Changes in ABA turnover and sensitivity that accompany dormancy termination of yellow-cedar (Chamaecyparis nootkatensis) seeds

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Received 2 April 2001; Accepted 16 August 2001

Abstract

Yellow-cedar (Chamaecyparis nootkatensis [D. Don] Spach) seeds exhibit prolonged coat-imposed dormancy following their dispersal from the parent plant. Analyses were undertaken using S-(-)-[3H]-abscisic acid (ABA) to monitor the capacity of embryos to metabolize ABA following their isolation from seeds subjected to various dormancy-breaking and control treatments. Radiolabelled phaseic acid (PA) and dihydrophaseic acid (DPA) were detected in embryos and, to a greater extent in the surrounding media, by 48 h regardless of whether the embryos had been excised from seed previously subjected to only a 3 d soak or to a full dormancy-breaking treatment. Of the two enantiomers of ABA, only the natural S-(-)-ABA effectively inhibited germination of isolated embryos. A metabolism-resistant synthetic ABA analogue S-(-)-d6-ABA, consistently slowed the germination rate of excised embryos to a greater extent than that caused by natural S-(-)-ABA. The deuterium-labelled ring methyl groups of the analogue made it more resistant to oxidation by yellow-cedar embryos and thus rendered the analogue more persistent and possessing greater activity. With increasing time of exposure to moist chilling, yellow-cedar embryos became increasingly insensitive to both ABA and to the analogue. Subjecting seed to chemical treatments (GA₃ in combination with 1-propanol) prior to moist chilling strongly enhanced the germinability of whole seeds. This treatment also had a relatively greater impact on ABA metabolism than did moist chilling alone, as indicated by a greater capacity of S-(-)-d6-ABA to inhibit the germination of embryos as compared to S-(-)-ABA. Moist chilling was most critical for reduced ABA sensitivity of embryos. A change in the embryo’s ability to metabolize ABA and reduced embryo sensitivity to ABA are two factors associated with dormancy termination of whole seeds of yellow cedar; a change in only one of these factors is insufficient to elicit high germinability.

Key words: Coat-imposed dormancy, embryo, abscisic acid metabolism, seed germination, yellow cedar.

Introduction

The natural range of yellow cedar (Chamaecyparis nootkatensis [D. Don] Spach) extends along the Pacific Northwest northward along the coast of Alaska, where it occurs at high elevations. After pollination, cones and seed take between 1.5 and 2 years to mature depending upon the elevation and environmental factors such as temperature (reviewed in Owens and Molder, 1984). Following dispersal from the parent plant, seeds are dormant. Only a low percentage of seeds will germinate the first year after seed dispersal; the remainder require another year to undergo moist chilling and break dormancy (Pawuk, 1993).
The mechanism underlying dormancy of yellow-cedar seeds is being investigated. Maintenance of dormancy is not a consequence of embryo immaturity at the metabolic and physiological levels (Xia and Kermode, 1999), as was previously suggested (Kurz et al., 1994).

Yellow-cedar embryos germinate when they are excised from mature dormant seeds and placed in water, indicating that the seed tissues enclosing the embryo (the testa, remnants of the nucellus and the megagametophyte) prevent radicle emergence (Ren and Kermode, 1999, 2000). This category of seed dormancy is typically referred to as coat-imposed or coat-enhanced and, as with embryo dormancy, various mechanisms may be involved (reviewed in Bewley and Black, 1982, 1994; Hilhorst, 1995; Bewley, 1997). In many cases, the enclosing seed tissues exert a powerful inhibitory effect of a chemical nature, which is often relieved by repeated washing (leaching) to remove the inhibitor (Bewley and Black, 1994). Maintenance of dormancy is hypothesized to be a consequence of a high abscisic acid (ABA) content in the dispersed (mature) seed and/or a heightened sensitivity of the embryo to this germination inhibitor (Walker-Simmons, 1987; Kermode, 1990, 1995). In some seeds, a high concentration of ABA is not necessary for dormancy maintenance, but rather is required only for dormancy imposition (reviewed in Bewley and Black, 1994). Several lines of evidence indicate that ABA is involved to some extent in the dormancy mechanism of yellow-cedar seeds. Fluridone, when used with GA₃, is effective in relieving the dormancy of whole mature seeds of yellow cedar, in the complete absence of moist chilling (Schmitz et al., 2001). This chemical is an inhibitor in the pathway of carotenoid biosynthesis (Bartels and Watson, 1978; Fong and Schiff, 1979) and has been used as a tool to decrease ABA within seeds (Fong et al., 1983; Xu and Bewley, 1995; Yoshioka et al., 1998; Le Page-Degivry and Garello, 1992). Yellow-cedar seeds that are treated with fluridone alone (i.e. with no GA₃ present) show a reduced capacity for germination as compared to their fluridone/GA₃-treated counterparts, which may indicate that a decline in ABA alone is insufficient to promote germination.

