RESEARCH PAPER

Genotype effects on ABA consumption and somatic embryo maturation in interior spruce (\textit{Picea glauca} \times \textit{engelmannii})

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Abstract

Abscisic acid (ABA) plays an important role during somatic embryo development and maturation in coniferous species. The purpose of this research was to study ABA utilization by genotypes with different embryo maturation capabilities in interior spruce. Cell lines ISP11 and ISP48 were of high embryo maturation capability. By contrast, the tissue of line ISP16 contained numerous immature embryos, but only a few mature embryos developed. Exogenous ABA, i.e. S-ABA [(+/-)\textit{cis}, \textit{trans}-ABA], racemic ABA, or ABA isomers were added into suspension cultures at a final concentration of 30 \(\mu\text{M}\). In comparison to racemic ABA and ABA isomers, S-ABA reduced tissue proliferation the most. In all cell lines, about half of the racemic ABA was used within 2 weeks; the remaining ABA was (–)\textit{cis}, \textit{trans}-ABA. The concentration of ABA showed little change thereafter. In the cultures supplied with ABA isomers, about half of (+/-)\textit{cis}, \textit{trans}-ABA was utilized during 22 d. By contrast, (+/-)\textit{trans}, \textit{trans}-ABA was hardly used, especially in line ISP16. S-ABA was almost completely metabolized by line ISP11. However, approximately 28% and 22% of the S-ABA remained in the culture of cell lines ISP16 and ISP48, respectively. Cell line ISP16 grew the fastest in culture. By 3 weeks, S-ABA consumption by ISP11 and ISP48 on the basis of tissue growth was, respectively, 2.2-fold and 3.4-fold greater than that of ISP16. A higher ratio of dihydrophaseic acid to phaseic acid existed with cell lines of higher embryo maturation capability, especially when the exogenously supplied ABA was chemically synthesized.

Key words: ABA consumption, ABA isomers, genotypes, interior spruce, maturation capability, somatic embryo.

Introduction

Conifers can be induced to form \textit{in vitro} cultures capable of producing somatic embryos (SEs). The capability of this tissue to develop mature embryos can vary widely. Since cell lines need to be multiplied to optimize their final yield, it is necessary to establish their embryogenic capability early on, if effort is not to be wasted. Unfortunately, capability of embryogenic tissue is not easily assessed from morphological criteria alone. Consequently, physiological and/or molecular characteristics are also employed (Jalonen and von Arnold, 1991; Fourré \textit{et al.}, 1997; Jourdain \textit{et al.}, 1997; Egertsdotter and von Arnold, 1998; Bishop-Hurley \textit{et al.}, 2003).

In conifers, changes in endogenous concentrations of abscisic acid (ABA) and other plant hormones are relevant to development and maturation of zygotic embryos (Kapik \textit{et al.}, 1995; Kong \textit{et al.}, 1997; Chiwocha and von Aderkas, 2002) or SEs (Kong \textit{et al.}, 1999; von Aderkas \textit{et al.}, 2001). Embryogenic tissue in coniferous species may not be able to synthesize ABA \textit{in vivo} (Label and Lelu, 2000) in response to exogenous ABA. It has also been suggested that they are only able to synthesize ABA at a low concentration when no exogenous ABA was supplied (Kong and Yeung, 1995). Exogenous ABA has been proved to be essential in promoting SE maturation in most coniferous species: a wide range of exogenous ABA concentrations (1–100 \(\mu\text{M}\)) has been supplied in gel-solidified or liquid media (reviewed by Attree and Fowke, 1993; Stasolla \textit{et al.}, 2002). Exogenous ABA was metabolized by embryogenic tissue into phaseic acid (PA) and dihydrophaseic acid (DPA) (Dunstan \textit{et al.}, 1992; Label and Lelu, 2000).

Currently, major ABA supplements for plant tissue culture include various combinations of ABA enantiomers.
or isomers (Fig. 1). Racemic ABA (R-ABA) is a chemically synthesized mixture of equal amounts of (+)-cis, trans-ABA and (–)-cis, trans-ABA. Mixed ABA isomers (M-ABA) are also chemically synthesized. M-ABA contains (+/–)-cis, trans-ABA and (+/–)-trans, trans-ABA in different ratios. S-ABA, a natural ABA [(+)-cis, trans-ABA] is synthesized in vivo. Differential uptake by plant tissues, as well as differing effects of the various exogenous ABA, have been reported (Lehmann et al., 1983; Balsevich et al., 1994; Cramer et al., 1998; Lin et al., 2005). In conifer somatic embryogenesis, previous research of exogenous ABA was mainly focused on its effects on in vitro culture, such as embryogenic tissue induction (Pullman et al., 2005) and SE quality (von Aderkas et al., 2002), or on its metabolism (Dunstan et al., 1992, 1994), its influence on concentrations of endogenous ABA (Label and Lelu, 2000; von Aderkas et al., 2001), or its effects on gene expression (Dong et al., 1997), etc. Generally these studies used only one genotype.

