Lodgepole pine: the first evidence of seed-based somatic embryogenesis and the expression of embryogenesis marker genes in shoot bud cultures of adult trees

SO-YOUNG PARK,1 KRYSTYNA KLIMASZEWSKA,2 JI-YOUNG PARK3 and SHAWN D. MANSFIELD3,4
1 Division of Forest Biotechnology, Korea Forest Research Institute, Suwon 441-847, South Korea
2 Natural Resources Canada, Canadian Forest Service, Laurentian Forestry Centre, 1055 rue du P.E.P.S., PO Box 10380, Station Sainte-Foy, Quebec City, QC, Canada G1V 4C7
3 Department of Wood Science, University of British Columbia, 4030-2424 Main Mall, Vancouver, BC, Canada V6T 1Z4
4 Corresponding author (shawn.mansfield@ubc.ca)

Received June 8, 2010; accepted August 16, 2010; published online October 8, 2010; handling Editor Gary Peter

Summary Of the various alternatives for cloning elite conifers, somatic embryogenesis (SE) appears to be the best option. In recent years, significant areas of lodgepole pine (Pinus contorta) forest have been devastated by the mountain pine beetle (MPB) in Western Canada. In an attempt to establish an SE propagation system for MPB-resistant lodgepole pine, several families displaying varying levels of resistance were selected for experimentation involving shoot bud and immature seed explants. In bud cultures, eight embryogenic lines were induced from 2 of 15 genotypes following various treatments. Genotype had an important influence on embryogenic culture initiation, and this effect was consistent over time. These lines were identified by microscopic observation and genetic markers. Despite the abundance of early somatic embryos, the cultures have yet to develop into mature embryos. In contrast, immature zygotic embryos (ZEts) cultured from megagametophytes initiated SE at an early dominance stage via nodule-type callus in 1 of 10 genotypes. As part of the study, putative embryogenesis-specific genes, WOX2 (WUSCHELL homeobox 2) and HAP3A, were analyzed in cultures of both shoot bud explants and ZEs. On the basis of these analyses, we postulate that PcHAP3A was expressed mainly in callus and may be involved in cell division, whereas WOX2 was expressed mainly in embryonal mass (EM)-like tissues. The findings from this study, based on molecular assessment, suggest that the cell lines derived from bud cultures were truly EM. Moreover, these experimental observations suggest that PcWOX2 could be used as an early genetic marker to discriminate embryogenic cultures from callus.

Keywords: gene expression, HAP3A, in vitro culture, Pinus contorta, recalcitrance, somatic embryos, somatic seedlings, WOX.

Introduction

Somatic embryogenesis (SE) entails a complex developmental reprogramming in which somatic cells are converted to embryogenic competent cells capable of becoming intact plants by achieving totipotency (Suprasanna and Bapat 2005, Zeng et al. 2007). SE is a biotechnological tool that has been developed for rapid and efficient cloning of elite tree genotypes, which has significantly aided in the deployment of forest trees (von Aderkas and Bonga 2000). However, in tree species, SE is difficult to achieve in tissues beyond the seedling stage, a consequence of the complex interplay of several physiological parameters and the appropriate regulation at the transcription level (reviewed in Bonga et al. 2010). Gymnosperms are generally more recalcitrant to propagation in vitro than most angiosperm trees and herbaceous plants (von Aderkas and Bonga 2000). As a consequence, SE has, most often, been achieved from immature embryos and juvenile tissues (Harvengt et al. 2001, Lelu-Walter et al. 2006, Park et al. 2006, Klimaszewska et al. 2007). It has proved extremely challenging to achieve SE from adult conifers by in vitro manipulation, and many attempts have been made to overcome the inherent phase change that is associated with aging (Gupta and Durzan 1985, Monteuuis 1991, Bonga 1997, Merkle et al. 1998, Andersone and Ievinsh 2002). Traditional methods of in vitro manipulation, including complex arrangements of plant growth regulators (PGRs) and media composition, have proved ineffective and many studies have, therefore, concluded that the choice of explants is important when attempting SE from mature trees (McCown 2000, von Aderkas and Bonga 2000), but not for conifers (Bonga and von Aderkas 1992).

