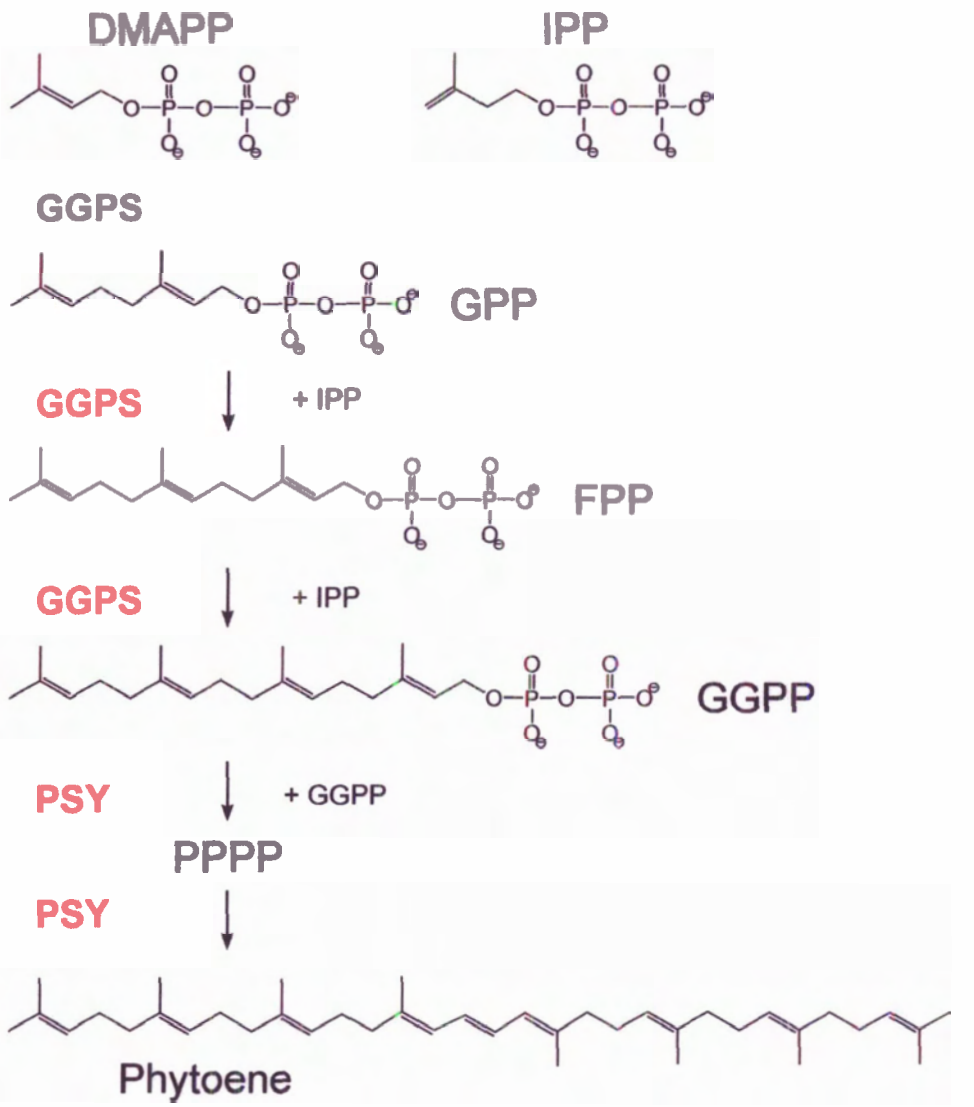

Fig. A2. Synthesis of phytoene from DMAPP and IPP in the plastid. GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, Geranylgeranyl pyrophosphate; PPPP, prephytoene diphosphate. GGPS, GGPP synthase; PSY, Phytoene synthase.