The dormancy mechanism of yellow cedar is complex and is not exclusively coat-imposed (although this is the primary mode of dormancy regulation) (Ren and Kermode, 1999, 2000). Furthermore, in addition to chemical inhibition, the megagametophyte also plays a role in the coat-imposed mechanism as a mechanical barrier to prevent radicle protrusion, a factor which may also involve regulation by ABA and other hormones such as gibberellins (through regulation of cell wall rigidity). For example, the micropylar megagametophyte decreases in mechanical strength following a dormancy-breaking treatment and, during germination, the cells of the megagametophyte in the area immediately surrounding the radicle exhibit a loss of their internal structure, that would represent significant weakening to allow radicle emergence (Ren and Kermode, 1999). More recent work suggests that the cell wall hydrolase, pectin methyl esterase, plays a role in weakening of the megagametophyte, allowing radicle emergence and the completion of germination (Ren and Kermode, 2000).

In many cases a reduction in endogenous ABA within seed tissues does not correlate well with dormancy termination (Bewley and Black, 1994, and references therein); rather, this process is more strongly correlated with a decline in embryo sensitivity to ABA (Walker-Simmons, 1987; reviewed in Kermode, 1990, 1995; Bewley, 1997). Embryos excised from yellow-cedar seeds subjected to dormancy-breaking treatments (e.g. combined warm/cold treatments) exhibit a decline in their sensitivity to increasing concentrations of natural S(+)-ABA as compared to their counterparts excised from untreated seed (Schmitz et al., 2000). This apparent insensitivity of the embryo may be due to an altered (i.e. reduced) perception of ABA, or a change in the embryo’s ability to metabolize ABA.

Synthetic ABA analogues have become powerful tools for studying changes in ABA metabolism and perception (Walker-Simmons et al., 1992; Wilen et al., 1993; Balsevich et al., 1994; Hill et al., 1995; Lamb et al., 1996; Abrams et al., 1997; Cutler et al., 1997; Qi et al., 1998). One such ABA analogue is S-[8',9'-,9'-,9'-,9'-hexadeuterobiosciscic acid, S(+)-d₆-ABA, in which the hydrogen atoms of the 8' and 9' methyl groups on the ring of S(+)-ABA are replaced by deuterium atoms. Enzymatic hydroxylation of ABA (the first step in its metabolism) involves cleavage of a C—H bond at the 8' position and since the C—D bond is stronger than the C—H bond, it is predicted that the chemically modified analogue would be metabolized more slowly (i.e. be more persistent in plant tissues and possess activity greater than that of the S(+)-ABA). For example, in some plant tissues (e.g. corn suspension cultures), in vivo oxidation rates are slower for S(+)-d₆-ABA than for S(+)-ABA and the biological activity of the deuterium-labelled analogue is higher with respect to delaying cress seed germination (Lamb et al., 1996).

The goal of the study is to elucidate the relative importance of ABA metabolism and reduced ABA perception (on the part of the embryo) in dormancy termination of yellow-cedar seeds. Toward this end, the effects of increasing concentrations of S(+)-ABA on the germination of embryos isolated from seeds subjected to various dormancy-breaking (and control) treatments were examined. Further, the effects of the unnatural ABA enantiomer, R-(−)-ABA, and a metabolism-resistant ABA analogue, S(+)-d₆-ABA, as compared to natural S(+)-ABA were studied. Labelling studies with S(+)-[³H]ABA (and thin-layer chromatography to
monitor accumulation of $^3$H-PA and $^3$H-DPA were carried out to examine changes in the embryo’s ability to metabolize ABA as a result of the seed being subjected to various dormancy-breaking (and control) treatments.

