J Plant Growth Regul (2007) 26:46–54
DOI: 10.1007/s00344-006-0035-4

J P G R
Journal of Plant Growth Regulation

Disrupting Abscisic Acid Homeostasis in Western White Pine (*Pinus monticola* Dougl. Ex D. Don) Seeds Induces Dormancy Termination and Changes in Abscisic Acid Catabolites

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Abstract

To investigate the role of abscisic acid (ABA) biosynthesis and catabolism in dormant imbibed seeds of western white pine (*Pinus monticola*), ABA and selected catabolites were measured during a combined treatment of the ABA biosynthesis inhibitor fluridone, and gibberellic acid (GA). Fluridone in combination with GA effectively disrupted ABA homeostasis and replaced the approximately 90-day moist chilling period normally required to break dormancy in this species. Individually, both fluridone and GA treatments decreased ABA levels in the embryos and megagametophytes of white pine seeds compared to a water control; however, combined fluridone/GA treatment, the only treatment to terminate dormancy effectively, led to the greatest decline in ABA content. Fluridone treatments revealed that a high degree of ABA turnover/transport occurred in western white pine seeds during the initial stages of dormancy maintenance; at this time, ABA levels decreased by approximately two-thirds in both embryo and megagametophyte tissues. Gibberellic acid treatments, both alone and in combination with fluridone, suggested that GA acted transiently to disrupt ABA homeostasis by shifting the ratio between biosynthesis and catabolism to favor ABA catabolism or transport. Increases in phaseic acid (PA) and dihydrophaseic acid (DPA) were observed during fluridone/GA treatments; however, increases in ABA metabolites did not account for the reduction in ABA observed; additional catabolism and/or transport of ABA and selected metabolites in all probability accounts for this discrepancy. Finally, levels of 7′ hydroxy-ABA (7′OH-ABA) were higher in dormant-imbibed seeds, suggesting that metabolism through this pathway is increased in seeds that maintain higher levels of ABA, perhaps as a means to further regulate ABA homeostasis.

Key words: Abscisic acid metabolism; Fluridone; Germination; Seed dormancy; *Pinus*

Received: 4 April 2006; accepted: 22 August 2006; Online publication: 16 February 2007

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Introduction

Western white pine (*Pinus monticola* Dougl. Ex D. Don) seeds exhibit pronounced primary dormancy at maturity. Under laboratory or nursery conditions, dormancy-breaking procedures for western white pine rely on a moist-chilling treatment of approximately 90 days, which is generally preceded by a water soak of up to 14 days (Feurtado and others 2003). Seed dormancy in this species is primarily ''coat-enhanced,'' being 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). Abscisic acid levels in embryos and megagametophytes decrease significantly during moist chilling, 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 because ABA levels remain high (Feurtado and others 2004).

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 and others 1995; Bianco and others 1997; Le Page-Degivry and others 1997; Yoshioka and others 1998; Grappin and others 2000; Ali-Rachedi and others 2004). To prove that ABA biosynthesis is required for dormancy maintenance, these studies used the herbicide fluridone [1-methyl-3-phenyl-5-(3-trifluoromethyl-(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). For instance, when dormant *Nicotiana plumbaginifolia* 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 after-ripened seed. In addition, exogenous application of gibberellic acid (GA_3) also initiates a decline in ABA levels in the dormant seed, although the decrease is not as pronounced as that induced by fluridone treatment (Grappin and others 2000).

The dynamic levels of ABA and ABA catabolites present in seeds are the result of the constant flux of biosynthesis versus catabolism (Nambara and Marion-Poll 2005). Catabolism of ABA can occur through a multitude of pathways, the nature of which often depend on the species, developmental stage 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 subfamily as ABA 8′ hydroxylases represents a major step forward in our knowledge of ABA catabolism (Kushiro and others 2004; Saito and others 2004). Further reduction of PA can take place to form dihydrophaseic acid (DPA). Abscisic acid 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 pathways include formation of 7′ hydroxy-ABA (7′OH-ABA) and 9′ hydroxy-ABA and its cyclized form neo-PA (Zeevaart 1999; Zhou and others 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?