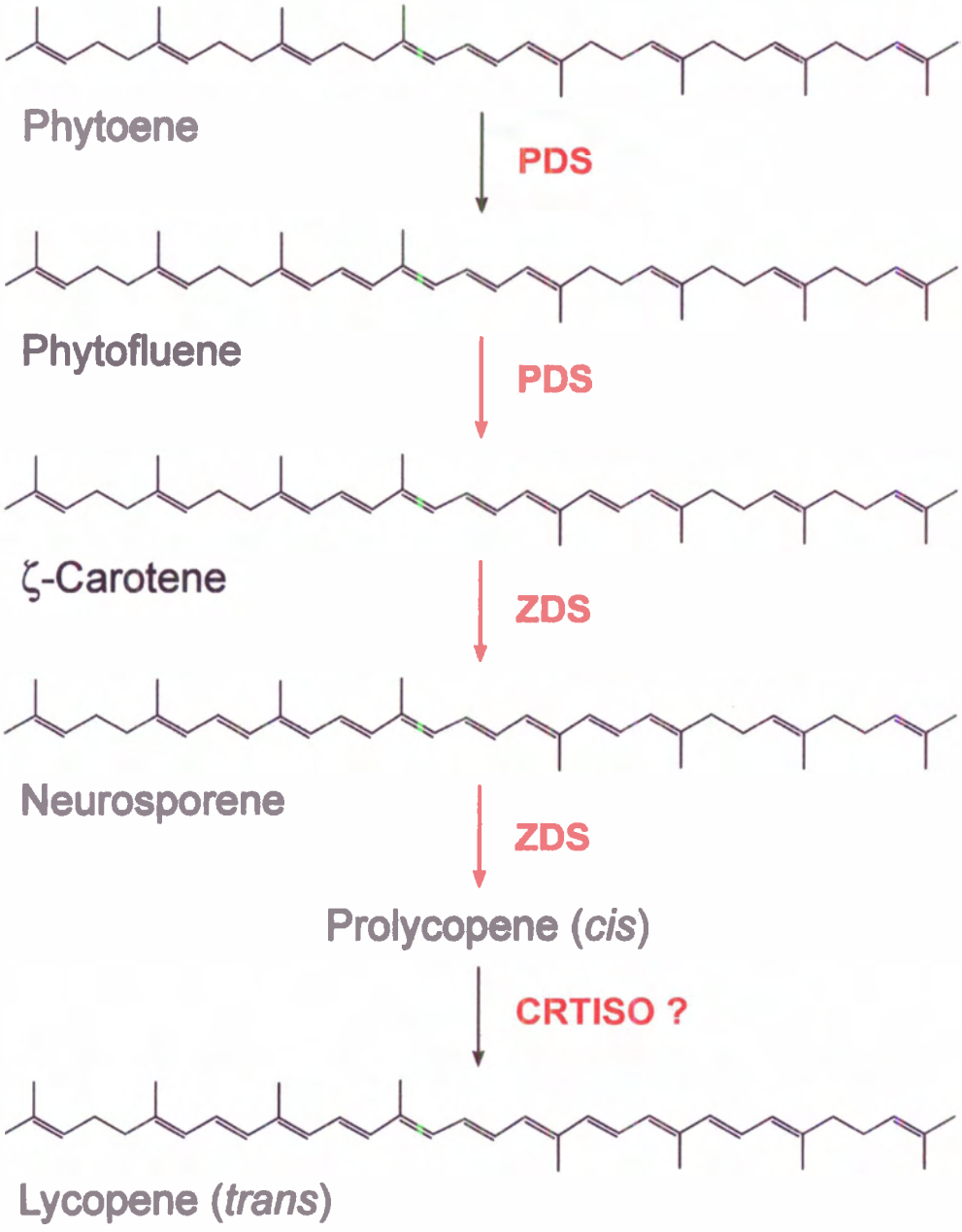

Fig. A3. Desaturation and isomerisation of phytoene to form lycopene. PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase.