**Materials and methods**

**ABA enantiomers and analogues**

$S$-($\pm$)-ABA (Fig. 1) was obtained by preparative HPLC resolution of racemic methyl acbisate followed by hydrolysis of the resolved esters; these procedures and those described for the resolution of ($R$-) and ($S$)-enantiomers of ABA have been published earlier (Dunstan et al., 1992). $S$-($\pm$)-$^3$H-ABA was synthesized and labelled according to an established procedure (Balsevich et al., 1994) and $S$-[8',8',9',9',9'-d$_6$]-hexadeuterobasabiscic acid, $S$-($\pm$)-$^6$-ABA (Fig. 1) was generated essentially as outlined earlier (Lamb et al., 1996) which describes the synthesis of $S$-($\pm$)-$^6$-ABA. The $d_6$ ester was hydrolysed with base in water and the deuterium atoms on the 7'-carbon were exchanged for hydrogens (S Abrams, unpublished data). Synthesis of the ABA metabolites (phaseic acid, PA and dihydrophaseic acid, DPA) which were used as standards on exchange for hydrogens (S Abrams, unpublished data). Synthesis of the ABA metabolites (phaseic acid, PA and dihydrophaseic acid, DPA) which were used as standards on the thin-layer chromatography plates (see below) was carried out as described previously (Balsevich et al., 1994); ($-$)-PA, the naturally occurring enantiomer (Fig. 1), was obtained from the medium of suspension cultures of corn (Zea mays L., cv. Black Mexican Sweet) that had been supplied with $S$-($\pm$)-ABA, according to the procedure of Balsevich et al. (Balsevich et al., 1994). DPA (Fig. 1) was prepared from the isolated PA (Zeevaart and Milborrow, 1976). 7'-HydroxyABA was synthesized chemically (Nelson et al., 1991) and 8'-hydroxyABA (Fig. 1: 8'-OHABA) was prepared as described previously (Zou et al., 1995). Epi-DPA and PBI 344 (dehydrovomifoliol, a product of bacterial metabolism of ABA; Hasegawa et al., 1984) were synthesized as described previously (Milborrow, 1975; Roberts et al., 1968).

**Seed materials and dormancy-breaking and control treatments**

Mature yellow-cedar (Chamaecyparis nootkatensis [D. Don Spuch]) seeds of seed lot 30156 (previously collected from natural stands by MacMillan Bloedel and obtained from the Tree Seed Centre in Surrey, BC, Canada) were used for all analyses because of their high viability (D Kolotelo, personal communication). A prolonged (~3 month) treatment in which yellow-cedar seeds are kept moist for a 4-week warm period (at 25–26 °C) followed by 8 weeks of moist chilling (at 4 °C) is effective in breaking the dormancy of yellow-cedar seed (Ren and Kermode, 1999). Also effective are treatments that subject the seed to moist chilling, but eliminate the previous warm moist period and, in its place, subject the whole seed to chemical treatments: [i] a 1 d incubation in 70 mM 1-propanol, followed by a 2 d incubation in 150 mg l$^{-1}$ GA$_3$ (Table 1, Treatment A) or [ii] a 4 d incubation in 10% polyethylene glycol [PEG] 8000, followed by 1 d of air-drying and then a 2 d incubation in 150 mg l$^{-1}$ GA$_3$ (Table 1, Treatment B). When these chemical treatments are followed by 4 or 8 weeks of moist chilling, they elicit comparable seed germination (~60–85% over 30 d) to that resulting from the longer warm/cold moist treatments (Xia and Kermode, 2000; Schmitz et al., 2001). Mature dry seeds of yellow cedar were subjected to the dormancy-breaking or control treatments presented in Table 1 (1–13), after which the embryos were dissected from the seeds. Mature seeds were subjected to a 72 h running water imbibition at 23 °C followed by a 3 d or 7 d chemical treatment (propanol/GA$_3$ or PEG/dry/GA$_3$, respectively) as noted in Table 1. Seeds were then transferred to 4 °C for moist chilling for 2, 4 or 6 weeks (Table 1). As controls for the chemical treatments, seeds were soaked and moist-chilled for identical periods, but were incubated in water in place of the chemical treatment (Table 1, water controls).

For all chemical treatments or their controls, seeds were treated with 20 ml solution at 25 °C in 9 cm diameter Petri dishes (100 seeds per dish), with agitation (100 rpm). Concentrations of chemicals were as follows: 10% PEG 8000 (w/v), 150 mg l$^{-1}$ GA$_3$ and 70 mM 1-propanol. To maintain high moisture conditions throughout the moist chilling treatments, seeds were placed in 9 cm diameter Petri dishes between two layers of prewetted Whatman No. 1 filter paper. At different time points throughout the dormancy-breaking and control treatments (Table 1), embryos were dissected from the seed and then placed in germination conditions in the presence of water, $S$-($\pm$)-ABA, $R$-($\pm$)-ABA or $S$-($\pm$)-$^6$-ABA as described in the section below. As noted in Tables 1 and 2, most of the controls involved a water treatment in place of the
Germination of isolated embryos to determine sensitivity to ABA enantiomers and ABA analogues