Materials and Methods

Fluridone/GA Treatments

Dry seeds (western white pine seed lot 08006, Feurtado and others 2004) were allowed to warm to room temperature for approximately 4–6 h from storage at –20°C before soaking. Seeds were soaked for 8 days at 25°C in a 10-l water bath with 5 l water exchanged daily. Sterilization was achieved by soaking seeds in 3% H_2O_2 (v/v) for 10 min and then rinsing them several times with de-ionized distilled H_2O. Seeds were then soaked in 50-ml Falcon tubes (under gentle agitation) with 1 of 4 different treatments. (1) Control: water; (2) GA: 50 μM GA_{4+7} (Imperial Chemical Industries, UK), (3) fluridone: 100 μM fluridone (LGC Promochem, Teddington, Middlesex, UK), (4) fluridone/GA: 100 μM fluridone and 50 μM GA_{4+7}. To avoid precipitation, fluridone, dissolved in DMSO, was added to rapidly-stirred water, and 0.05% Tween 20 (Sigma-Aldrich, Oakville, Ontario, Canada) was added as a surfactant. GA_{4+7} was dissolved in ethanol. Controls contained the appropriate amounts of ethanol and DMSO and

all solutions contained 0.05% Tween 20. After 3 days, seeds were surface-dried and placed on Whatman 3MM paper supported by 15-ply K-22 germination paper (Seedburo Equipment Company, Chicago, IL, USA) in deep-dish 9-cm Petri plates that had been moistened with approximately 23 ml of the aforementioned treatment solutions. Seeds were then transferred to germination conditions (23°C, 16-h photoperiod, and light intensity ~100 μmol m^{-2} s^{-1}). Germination was monitored for 30 days.

Extraction of ABA and ABA Metabolites

Seeds were dissected into embryo and megagametophyte at various times during fluridone and/or GA treatments. Three replicate extractions were carried out on independent seed batches of seed lot 08006. During dissection, seedcoats were discarded because it had previously been determined that the amounts of ABA and ABA metabolites in the seedcoat are low after prolonged water soaks (Feurtado and others 2004). Collection of material and extraction of ABA and metabolites followed procedures outlined in Feurtado and others (2004), with the following modifications. Twenty embryos were used for extraction; ungerminated samples were approximately 20 mg DW (dry weight), whereas germinated samples were approximately 40 mg DW. Megagametophyte samples were weighed into 150 mg DW aliquots (20 megagametophytes were approx. 200 mg DW). Ungerminated seeds were collected at all stages except for during 7–9 days in germination conditions when germinated seeds with 1–1.5 cm radicles were collected.

Quantification of ABA and ABA Metabolites

Quantification of ABA and ABA metabolites was achieved as described in Feurtado and others (2004), with additional modifications necessary to incorporate neo-PA, a new metabolite produced by 9′ hydroxylation of ABA, into the method. Synthesized (–)-neo-PA was used to generate a standard curve along with its deuterium-labeled internal standard, (–)-8′,8′,8′-d_3 neoPA (Zhou and others 2004).

Quantitative analysis was performed by high performance liquid chromatography (HPLC) and electrospray ionization-tandem mass spectrometry (ESI-MS/MS) with multiple reaction monitoring (MRM). Electrospray parameters were as follows: 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 74 l/h; desolvation gas flow 700 l/h. Tandem mass spectrometry conditions were as follows: low and high mass resolution 12.0 for both mass analyzers; ion energies 1 and 2, respectively, 1.0 and 2.0 V; entrance and exit potentials 10 V; detector (multiplier) gain 650 V. Multiple reaction monitoring was performed using a collision cell (Ar) gas pressure of approximately 5.4×10^{-3} mbar and collision energies of 10 (ABA), 12 (ABA-GE), 13 (PA and neo-PA), or 18 V (DPA and 7′OH-ABA) to induce dissociation of the corresponding deprotonated molecular ions. Three MRM scan functions were used during the 25 min HPLC run. Multiple reaction monitoring Function 1 contained DPA/d_3 DPA (eluting at 4.3 min; with precursor- to product-ion transitions 281 > 171/284 > 174, respectively); Function 2 contained ABA-GE/d_5 ABA-GE (8.9 min; 425 > 263/430 > 268), PA/d_3 PA (9.6 min; 279 > 139/282 > 142), 7′OH-ABA/d_4 7′OH-ABA (10.7 min; 279 > 151/283 > 154), and neo-PA / d_3 neo-PA (11.7 min; 279 > 205/282 > 208); and Function 3 contained ABA/d_4 ABA (13.6 min; 263 > 153/267 > 156). To achieve accurate quantification, calibration curves were generated using standard solutions containing 0, 2.5, 5, 10, 25, 50, 100, 200, 500, 750, or 1,000 pg/μl of each analyte, together with 100 pg/μl of each deuterated internal standard. Regression curves were then plotted, and samples were quantified, using MassLynx v.4.0 software (Waters, Milford, MA, USA).