However, somatic embryogenesis is known to be genotype-dependent in response to exogenous ABA (Jalonen et al., 1992; Find et al., 1998). In addition, extracellular proteins may play a crucial role in this process (von Arnold et al., 1996). At present no information is available to compare ABA utilization among genotypes that have demonstrated differences in embryo maturation capability. The goal of this research is to study ABA utilization in suspension cultures in three selected embryogenic cell lines that differ in morphology, growth rates, and embryo maturation capabilities. Interior spruce [Picea glauca (Moench) Voss × engelmannii Parry ex Engelm.] was chosen for this study as it is a relatively easy species in which to induce somatic embryogenesis. This research also compares the effects of exogenously supplied ABA, in particular S-ABA, R-ABA, and M-ABA.

**Materials and methods**

**Induction of embryogenic tissue in interior spruce**

Mature cones of hybrid crosses were obtained from the seed orchard of Kalamalka Research Station (Vernon, BC, Canada) in September 2005. Cones were sprayed with 70% ethanol and kept in a laminar flow hood for about 10 min. Seeds were then dissected from the cones and sterilized in 20% (v/v) bleach (7% NaOCl) for 15 min. This step was followed by three rinses with sterile water. Embryos were dissected from megagametophytes under a dissecting microscope and placed on gel-solidified medium for the induction of embryogenic tissue. Half-strength LV medium (Litvay et al., 1985) was used as the basal medium with additives of 10 g l⁻¹ sucrose, 0.8 g l⁻¹ casein hydrolysate, and 0.4 g l⁻¹ glutamine. In addition, 20 µM 2,4-D and 10 µM 6-benzyladenine were added to the basal medium. This medium was solidified with 3.8 g l⁻¹ phytagel (Sigma, St Louis, MO, USA). Twelve embryos were placed on induction medium in each Petri dish. Induction plates were kept in darkness at 22 ± 1 °C for 3 weeks.

**Embryogenic tissue maintenance**

Modified half-strength LV medium (mLV) was used as the basal medium plus additives as in the induction medium. In mLV medium, CaCl₂ was increased by 20-fold and no NH₄NO₃ was used. In maintenance medium, plant growth regulators were reduced to half the strength of those in the induction medium. Embryogenic tissue was selected, based on morphology, under a dissecting microscope and transferred onto maintenance medium. Maintenance cultures were kept in darkness at 22 ± 1 °C and subcultured biweekly.

For initiation and maintenance of suspension cultures, 1 g embryogenic tissue was transferred into a 250 ml flask containing 100 ml liquid maintenance medium. The medium composition was identical to that in solid maintenance medium but minus gelling agent. Flasks were placed on an incubator shaker (G25, New Brunswick Scientific Co., Inc.) set at 100 rpm, with no light at 22 ± 1 °C. Suspension cultures were sub-cultured weekly with 1% (w/v) initial cell inoculation density.

**Embryo maturation culture**

Since SEs could not mature in suspension cultures, embryogenic tissue had to be transferred onto a gel-solidified maturation medium to test embryo maturation capability. In order to initiate maturation cultures, tissue in liquid maintenance culture was drained with a cell collector and resuspended in liquid medium at 12% density (w/v). Resuspended tissue (0.8 ml) was spread on a filter paper that was placed on gel-solidified maturation medium. The basal medium for embryogenesis was mLV medium containing 60 µM R-ABA, 30 g l⁻¹ sucrose, 50 g l⁻¹ PEG 4000, and 0.2 g l⁻¹ NH₄NO₃. Maturation medium was solidified with 8 g l⁻¹ phytagel. Casein hydrolysate, glutamine, and ABA were sterilized through a 0.22 µm filter and added to the autoclaved media. Cultures were evaluated 8 weeks after the initiation of maturation culture. Mature SEs with well-developed cotyledons were counted. Previous experiments showed that the selected cell lines were unable to produce mature embryos in the absence of exogenous ABA.