Germination assays were based on three replicates of 10 embryos each. To examine sensitivity to different concentrations of S- (+)-ABA, the embryos were placed in 6 cm diameter Petri dishes on one layer of Whatman No. 1 filter paper prewetted with 3 ml of water or S- (+)-ABA solutions (10⁻², 10⁻⁶ and 10⁻⁸ M). To examine the effects of the unnatural ABA enantiomer, R-(-)-ABA and the metabolism-resistant ABA analogue, S- (+)-d⁶-ABA, on the germination of isolated embryos (as compared to natural S- (+)-ABA), embryos were incubated in water or in S- (+)-ABA, R-(-)-ABA or S- (+)-d⁶-ABA (all at 10⁻⁶ M). Solutions of S- (+)-ABA, R-(-)-ABA and the ABA analogue were prepared by dissolving known weights (2–4 mg) into 1 ml methanol and then diluting them further with the appropriate amount of sterile water. All solutions, including the control, contained equal amounts of methanol and were adjusted to a pH of 5.5. Germination was monitored daily for a total of 18 d. Germination conditions were 25.24 °C day/night with a 15 h photoperiod; light intensity at 100 μmol m⁻² s⁻¹, PAR 400–700 nm. All solutions were replaced on day 6 and day 12 of the germination assay. Germination was defined as elongation of the radicle, which was also accompanied by the opening and greening of the cotyledons.

Data in Figures 3–7 are based on an average of three replicates of 10 embryos each (±SE). Data in Table 2 are based on an average of 3–6 replicates of 30 seeds each (±SE). SE was calculated using InStat for MacIntosh © 1992, 1993 GraphPad Software (Version 2.01).

Metabolism studies using S- (+)-[³H]ABA

Treatment and sampling: Embryos (50 mg) excised from variously treated seed (Table 1) were fed with 40 μM S- (+)-[³H]ABA in 1 ml of medium in the dark; two replicates were carried out per treatment. In time-course experiments, the embryos were collected at 0, 6, 12, 24, and 48 h following the addition of radiolabelled ABA, rinsed thoroughly with distilled water and immediately flash frozen and stored at −80 °C, prior to metabolite analysis. The medium at each time point was also stored at −80 °C for further analysis.

Extraction and analyses of S- (+)-[³H]ABA and its metabolites: Frozen embryos (50 mg) were ground in 2 ml 95% isopropanol containing 5% glacial acetic acid and allowed to extract overnight on a shaker (50 rpm) in the dark at 23 °C. Following centrifugation, the bulk filtrate was concentrated under nitrogen gas. The procedure to extract ABA and its metabolites was carried out as outlined earlier (Qi et al., 1998). Following extraction, aliquots containing equal counts (dpm) were applied to silica gel GF254 thin-layer chromatography (TLC) plates for separation of ABA and its metabolites. The TLC plates were developed with toluene:EtOAc:acetic acid (25:15:2, by vol.). Radioactive bands were detected by autoradiography and the bands identified by co-chromatography with known standards (i.e. ABA, 7'-hydroxyABA, 8'-hydroxyABA, PA, epi-DPA, DPA and dehydrovomifoliol, PBI 344).

ABA and its metabolites present in the medium (1 ml) were purified using Waters Oasis HLB extraction cartridges according to the manufacturer’s instructions (Waters, Milford, MA, USA). Following extraction, aliquots containing equal counts were applied to silica gel GF254 thin-layer chromatography (TLC) plates for separation of ABA and its metabolites as described above.

Results and discussion

Treatments effective in terminating the dormancy of whole seeds of yellow cedar

To terminate coat-imposed dormancy, seeds must be subjected to treatments that alleviate the block to germination (radicle emergence) that is imposed by seed tissues surrounding the embryo (the megagametophyte,
Metabolism of S-(+)-ABA in yellow-cedar embryos