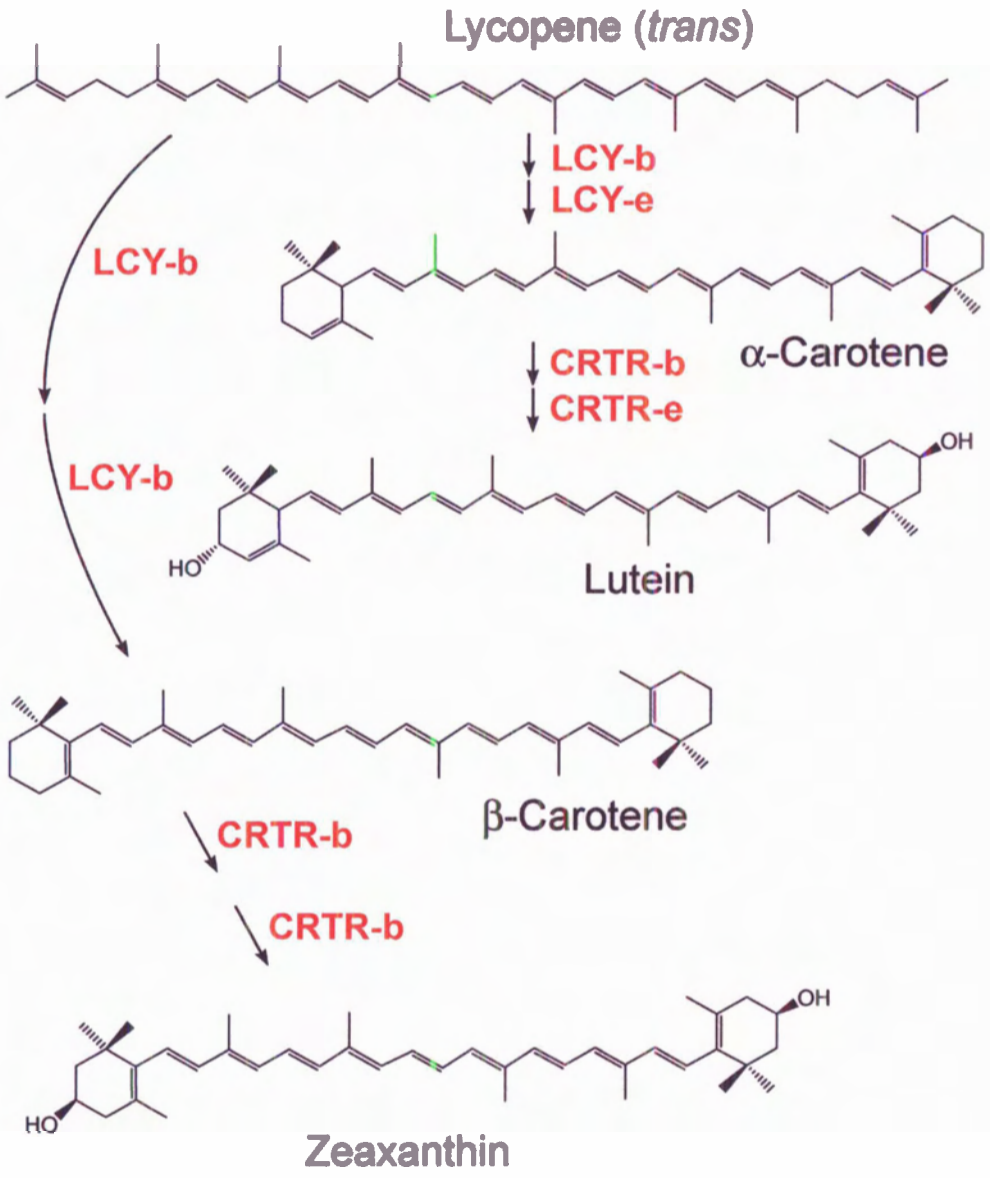

Fig. A4. Cyclisation of lycopene via the α- and β-branchpoints. The branch devoted to ABA synthesis forms β-carotene (2 steps via γ-carotene) and zeaxanthin (2 steps via β-cryptoxanthin). LCY-b, lycopene-β-cyclase; CRTR-b, β-ring hydroxylase; LCY-e, lycopene-ε-cyclase; CRTR-e, ε-ring hydroxylase.