Results and Discussion

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-day soak in water at 25°C yielded 17% germination (Figure 1; Control). This lengthy pre-soak is needed for efficient dormancy breakage during subsequent moist chilling (Feurtado and others 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, respectively. Effective dormancy-termination was elicited by the combination of GA_{4+7} and fluridone, in which germination was 77% after 30 days (Figure 1, fluridone/GA). However, even this combined treatment was not as efficient as 98 days of moist chilling, which elicits approximately 90% germination, and seeds germinate faster and more synchronously (Feurtado and others 2004).

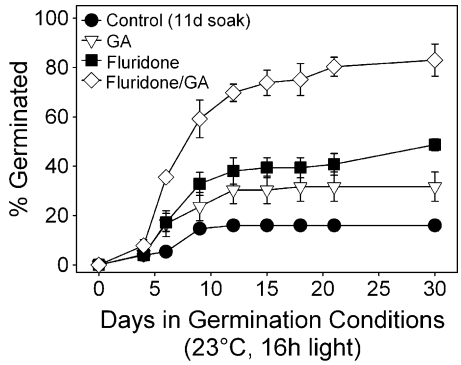

Figure 1. Germination of western white pine seeds after fluridone and/or gibberellic acid (GA) treatment. Seeds were soaked for 8 days in water and then for 3 days in 100 μM fluridone and/or 50 μM GA prior to incubation in germination conditions on the same solutions. Germination was monitored for 30 days 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).

The initial finding that GA did not stimulate germination appreciably was not surprising because exogenous GA 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 and others 2004). As noted at the beginning of this article, 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 and others 2000; Ali-Rachedi and others 2004). However, fluridone prevents thermodormancy in lettuce seeds at 28°C; but when the temperature is increased to 33°C fluridone alone is no longer effective, and the addition of 2 mM GA_3 is needed to restore germination (Gonai and others 2004). Although it is unknown why such a high level of GA_3 (2 mM) is required, the authors suggest that an increased sensitivity to ABA may account for the requirement for GA at 33°C (Gonai and others 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 (for example, 90 days) to fully terminate dormancy (Feurtado and others 2004). Similarly, in yellow cedar (*Chamaecyparis nootkatensis*) seeds, which normally require a 90-day dormancy-breaking treatment (30 days of warm, moist conditions followed by 60 days 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 and others 2001).

Changes in ABA During Dormancy Maintenance, Termination, and Germination

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 remained constant throughout the initial treatment soak in the embryo, and this was similar to the pattern in the megagametophyte (Figure 2). However, ABA levels in megagametophytes were approximately fivefold 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 (that is, at 3 days in germination conditions) (Figure 2). After the peak in germination, as seed populations returned to maintain a ''dormant'' state, ABA levels in embryo and megagametophyte tissues increased in all treatments except in those seeds treated with fluridone (Figure 2).

The transient decline in ABA levels in embryos and megagametophytes of dormant-control water-soaked seeds of western white pine by approximately one-half by 3 days in germination conditions is noteworthy and has been found in numerous species (however, the decline in dormant-imbibed seeds is often far less than that of their nondormant counterparts; see further discussion below). A decrease in ABA upon imbibition (or placement in conditions normally conducive to germination) occurs in dormant seeds of barley, *Arabidopsis* Cvi and C24 ecotypes, Douglas fir, as well as in thermodormant lettuce seeds imbibed at 33°C (Bianco and others 1997; Corbineau and others 2002; Ali-Rachedi and others 2004; Gonai and others 2004; Millar and others 2006). In species where ABA has been assayed at later time points in the dormant-imbibed seeds (*Arabidopsis* Cvi, Douglas-fir, and thermodormant lettuce seeds), the decrease in ABA