Labelling studies with S-(+)-[^3]H]ABA were carried out to examine whether yellow-cedar embryos metabolize ABA more efficiently as a result of the seed being subjected to dormancy-breaking treatments. Within leaves, developing seeds and seedlings, ABA is metabolized through oxidation to form 8'-hydroxyABA (Walton, 1980; Loveys and Milborrow, 1984) (Fig. 1A); this first step is catalysed by an 8'-hydroxylase enzyme. Subsequently, 8'-hydroxyABA cyclizes to form phaseic acid (PA) (Loveys and Milborrow, 1984; Zeevaart and Creelman, 1988; Balsevich et al., 1994) which may be further reduced to dihydrophaseic acid (DPA) in some tissues (Gillard and Walton, 1976; Zeevaart and Creelman, 1988; Parry, 1993). Following feeding of embryos excised from the variously treated seed (Table 1; see Fig. 2 legend) with S-(+)-[^3]H]ABA for 0, 6, 12, 24, and 48 h, extracts from embryos and media were subjected to TLC to monitor the accumulation of ^3H-PA and ^3H-DPA (Fig. 2; 0, 24 and 48 h shown). Metabolism of ABA into PA and DPA occurred by 48 h regardless of whether the embryos had been excised from seed previously subjected to only a 3 d soak or to a full dormancy-breaking treatment (Fig. 2; compare lane 1 with lane 4). However, the majority of ABA metabolites were not detected intracellularly (i.e. within embryos, Fig. 2A), but rather were present in media (Fig. 2B). An unidentified metabolite also accumulated in both embryos and media (Fig. 2, open arrow); running several standards on the TLC plates concurrently with this metabolite ruled out likely candidates (PA, DPA, 8'-hydroxyABA,
7'-hydroxyABA, dehydrovomifoliol, and ep-DPA) (data not shown) and its significance remains to be determined. The experiment, in its present design, does not demonstrate that the seed treatments are causing differential metabolism of ABA in the embryo. This is similar to embryos of Fraxinus excelsior (ash) seeds in which both dormant and moist chilled embryos rapidly metabolize ABA to PA, DPA, and an unidentified polar metabolite apparently derived from DPA (Sondheimer et al., 1974).

Effects of S(+)-ABA and R(-)-ABA on embryo germination

Most studies of the inhibitory effects of ABA on embryo and seed germination have used racemic ABA, despite the problems associated with the differential effects of the two enantiomers, S(+)-ABA (the natural hormone) and R(-)-ABA (the unnatural enantiomer). Natural S(+)-ABA (10⁻⁶ M) was inhibitory to the germination of yellow-cedar embryos excised from dormant seed in contrast to its unnatural enantiomer, R(-)-ABA (Fig. 3). Both enantiomers equally inhibit barley and wheat embryo germination (Abrams et al., 1993); however, there are several reports of their differential effects on other species (e.g. cress seed germination) and on other processes (e.g. gene expression in barley and transgenic tobacco embryos, freezing tolerance in bromegrass cells, and induction of stomatal closure), in which the natural ABA has the greatest effect (Walker-Simmons et al., 1992; Churchill et al., 1992; Gusta et al., 1992; Cummins and Sondheimer, 1973; Jiang et al., 1996).

Sensitivity of embryos to increasing concentrations of S(+)-ABA following exposure of whole seeds to dormancy-breaking and control treatments

To examine changes in embryo sensitivity to ABA associated with dormancy breakage of whole seeds, embryos were isolated from seeds at various times throughout the dormancy-breaking treatments (Table 1) and were incubated in water or in different concentrations of S(+)-ABA (Fig. 4). Embryos excised from mature dormant seeds (that had received only a 3 d soak) (Fig. 4, treatment 1) were relatively sensitive to ABA at 10⁻⁵ and 10⁻⁶ M. As seeds were subjected to longer lengths of the dormancy-breaking protocol, the excised embryos became increasingly insensitive to ABA. For example, embryos of seed subjected to treatments effective in eliciting high germinability of whole seeds (i.e. 60% or greater) (in Fig. 4 and Table 1, treatments 4, 8 and 10) were relatively insensitive to all but the highest concentration of ABA tested (10⁻³ M).

Effects of S(+)-ABA and S(+)-d6-ABA on the germination of yellow-cedar embryos

As another approach to examining changes in ABA metabolism as a result of dormancy-breakage, the effects of the metabolism-resistant ABA analogue S(+)-d6-ABA on the germination of isolated embryos as compared to natural S(+)-ABA were examined. Yellow-cedar embryos were isolated from seeds at various times throughout the dormancy-breaking treatments or their controls (Table 1; treatments 1–11) and were incubated in water, S(+)-ABA (10⁻⁶ M) or S(+)-d6-ABA (10⁻⁶ M). Germination was monitored daily for a total of 18 d (Figs 5, 6); the number of days required before 50% of the embryos achieved germination (G50) in the presence of water, natural S(+)-ABA and the ABA analogue are noted in Table 2. Table 2 also indicates the germinability of whole seeds of yellow cedar following their exposure to some of the dormancy-breaking or control treatments. A faster rate of germination of excised embryos in the presence of both natural ABA and the ABA analogue as a result of seeds being subjected to a dormancy-breaking treatment indicates a relative insensitivity to the hormone. Further, a delayed germination rate (G50 in Table 2) in the presence of the S(+)-d6-ABA analogue as compared to natural ABA indicates its greater biological activity. This in turn indicates that persistence of the hormone is the limiting factor (accounting for enhanced germinability in natural ABA).