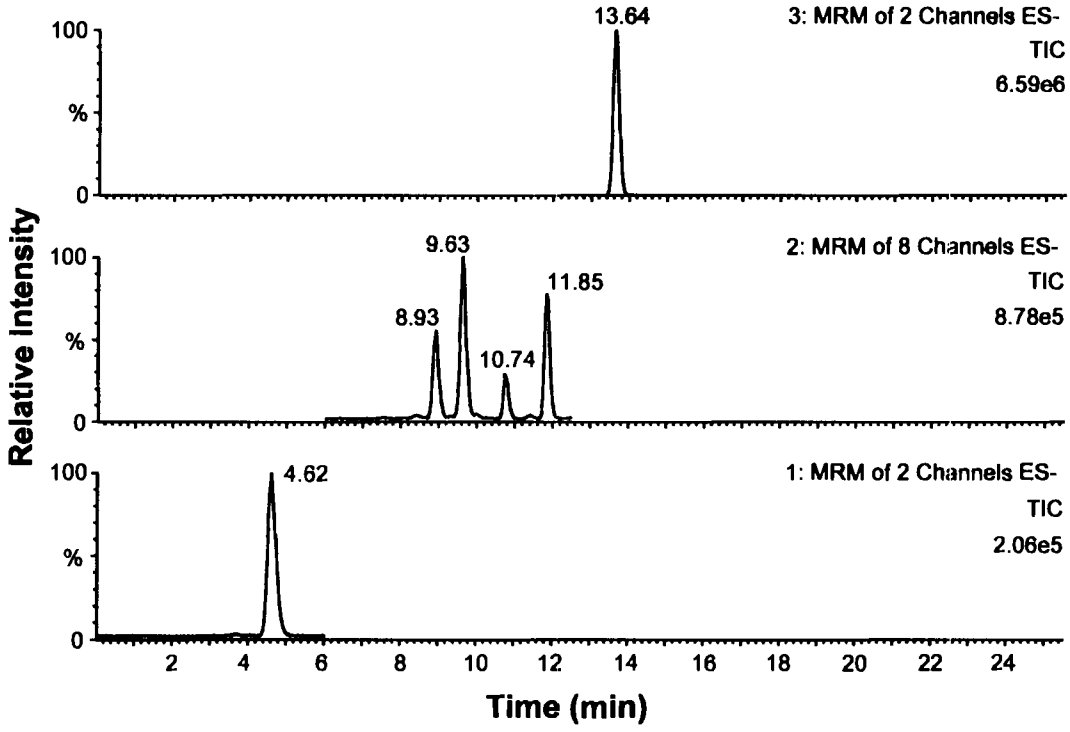

Fig. A5. Total ion current chromatogram for ABA and ABA metabolites and their respective deuterium-labeled analogs separated by reversed phase liquid chromatography (RP-LC). Relative intensity is plotted on the y-axis. The retention times for the compounds are: 4.62 min for DPA/d3-DPA, 8.93 min for ABA-GE/d5-ABA-GE, 9.63 min for PA/d3-PA, 10.74 min for 7'OH ABA/d4-7'OH ABA, 11.85 min for neo-PA/d3-neo-PA, and 13.64 min for ABA/d4-ABA (see section 4.2.6).