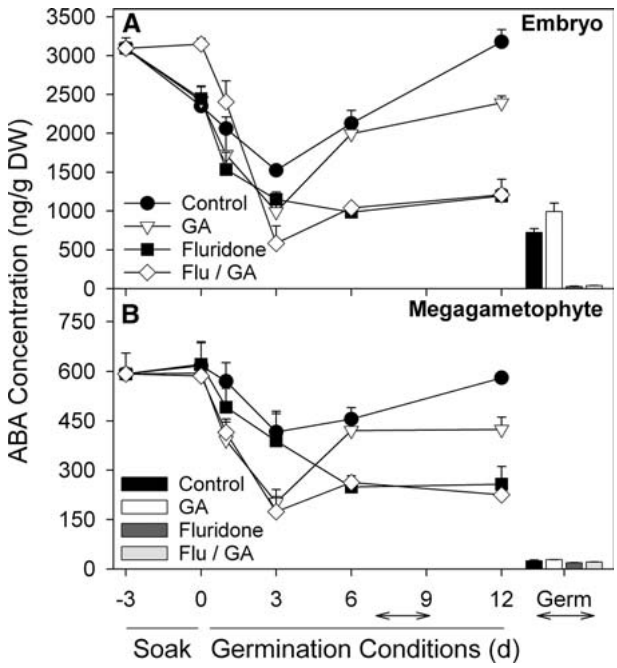

Figure 2. Changes in abscisic acid (ABA) within the embryo (**a**) and megagametophyte (**b**) during fluridone/GA treatments. Ungerminated seeds were analyzed before and after a 3-day soak in 100 μM fluridone and/or 50 μM GA and throughout 12 days in germination conditions (15-day total treatment time). Germinated seeds (Germ, bars) were analyzed between 7 and 9 days in germination conditions, as 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).

is transient, and there is an increase in ABA levels as dormancy is maintained (Bianco and others 1997; Corbineau and others 2002; Ali-Rachedi and others 2004; Gonai and others 2004). Thus, at least for some species, this pattern 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 (which for some seeds represents the intial stages of imbibition), there is a shift in ABA homeostasis to favor ABA biosynthesis, and ABA levels increase as dormancy is maintained.

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 by the fluridone/GA treatment (megagametophyte) until 3 days into germination conditions (Figure 2). After 3 days in germination conditions, ABA in embryos and megagametophytes increased in GA-treated 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. This may be because the GA effect on ABA metabolism (for example, one that promotes catabolism) was only transitory (until 3 days) and because a later shift in ABA homeostasis to favor ABA biosynthesis (from 3 to 6 days) prevented further germination. This increased germination did not occur in fluridone-treated seeds because 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 thermodormant lettuce seeds (Ali-Rachedi and others 2004; Gonai and others 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). Abscisic acid 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 that ABA was simply being catabolized 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 metabolized (transported) during the first day in germination conditions. During days 1–3, a total of 200 ng/g DW was metabolized per day; during days 3 to 6, the catabolism rate declined to only 50 ng/g DW per day, and it did not decrease thereafter (Figure 2a). Similar trends were revealed in the megagametophyte (Figure 2b). Thus, it seems that ABA catabolism (or transport) ceases by 6 days in germination conditions. The fluridone data support the notion that under normal circumstances, that is, in dormant imbibed (control) seeds, there is a shift in ABA homeostasis from a catabolic state toward one where ABA biosynthesis predominates as dormancy maintenance is continued.

The fluridone/GA-treated seeds further reveal the nature of the dormancy mechanism in western white pine, as well as a more precise action of GA. Because little or no ABA is synthesized during fluridone treatment, GA must have an effect on ABA catabolism or transport, because 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