Compared to embryos excised from seeds that received only a 3 d soak, embryos isolated from seeds subjected to moist chilling (regardless of whether or not they had received a chemical or water treatment interpolated between the 3 d soak and the moist chilling) showed an enhanced capacity to germinate in the presence of both S(+)-ABA and its metabolism-resistant analogue, S(+)-d6-ABA. Further, the relative insensitivity of the embryo to ABA and the ABA analogue as a result of moist chilling became more pronounced as the length of the moist chilling treatment was increased (Figs 5, 6; Table 2).
Fig. 4. (A–F) Effects of increasing concentrations of S-(+)-ABA on the germination of embryos excised from variously treated seed. Natural S-(+)-ABA was used at concentrations of 10^{-5} to 10^{-7} M and germination of embryos in the presence of the hormone is compared to that exhibited by embryos incubated in water. Embryos were excised from seed that had been subjected to part or all of a dormancy-breaking treatment (Treatments are as numbered in Table 1). Data are based on the average of three replicates of 10 embryos each (±SE; InStat, Version 2.01).
Fig. 5. Effects of $S$-($\alpha$)-ABA and the metabolism-resistant ABA analogue, $S$-($\alpha$)-hexadeutero ($d_6$)-ABA, on the germination of embryos excised from variously treated seed. Seed treatments are as numbered in Tables 1 and 2. The natural ABA and ABA analogue were used at a concentration of $10^{-6}$ M and germination of embryos in the presence of the hormones is compared to that exhibited by embryos incubated in water. Data are based on the average of three replicates of 10 embryos each (± SE; InStat, Version 2.01). Also refer to Table 2.
Fig. 6. Effects of S(+)-ABA and the metabolism-resistant ABA analogue, S(+)-hexadeutero (d6)-ABA, on the germination of embryos excised from variously treated seed as indicated. Seed treatments are as numbered in Tables 1 and 2. The natural ABA and ABA analogue were used at a concentration of $10^{-6}$ M and germination of embryos in the presence of the hormones is compared to that exhibited by embryos incubated in water. Data are based on the average of three replicates of 10 embryos each ($\pm$ SE; InStat, Version 2.01). Also refer to Table 1.
Table 2. Whole seed germinability and the number of days required for isolated embryos to achieve 50\% germination (G50)

<table>
<thead>
<tr>
<th>Seed treatments\textsuperscript{a,b}</th>
<th>Whole seed germination percentage (after 30 d)\textsuperscript{c}</th>
<th>D for 50% embryo germination (G50)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Water</td>
</tr>
<tr>
<td>(1) 3 d soak</td>
<td>0 ± 0</td>
<td>7.6</td>
</tr>
<tr>
<td>3 d soak; 2 weeks chilling after:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2) 3 d propanol/GA\textsubscript{3}</td>
<td>–</td>
<td>8.0</td>
</tr>
<tr>
<td>(3) 3 d water\textsuperscript{d}</td>
<td>–</td>
<td>7.2</td>
</tr>
<tr>
<td>3 d soak; 4 weeks chilling after:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(4) 3 d propanol/GA\textsubscript{3}</td>
<td>61.5 ± 3.6</td>
<td>6.0</td>
</tr>
<tr>
<td>(5) 3 d water\textsuperscript{d}</td>
<td>16.0 ± 4.2</td>
<td>5.6</td>
</tr>
<tr>
<td>3 d soak; 2 weeks chilling after:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(6) 7 d PEG dry/GA\textsubscript{3}</td>
<td>–</td>
<td>7.0</td>
</tr>
<tr>
<td>(7) 7 d water\textsuperscript{d}</td>
<td>–</td>
<td>7.0</td>
</tr>
<tr>
<td>3 d soak; 4 weeks chilling after:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(8) 7 d PEG dry/GA\textsubscript{3}</td>
<td>62.0 ± 3.3</td>
<td>5.8</td>
</tr>
<tr>
<td>(9) 7 d water\textsuperscript{d}</td>
<td>40.0 ± 8.5</td>
<td>6.0</td>
</tr>
<tr>
<td>3 d soak; 6 weeks chilling after:</td>
<td></td>
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<tr>
<td>(10) 7 d PEG dry/GA\textsubscript{3}</td>
<td>–</td>
<td>5.0</td>
</tr>
<tr>
<td>(11) 7 d water\textsuperscript{d}</td>
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<td>5.0</td>
</tr>
<tr>
<td>3 d soak; 4 weeks warm after:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(12) 3 d water\textsuperscript{a,c}</td>
<td>0 ± 0</td>
<td>7.2</td>
</tr>
<tr>
<td>(13) 3 d propanol/GA\textsubscript{3}\textsuperscript{a,c}</td>
<td>31.0 ± 2.1</td>
<td>5.6</td>
</tr>
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</table>

\textsuperscript{a}Treatments are numbered as in Table 1 and Figs 5 and 6.