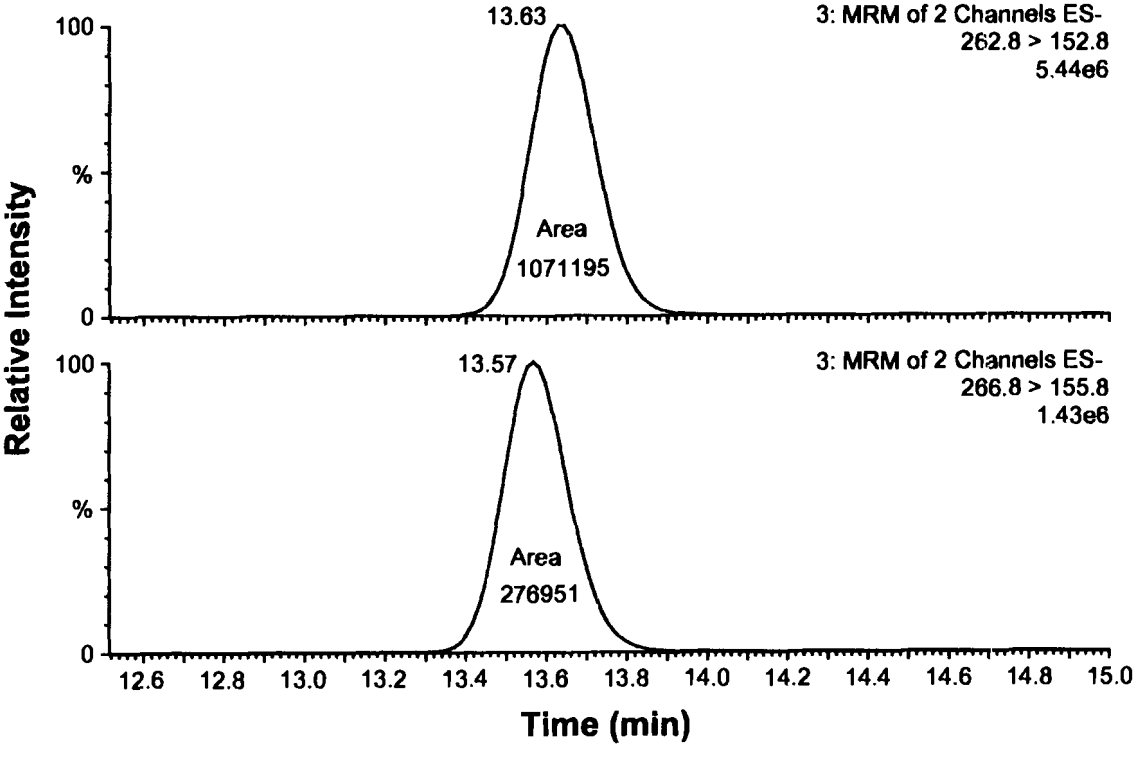

Fig. A6. RP-LC ES-/MS/MS chromatogram for ABA and the internal standard d4-ABA under multiple reaction monitoring (MRM) conditions (see section 3.2.4 and 4.2.6). By monitoring the deprotonated parent (product) ion to predominant daughter ion transition in MRM mode, co-eluting peaks for ABA (*m/z* 262.8 > 152.8) and d4-ABA (*m/z* 266.8 > 155.8) are resolved.

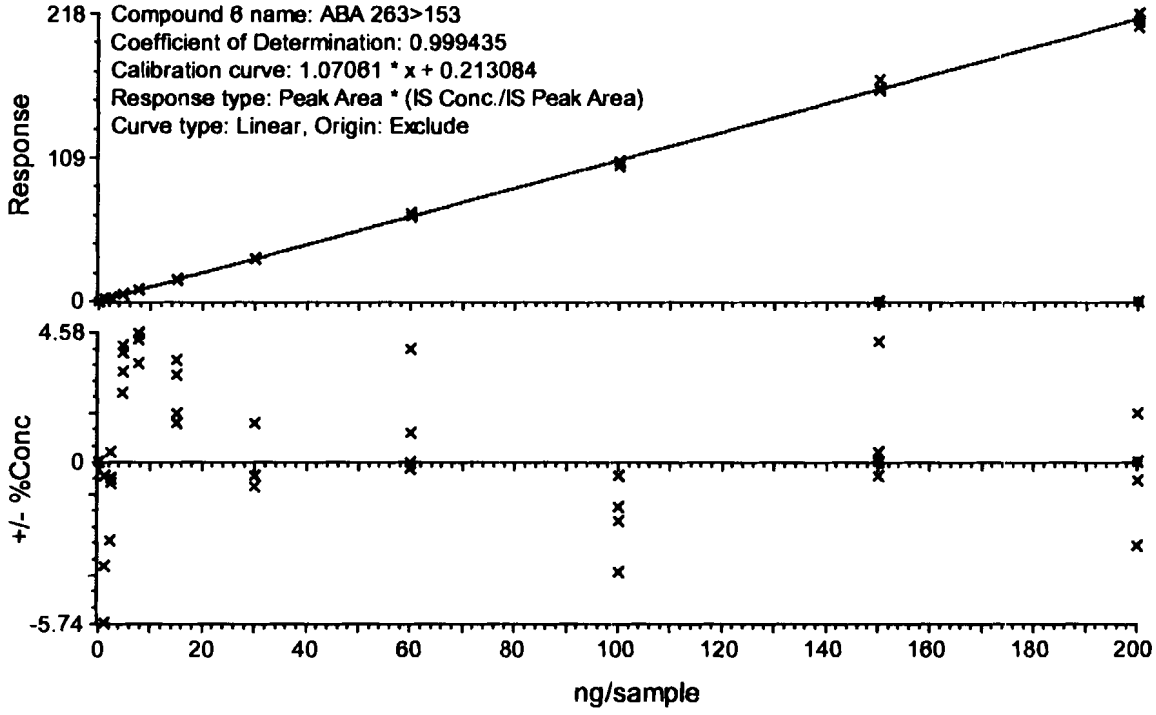

Fig. A7. Standard curve for the quantification of ABA. The area beneath the MRM product ion peak was determined for each analyte and internal standard in a dilution series (see Fig. A6). Standard curves were created for the 6 different compounds by plotting the known concentration of each unlabeled compound against the calculated response for each standard solution. Standard curves for each compound were generated using the proprietary MassLynx software (Waters, Milford, MA).