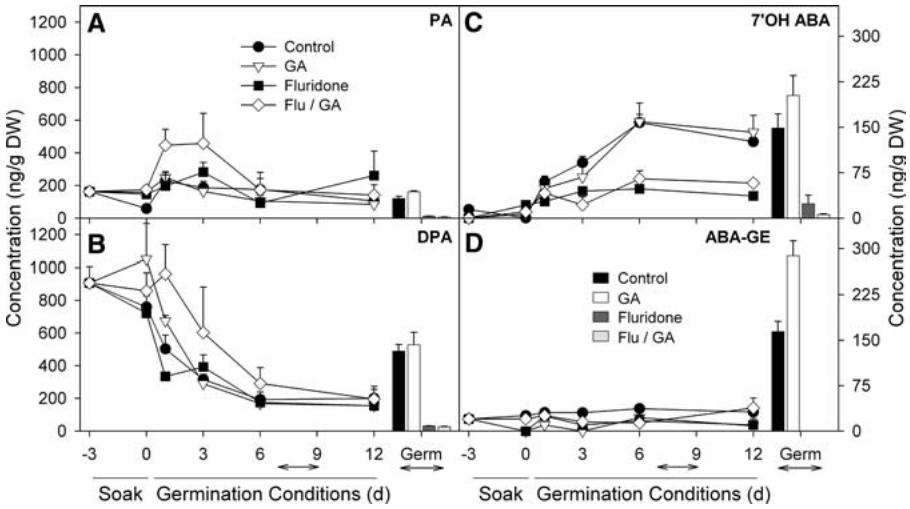

Figure 3. Changes in ABA metabolites within the embryo during fluridone/GA treatments. (**a**) PA; (**b**) DPA; (**c**) 7′OH ABA; (**d**) ABA-GE. Embryos of ungerminated seeds were analyzed before and after a 3-day soak in 100 μM fluridone and/or 50 μM GA and throughout 12 days in germination conditions (15 days total treatment time). Germinated seeds (Germ, bars) were analyzed between 7 and 9 days in germination conditions, as 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).

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 the stimulation of ABA metabolism toward 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 thermodormant lettuce seed (Gonai and others 2004). Thus an important determinant of whether a seed remains dormant or germinates is represented by its ability to reduce ABA levels during imbibition (incubation in germination conditions); this ability or capacity is modulated through treatments that break dormancy, such as moist chilling and after-ripening (Feurtado and others 2004; Millar and others 2006). Recent experiments in the *Arabidopsis* C24 ecotype and barley suggest that ABA 8′-hydroxylase gene expression (for example, the CYP707A2 enzyme) is a key determinant in the regulation of ABA levels during the transition from dormancy to germination (Millar and others 2006).

Changes in ABA During Germination and Early Seedling Growth

In embryos of germinated seeds, ABA and ABA metabolite levels were higher in seeds capable of ABA biosynthesis (control and GA) and virtually nonexistent in germinated seeds that had been treated with fluridone (Figure 2 and 3). Similarly, in seeds moist-chilled for 98 days, ABA and metabolite levels were low through 3 days in germination conditions, prior to radicle protrusion; following germination, ABA and metabolite levels increased in seedling (embryo) tissue (Feurtado and others 2004). Taken together, these results suggest that (1) through dormancy termination and early germination, ABA and subsequent metabolite levels decrease to low levels (perhaps as a prerequisite to germination completion) and (2) there is *de novo* ABA synthesis taking place during early seedling growth (or late germination). The *de novo* synthesis of ABA is conceivably needed to sustain optimal seedling growth and stress responses (Finkelstein and Rock 2002; Feurtado and others 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.

Catabolism of ABA via 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 days (Figure 3a). Dihydrophaseic acid, which decreased in all treatments over time, decreased at the slowest rate in the fluridone/GA treatment (Figure 3b). In the megagametophyte, similar trends were

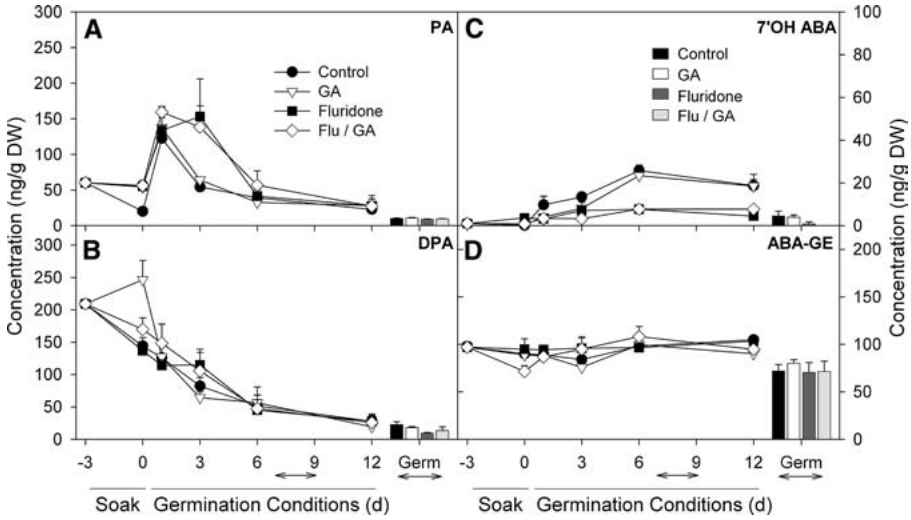

Figure 4. Changes in ABA metabolites changes within the megagametophyte during fluridone/GA treatments. (**a**) PA; (**b**) DPA; (**c**) 7′OH ABA; (d) ABA-GE. Megagametophytes of ungerminated seeds were analyzed before and after a 3-day soak in 100 μM fluridone and/or 50 μM GA and throughout 12 days in germination conditions (15 days total treatment time). Germinated seeds (Germ, bars) were analyzed between 7 and 9 days in germination conditions, as 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).