\textsuperscript{b}Chemical treatments were as follows: (i) 4 d 10\% PEG 8000 (w/v), 1 d air-drying at 23 °C, 2 d 150 mg l\textsuperscript{-1} GA\textsubscript{3} and (ii) 1 d 70 mM 1-propanol, 2 d 150 mg l\textsuperscript{-1} GA\textsubscript{3}.

\textsuperscript{c}Treatments that are controls for the chemical treatments. Note that the seeds subjected to the experimental and control treatments receive the same length of moist chilling.

\textsuperscript{d}Treatments that represent controls for the moist chilling (warm moist conditions).

\textsuperscript{e}Based on an average of 3–6 replicates of 30 seeds each (±SE).

The chemical treatments (i.e. PEG/re-dry/GA\textsubscript{3} and propanol/GA\textsubscript{3}) had very little effect on reducing the sensitivity of the embryo to \textit{S}(\textit{+})-ABA. In the great majority of cases, embryos treated with water for an equivalent period (as a substitution for the chemical treatment preceding moist chilling), showed comparable sensitivities to the hormone (Figs 5, 6; Table 2). This was also evident when embryos from these control seeds were placed in increasing concentrations of \textit{S}(\textit{+})-ABA, as per Fig. 4 (data not shown). Thus, it is moist chilling that is most critical for a reduced ABA sensitivity of the embryo.

Embryos exposed to the metabolism-resistant \textit{S}(\textit{+})-\textit{d6}-ABA analogue consistently germinated more slowly that those incubated in natural \textit{S}(\textit{+})-ABA. This analogue-specific effect, is greater when embryos have been excised from seeds that have received some moist chilling. Nonetheless, while a reduced sensitivity of the embryo to ABA was highly correlated with exposure of the seed to moist chilling, differences between the ability of the \textit{S}(\textit{+})-\textit{d6}-ABA analogue to delay germination of isolated embryos as compared to \textit{S}(\textit{+})-ABA were consistently greater in embryos excised from seeds which had been exposed to the propanol treatment (instead of water or PEG) prior to moist chilling (In Figs 5 and 6 and in Table 2, compare treatments 2 versus 3, 4 versus 5, 6 versus 7, 8 versus 9, and 10 versus 11). Thus, the imposition of propanol treatments on the seed prior to moist chilling appears to have a greater impact on ABA metabolism than moist chilling alone.

Embryos excised from seeds which did not receive moist chilling but were subjected instead to a moist warm period (25 °C) of equivalent duration (Fig. 7A) showed a decreased capacity to germinate in the presence of both \textit{S}(\textit{+})-ABA and \textit{S}(\textit{+})-\textit{d6}-ABA as compared to those which had experienced moist chilling (compare Fig. 7A with Fig. 5, treatment 5). Both ABA and the analogue were equally effective in delaying germination of these embryos (Fig. 7A). These results are consistent with the data presented above and further demonstrate the importance of moist chilling in effecting a reduction of the embryo’s responsiveness to ABA as far as germination is concerned. Embryos excised from seeds that underwent a 4 week warm moist period, after being subjected to a 1-propanol/GA\textsubscript{3} treatment also retained some sensitivity to ABA (compare Fig. 7B with Fig. 5, treatment 4), and the differences in their responsiveness to the \textit{S}(\textit{+})-\textit{d6}-ABA analogue versus the natural ABA were more pronounced (Fig. 7B), as a result of the chemical treatment (compare A with B in Fig. 7).
Data are based on the average of three replicates of 10 embryos each (Table 1) or to a chemical treatment (B; treatment 13 in Table 1). Excised from seed subjected to a 30 d warm moist period instead of 30 d moist chilling, after first being subjected to either water (A; treatment 1) or to a chemical treatment (1-propanol; treatment 12 in Table 1). Data are based on the average of three replicates of 10 embryos each (±SE; InStat, Version 2.01). Also refer to Table 2.

Further discussion

Altered ABA metabolism and reduced embryo sensitivity to ABA: critical factors for dormancy termination of the whole seed?