**ABA supplements in suspension cultures**

Exogenous ABA of high purity, i.e. S-ABA (Lomon Bio Technology Co., Ltd, Deyang, PR China), R-ABA (Sigma), or M-ABA (Sigma) was dissolved in a small quantity of 1 N NaOH before bringing up to the desired volume with distilled water. For ABA stock solution, pH was adjusted to 5.8 with 0.1 N HCl. Freshly made ABA stock solutions were filter-sterilized and added to suspension media for a final concentration of 30 µM. Culture
medium for ABA treatments was mLV plus the additives for liquid maintenance omitting 2,4-D and 6-benzyladenine. Erlenmeyer flasks (150 ml) containing 50 ml liquid medium were used in this experiment. One millilitre of settled tissue was transferred from liquid maintenance culture into each flask containing ABA-supplied media. Three flasks were used for each treatment as replicates. All flasks for ABA experiments were kept under the same culture conditions as other suspension cultures. One millilitre of culture medium was taken from each flask at different time points during a 3-week period of culturing. The samples were either immediately processed for analysis or temporarily stored at −20 °C until required for further analysis.

Measurement of tissue growth in suspension culture

Tissue growth was measured by settled culture volume (SCV). Briefly, culture flasks were taken off the shaker and placed in the hood with caps on for 15 min to let tissue settle down before the culture volume was measured. Tissue growth was calculated using the following equation:

\[
\text{Tissue growth (\%)} = \frac{\text{ml SCV (final culture) } - \text{ ml SCV (initial culture)}}{\text{ml SCV (initial culture)}} \times 100
\]

Analyses with high performance liquid chromatography (HPLC)

For preparation, liquid samples of 500 μl were added into 2 ml centrifuge tubes and centrifuged in an Eppendorf centrifuge (5415C) at 9800 g for 10 min. Supernatant (250 μl) was then taken from each sample. In order to remove potential particles in the sample, samples were filtered through a 5 µm mini-filter fitted to a syringe (2 ml). All samples were transferred into vials of HPLC auto sampler (System 508). Vial inserts of 200 μl were used for samples of small quantity. As a control, exogenous standards of R-ABA (Sigma), M-ABA (Sigma), S-ABA (Lomon Bio Technology), PA, and DPA (Plant Biotechnology Institute, Saskatoon, SK, Canada) were added to the suspension culture medium as appropriate. Control standards were processed through the same steps as for other samples.

The prepared sample was injected into reversed-phase HPLC (Beckman Coulter, System Gold, Fullerton, CA, USA) with a Luna C18 column (250×4.6 mm i.d., 5 µm, 100 Å; Phenomenex, Torrance, CA, USA) protected by a security guard cartridge (4×3 mm). Binary gradient elution was performed at 1 ml min⁻¹ with methanol (MeOH) and water; both were acidified with 0.1% acetic acid. The column was equilibrated with 50% MeOH for 5 min. After sample injection, MeOH was raised from 50% up to 80% during a period of 14 min and then raised instantly to 100% and maintained for 4 min before decreasing to 50% for equilibration before the next sample injection. Column elution was monitored using a UV detector at a wavelength of 254 nm. All solvents used were of analytical grade. Retention times of (+/-) cis-ABA, trans-ABA, (+/-) trans-ABA, PA, and DPA, were, respectively, c. 9.7 min, 8.8 min, 6.8 min, and 5.4 min. Identification and quantification of ABA, PA, and DPA was obtained by co-chromatography of exogenous standards in HPLC conditions using retention time and peak area.

For chiral chromatography, a 500 μl sample was brought to dryness, after filtration and centrifugation, by vacuum evaporator. The sample was then dissolved with 50 μl 100% MeOH and transferred into inserts of auto-sampler vials. Samples were injected into a chiral HPLC column [Pirkle Covalent (R, R)-Whelk-O1, 25 cm×4.6 mm i.d., 5 µm, 100 Å, ReGIS Technologies Inc., Morto Grover, IL, USA] with a security guard cartridge (C18, 4×3 mm). ABA was separated by isocratic elution of hexane/IPA (85/15, v/v) acidified with 0.1% acetic acid at a flow rate of 1.5 ml min⁻¹. ABA was detected with a UV detector as described previously. Retention times of (+/-) cis, trans-ABA and (–) cis, trans-ABA were c. 6.9 min and 8.1 min, respectively.

Experimental design and statistical analysis

Three flasks were used for each treatment per cell line in suspension culture and five Petri dishes were used for maturation culture of each cell line. Three replicates were analysed by HPLC for each time point per cell line. Statistical analysis was performed with analysis of variance (ANOVA) using MINITAB statistical software (MINITAB Inc., State College, PA, USA) and setting significance at \( P < 0.05 \).