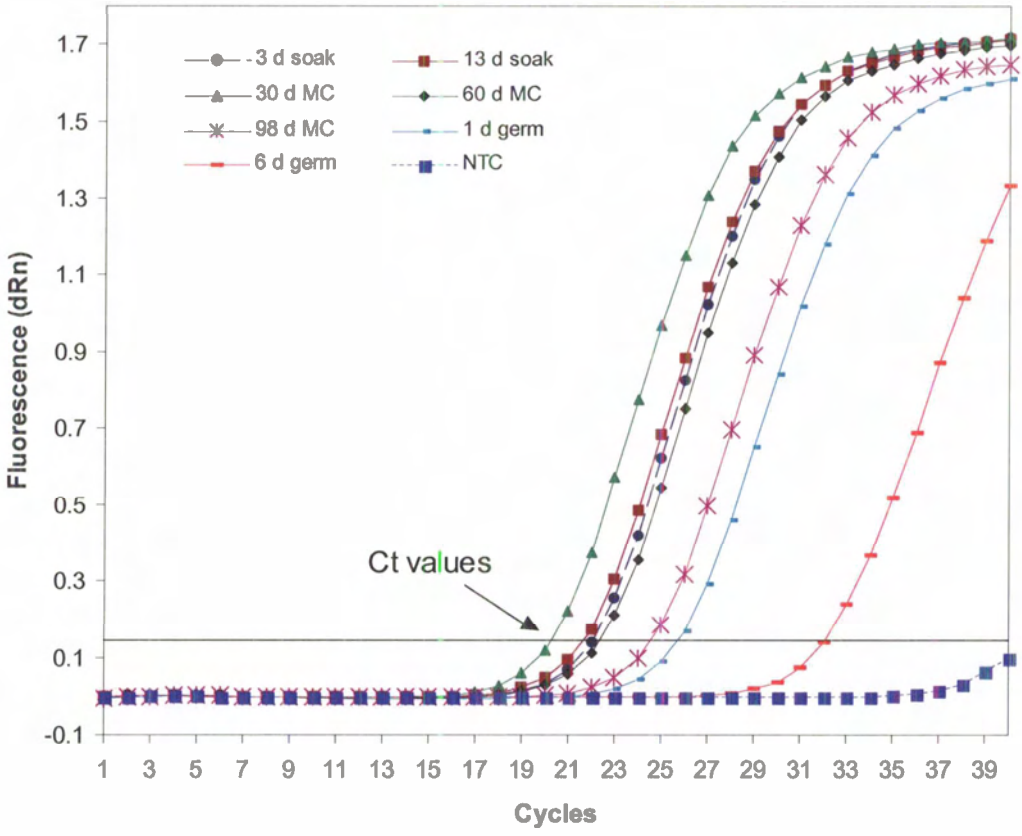

Fig. A8. Amplification plot for the *PmNCED1* 113 bp amplicon. PCR cycle number is plotted against fluorescence (dRn). dRn is the baseline subtracted fluorescence reading normalized to the reference dye ROX. germ, days in germination conditions; MC, moist chilling; NTC, no template (cDNA) control.