As mentioned previously, the critical question pertaining to the present study is whether changes in the embryo’s ability to metabolize ABA and/or reduced embryo sensitivity to ABA can be correlated with dormancy breakage, i.e. the ability of whole seeds to germinate. Although moist chilling was effective in reducing the embryo’s sensitivity to the germination inhibitor \( S' \) (as described above), high germinability of whole seeds requires more than moist chilling alone; the above-noted combination of chemicals and subsequent moist chilling are also necessary. As noted in Table 2, in the present study, germination of whole seeds was \(~60\%\) after seeds were subjected to dormancy-breaking treatments that incorporated 1-propanol or PEG, in combination with the 2 d \( \text{GA}_3 \) incubation and 4 weeks of moist chilling (treatments 4 and 8, respectively). The response of embryos excised from these seeds to \( S' \)-ABA and to the metabolism-resistant ABA analogue, as far as germination is concerned, indicated both a decline in embryo sensitivity to ABA (primarily as a result of moist chilling) as well as potential changes in ABA metabolism (primarily as a result of the chemical treatments). One set of control seeds which received 3 d of incubation in water (in place of the 1-propanol; \( \text{GA}_3 \) treatment), but the same duration of moist chilling (4 weeks), exhibited low germinability (\(~16\%\)); the embryos from these seeds exhibited a change in ABA sensitivity (as compared to those excised from seeds receiving only a 3 d soak; treatment 1), but did not exhibit the delayed germination specifically caused by the ABA analogue as compared to natural ABA (Fig. 5, treatment 5 versus 4). Interestingly, a second control (treatment 9) in which seeds received a prolonged (7 d) incubation in water (in place of the PEG/air-drying \( \text{GA}_3 \) treatment), but the same duration of moist chilling (4 weeks), exhibited moderate germinability (\(~40\%\)); the embryos from these seeds exhibited a greater change in ABA sensitivity and also showed delayed germination specifically caused by the ABA analogue, albeit not as prominent as those embryos from seeds in treatment 8 that received the chemical treatment (Fig. 6). The other controls substituted moist chilling with a warm moist treatment (25–26 °C) after previously subjecting seed to water (treatment 12) or to a chemical treatment (1-propanol; \( \text{GA}_3 \) ) (treatment 13). Seed incubated in water and subsequently exposed to 4 weeks of warm moist conditions did not germinate (Table 1); embryos from these seeds remained relatively sensitive to ABA and there were no effects on delayed germination specifically caused by the ABA analogue (Fig. 7A). In comparison, embryos excised from seeds that were chemically treated but subjected to warm stratification exhibited somewhat intermediate behaviour (Fig. 7B). Here, germinability of whole seeds was low to moderate (\(~30\%\)); the most pronounced change in embryo germinability was specific to the \( S' \)-d6-ABA analogue.

ABA turnover (and 8’ hydroxylase) is a key factor in controlling ABA responses in planta. In yellow-cedar embryos, \( S' \)-ABA metabolism occurs by oxidative catabolism to PA and DPA (via 8’-hydroxy-ABA). Other work indicates that PA is the effective product of ABA 8’ hydroxylase and that this is the rate-limiting step for hormone inactivation (Cutler et al., 1997). The observed isotope effect in yellow-cedar germination experiments indicates that the conversion of the C—H bond to the C—OH bond becomes rate-limiting as a result of yellow-cedar dormancy-breaking treatments and that a change in ABA metabolism is associated with dormancy termination. In cress seeds, a deuterated analogue slowed germination more than \( S' \)-ABA when applied in equivalent concentrations (Lamb et al., 1996), clearly indicating the importance of ABA metabolism and ABA 8’ hydroxylase in the modulation of germination rates in vivo.
The results discussed in this paper demonstrate that dormancy termination is accompanied by a change in the ability of the embryo to metabolize ABA (8’-hydroxylation becomes rate-limiting). These changes, and the reduced embryo sensitivity to ABA, are two factors associated with dormancy termination of whole seeds of yellow cedar; a change in only one of these factors is insufficient to elicit high germinability. It is speculated that the chemical treatments have some direct effect on the 8’ hydroxylase enzyme, while moist chilling causes changes that affect ABA reception or possibly downstream signal transduction events.

Acknowledgements

We are grateful to Garth Abrams, Adrian Cutler, Joan Krochko, Qunqang Qi, Patricia Rose, and Mary Loewen of the Plant Biotechnology Institute (PBI), Saskatoon, SK, Canada for helpful discussions or technical assistance. Stan Wheat and Mike Gerhard (MacMillan Bloedel, Nanaimo, BC, Canada), John Russell (BC Forest Service, Lake Cowichan, BC) and Dave Kolotelo (BC Ministry of Forests, Tree Seed Centre, Surrey, BC) are thanked for their help in obtaining mature seed of yellow cedar. This research was supported by a Forest Renewal BC grant HQ96232-RE to ARK. NS is a recipient of a BC Science Council GREAT award.

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