Results

Tissue growth, morphology, and embryo maturation capability

After 22 d in suspension cultures supplied with exogenous ABA, the highest tissue growth was obtained with cell line IPS16 and the lowest one was with cell line ISP48 (Fig. 2). In those cultures supplied with ABA from three different sources, S-ABA slowed down tissue growth significantly in the cultures of cell lines IPS16 or ISP11 when compared with cultures supplied with chemically synthesized ABA, i.e. R-ABA and M-ABA (Fig. 2). Within cell line ISP16, significantly higher tissue growth was achieved in the culture supplied with M-ABA than other cultures (Fig. 2). After 3 weeks in the ABA-containing suspension cultures, most embryos in cell lines ISP11 and ISP48 developed to the pre-cotyledonal stage. The embryo proper increased in width and length and the colour turned a little yellow (Fig. 3A, C). Although numerous immature embryos developed in the culture of cell line ISP16, little change could be found in embryo size, especially in the width of the embryo proper. They were still tiny and of a semi-transparent white colour (Fig. 3B), similar to those in previous tests in the absence of exogenous ABA. It
indicated little response of the embryos to exogenously added ABA. Embryos of two out of the three cell lines developed cotyledons in 3–4 weeks after transfer from liquid maintenance culture to gel-solidified maturation medium; the exception was cell line ISP16. Cultures were evaluated and mature embryos were counted after 8 weeks in maturation culture (Fig. 3D–F). On the basis of initial tissue quantity, cell line ISP11 produced the highest number of mature SEs, i.e. 212±34 SEs (100 mg tissue)^{-1} for ISP48. By contrast, the tissue of ISP16 contained fewer mature embryos, i.e.14±8 SEs (100 mg tissue)^{-1}. Furthermore, the tissue of ISP16 kept proliferating during maturation culture (Fig. 3E). Compared with ISP16, the yield of mature embryos was 15.1-fold and 10.4-fold higher with ISP11 and ISP48, respectively.

Changes of total ABA concentration in suspension cultures with different cell lines

In the cultures supplied with S-ABA, little change occurred in ABA concentrations during the first week (Fig. 4).

After 14 d, total ABA concentrations in the media declined by approximately 49.2, 33.2, and 39.4% in the cultures with ISP16, ISP11, or ISP48, respectively. At the end of 22 d, exogenously supplied ABA was almost used up in the culture of ISP11. Meanwhile 28.2% or 22.3% ABA was retained in the cultures with cell lines ISP16 and ISP48, respectively.

In the culture supplied with R-ABA, concentrations of total ABA declined to approximately 50% in 2 weeks in all three cell lines. ABA concentrations in the culture showed little change thereafter. ABA remaining in the media was identified as (−)-ABA by chiral chromatography. Patterns of ABA concentration changes were similar in all the three cell lines except for a faster decline of ABA concentration in the culture of ISP48 over the first week of the culture (Fig. 5).

In the cultures supplied with M-ABA, about half of (+/−)-cis, trans-ABA was utilized during a 3 week period in all three cell lines (Fig. 6). However, concentrations of (+/−)-trans, trans-ABA showed little decrease in the culture during the first 10 d. The concentration of (+/−)-trans, trans-ABA even increased a little in the culture of ISP16.

Fig. 3. Response of genotypes to exogenous ABA in suspension and maturation cultures. (A–C) showing embryogenic tissue in suspension cultures supplied with 30 µM R-ABA for 22 d. Embryos increased in size in cell lines ISP48 (A) and ISP11 (C), but not in cell line ISP16 (B). (D–F) Embryogenic tissue cultured on gel-solidified maturation media supplied with 60 µM R-ABA for 8 weeks. Many embryos matured with cell lines ISP48 (D) and ISP11 (F) with little additional tissue proliferation. Tissue of cell line ISP16 was proliferated with little embryo maturation (E). All scale bars = 2 mm.

Fig. 4. Changes of ABA concentrations in culture media supplied with S-ABA. Embryogenic tissue of three genotypes was cultured in suspension cultures supplied with 30 µM S-ABA. Mean ±SE, n=3.

Fig. 5. Changes of ABA concentrations in culture media supplied with R-ABA. Embryogenic tissue of three genotypes was in suspension cultures supplied with 30 µM R-ABA. Mean ±SE, n=3.
At the end of 3 weeks, concentrations of (+/–)-trans, trans-ABA were slightly lower than their initial concentrations in the cultures of either cell lines ISP11 or ISP48 (Fig. 7).