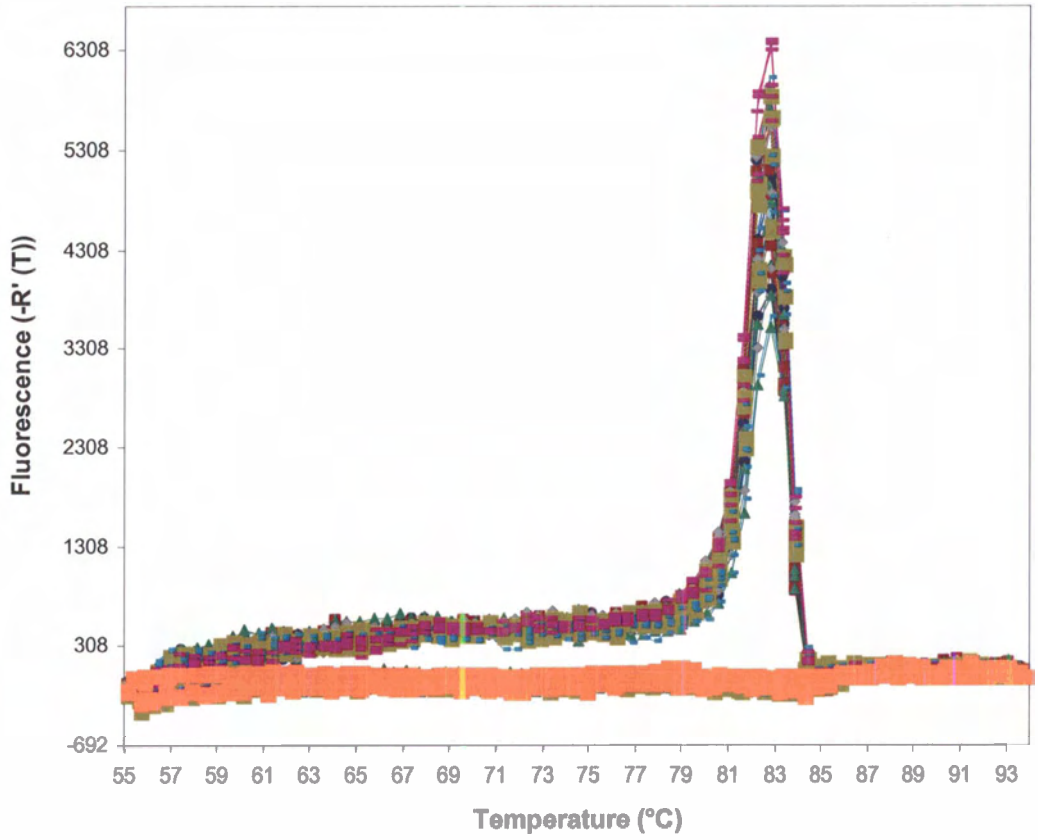

Fig. A9. Product dissociation curve for *PmCYP707A1* 235 bp amplicon. The first derivative of raw fluorescence is plotted against temperature. The single peak at approx. 83°C indicates a single PCR product is being amplified in these samples.

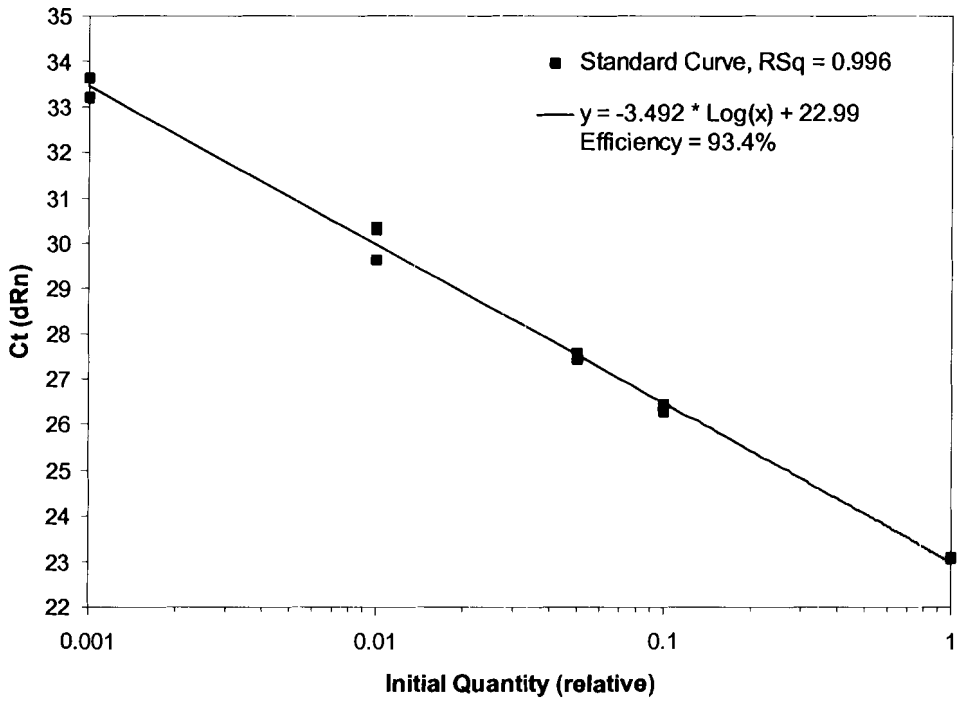

Fig. A10. Standard curve for *PmCYP707A2* 250 bp amplicon generated from a dilution series of 13 d soak cDNA. Standard curves were generated to calculate amplification efficiency of primer pairs. In this example amplification efficiency was calculated for primers CYP_q5 and CYP_q6.

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