observed. However, a peak in PA occurred in all treatments between 0 and 3 days in germination conditions and continued to peak until 6 days in the fluridone and fluridone/GA treatments (Figure 4a). The data suggest that ABA was metabolized via the 8′ hydroxylation pathway to PA and DPA. This is especially evident in the fluridone/GA treatment, where the largest decrease in, or catabolism of, ABA was seen. However, the rise in metabolites such as PA or decrease in DPA is not consistent with the observed decrease in ABA in any of the four treatments (that is, control, fluridone, GA, or fluridone and GA in combination). Several possible events exist to explain this result:

1. Abscisic acid is being metabolized to other unknown metabolites that were not measured in this study.

2. If 8′-hydroxylation is the major pathway for ABA catabolism in western white pine seeds, flux may continue through PA and DPA to a further metabolite such as DPA-glucoside. Dihydrophaseic acid-glucoside has been reported in seeds of soybean (*Glycine max*) and in sunflower embryos (Setter and others 1981; Barthe and others 1993).

3. Transport of ABA or any of the metabolites out of the seed into the surrounding medium may contribute to the observed levels within the seeds.

In addition, it must be noted that this study looked at static levels of metabolites at select time points. However, at any given time, ABA and each of its metabolites are the sum of the flux through the pathway. For instance, a given level of ABA in a particular tissue is the ratio of its biosynthesis compared to its catabolism and/or transport. Thus, the best method for ABA metabolite analysis would be to measure the flux through each of the various metabolic pathways. At present, an available option for analysis of flux includes feeding of a labeled form of ABA (for example, ^{3}H(+)-ABA). This ultimately leads to an undesirable effect, because the compound adds an external variable that perturbs downstream *in vivo* physiological events.

Hydroxylation of ABA at the 7′OH position was an apparent catabolic pathway in the 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 days 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 (Figure 3c). Similar trends were seen in the megagametophyte tissues, except that the levels of 7′OH-ABA were approximately sevenfold lower (Figure 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 and others 2004). The increases of 7′OH-ABA in tissues actively

biosynthesizing ABA and maintaining higher ABA levels (that is, control, GA-treated, and germinated seeds) suggest that ABA accumulation may activate its own catabolism (through the 7′-hydroxylation pathway), thereby providing a means to self-regulate ABA levels.

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

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. Abscisic acid-GE was present at very low levels in embryos of ungerminated seeds regardless of whether fluridone or GA was used (Figure 3d). This contrasts with 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 and others 2004). Abscisic acid-GE in the megagameophytes was present at constitutive levels and remained unchanged, even in megagametophytes from germinated seeds (Figure 4d). Identical findings were observed in megagametophytes of seeds that had been subjected to moist-chilling as the dormancy-breaking treatment (Feurtado and others 2004). Abscisic acid-GE did, however, increase significantly in embryos of germinated seeds in which ABA biosynthesis was not inhibited (Figure 3d, Control and GA; Feurtado and others 2004). Thus, it seems that 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). The significance of ABA-GE accumulation in germinated seedlings of white pine remains unclear, but it has been suggested in *Arabidopsis* 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 and others 2005).

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 and others 2004). Here, disrupting ABA homeostasis through the use of fluridone and GA has shown 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 favor ABA biosynthesis, presumably helping to maintain a dormant state. Future work should concentrate on (1) determining the extent of transport and leaching of ABA and metabolites; (2) determining how key regulatory genes/enzymes such as 9-*cis* epoxycarotenoid dioxygenase (NCED) and ABA 8′-hydroxylase (CYP707A) change in response to different dormancy-breaking procedures, as well as in the dormant imbibed seed; and (3) how endogenous GA may contribute to dormancy termination.

ACKNOWLEDGMENTS

The authors thank Dave Kolotelo and Dean Christianson of the British Columbia Ministry of Forests, BC, Canada, for helping obtain mature seed of western white pine. The authors also thank Dr. Andrew R. S. Ross for suggested improvements to the manuscript. This research was supported by Natural Sciences and Engineering Research Council of Canada (Strategic) and Protein Engineering Network of Centres of Excellence (PENCE) grants awarded to A.R.K., S.R.A., A.J.C., A.R.S.R., and other investigators.

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