Quantification of S-ABA turnover on the basis of tissue units
When the culture was supplied with S-ABA, a significant difference in ABA consumption on the basis of per millilitre of SCV existed among the three genotypes (Fig. 8). Ability to consume ABA was consistently higher with cell line ISP48 than the rest. Within 22 d, consumption of S-ABA with cell lines ISP11 and ISP48 was 2.2-fold and 3.6-fold greater, respectively, than that of ISP16.

ABA metabolites in culture media
Concentrations of PA and DPA increased with time in the culture. After 22 d, the total concentrations of ABA metabolites, i.e. PA plus DPA, differed with ABA source (Fig. 9). The highest concentration was observed in media supplied with S-ABA and the lowest with M-ABA, regardless of genotype. Neither PA nor DPA was observed in the media without inoculating embryogenic tissues over a 22 d period (results not shown). Concentrations of DPA in the media were usually lower than the concentrations of PA except when ISP11 was cultured with either R-ABA or M-ABA. Compared with ISP16, a poor producer of mature SEs, ISP11 and ISP48 had higher ratios of DPA to PA. The ratio was higher in a manner independent of ABA source.

Discussion
Based on this study, the ability of embryogenic tissue to use exogenous ABA may reflect the capability of embryo

Fig. 6. Changes of (+/–)-cis, trans-ABA concentrations in culture media supplied with M-ABA. Embryogenic tissue of three genotypes was cultured in suspension cultures supplied with 30 μM M-ABA. Mean ± SE, n=3.

Fig. 7. Changes of (+/–)-trans, trans-ABA concentrations in culture media supplied with M-ABA. Embryogenic tissue of three genotypes was cultured in suspension cultures supplied with 30 μM M-ABA. Mean ± SE, n=3.

Fig. 8. S-ABA consumption on the basis of tissue units. Embryogenic tissue of three genotypes was cultured in suspension culture for 22 d. These cultures were supplied with 30 μM S-ABA. Mean values of three independent replicates with standard errors are shown. Significant differences at the P < 0.05 level are indicated by different letters.

Fig. 9. Concentrations of PA and DPA in suspension cultures. Embryogenic tissue of three genotypes was cultured in suspension cultures for 22 d. These cultures were supplied with 30 μM S-ABA, R-ABA, or M-ABA. Mean ± SE, n=3.
maturation in different genotypes. Past studies mainly focused on ABA consumption in cultures; they were often based on a single cell line for each species (Dunstan et al., 1992, 1994; Label and Lelu, 2000). In the present study, ABA consumption per unit of tissue was used in order to quantify the capability of tissue to utilize exogenous ABA. In the present experiments, cell density, culture volume, and ABA concentration were initially kept identical. In addition, culture conditions and culture age were also kept consistent. In this way, a fair comparison of ABA consumption was expected. In this study, cell line ISP16 demonstrated the highest tissue proliferation and the lowest ability to consume exogenous ABA on the basis of per unit of tissue.

By contrast with embryogenic tissue, non-embryogenic callus could not metabolize exogenous ABA in larch (Label and Lelu, 2000). This indicated that the ability of tissue to utilize exogenous ABA correlated with the capability of the tissue for somatic embryogenesis. On the other hand, embryo maturation is a process that may not be under the strict control of a single plant hormone such as ABA (Jourdain et al., 1997; von Aderkas et al., 2001). In order to obtain mature embryos in conifers, three major culture steps are usually required, including induction of embryogenic tissue, maintenance of the tissue, and stimulation of embryo maturation. In the first two steps, auxin and cytokinin are commonly used to proliferate embryogenic tissue. On induction/proliferation media, immature embryogenic tissue, maintenance of the tissue, and stimulation of embryo maturation depends on endogenous hormones, especially auxin, and the ability of tissue to reduce PA further into DPA.

Furthermore, limitation of ABA availability may promote the capability of embryo maturation was observed. The ratio of DPA to PA relationship between the ratio of DPA to PA and the capability of embryo maturation was expressed as the linear regression of the ratio of DPA to PA and the ability of tissue to reduce PA further into DPA. Furthermore, limitation of ABA availability may promote tissue to oxidize PA into DPA. This was supported by the fact that higher ratios of DPA to PA existed in the cultures supplied with chemically synthesized ABA.

A slight decrease in (+/–)-trans, trans-ABA concentrations in the cultures of ISP11 and ISP48 were observed, indicating a possible utilization of trans, trans-ABA after 22 d in the culture when (+)-cis, trans-ABA, the preferred isomer, was possibly used up in the media. There have been no reports on utilization of (+/–)-trans, trans-ABA by conifer embryogenic tissue. In order to check utilization of (+/–)-trans, trans-ABA, in future experiments, this ABA should be supplied as the sole ABA source to enhance its possible turnover.
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References