Over the past decade, considerable progress has been made to improve our understanding of the recalcitrant
physiology and cellular totipotency reprogramming during SE in plants (Zeng et al. 2007). Embryogenic cells are totipotent stem cells and can, by definition, give rise to any type of cell or tissue, whereas meristematic (apical and root) and cambial cells are pluripotent stem cells and can, therefore, give rise to most, but not all, kinds of cell or tissue (Verdeil et al. 2007, Elo et al. 2009). Recently, stem cell-containing tissues have been identified as likely target tissue for the induction of embryogenic culture from mature gymnosperms (Aronen et al. 2008, Klimaszewska et al. 2009). However, to date, there is no evidence confirming this hypothesis.

There has been extensive research investigating the role of genes involved in SE processes, which has shown that three categories of gene are expressed: (i) genes involved in cell division, (ii) genes involved in organ formation and (iii) genes specific for the process of SE (Komamine et al. 2005). The latter group of genes has been the primary target of several studies in model species, e.g., somatic embryogenesis receptor-like kinase (SERK) in carrot (Schrader et al. 1997), leafy cotyledon (LEC) genes in Arabidopsis (Gaj et al. 2005), WUSCHELL homeobox (WOX) in Picea abies (Palovaara and Hakman 2008) and WUS in ginseng (Kiselev et al. 2009). Although it is possible to identify embryogenic from non-embryogenic cultures by visual inspection, reliable expression markers associated with early SE that enable discrimination among cultures containing a high percentage of embryogenic cells when the culture is a mixture of embryogenic and non-embryogenic cells would be extremely valuable (Palovaara and Hakman 2008). Apart from their use in early embryogenic culture discrimination, molecular markers would also be invaluable to study the underlying molecular mechanisms regulating the change of somatic cells to embryogenic cells. Among several putative marker genes, two transcription factors, WOX2, which is one of 15 WOX family proteins, and HAP3 (heme-activated protein 3), which is encoded by LEC genes, are well known to play a key role in controlling many aspects of plant SE (Yazawa et al. 2004, Gaj et al. 2005, Palovaara and Hakman 2008, Palovaara et al. 2010). However, relatively little is known about the molecular events that occur in conifer cultures.

Since 1997, lodgepole pine (Pinus contorta Dougl. ex Loud. Engelm) genotypes (Fig. 3a), which had previously been selected based on the level of resistance to MPB attack, from the British Columbia Ministry of Forests and Range seed orchard 307 (British Columbia, Canada), were collected bi-weekly from February to July in 2008 and 2009.

Apical shoot buds (Fig. 3b) were rinsed with 95% ethanol briefly to remove resin from the surface and then immersed in 30% H2O2 for 1 h. Afterward, the buds were soaked in 70% ethanol for 15 min and then rinsed three times with sterilized deionized water. The scales of the bud were then removed, and five transverse slices (0.5–1 mm thick) were taken sequentially from the tip of each bud. Approximately 25 slices were placed in Petri dishes (90 × 15 mm2) containing a Gupta and Durzan (DCR) medium (Gupta and Durzan 1985) supplemented with 3 g L−1 activated charcoal (AC; Sigma, St Louis, MO, USA) and 0.2 g L−1 polyvinylpyrrolidone (PVP) for pre-culture (AC medium, Table 1). The explants were cultured on either AC medium following alternative temperature treatment at 38°C for 4 h and then transferred to 4°C for 3 days or DCR induction medium (IM; Table 1) without pre-culture as control. All experiments were conducted with four to six replicate Petri dishes per treatment, arranged in a fully randomized design. After preculture, the explants were transferred to IM containing 2,4-Dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA) and 6-benzyladenine (BA), all obtained from Sigma. The pH of the media was adjusted to 5.7 with 1 M HCl after the addition of all ingredients, except gelling agent or filter-sterilized nutrients. Gellan gum (Phytage™; Sigma) was added prior to autoclaving at 121 °C for 20 min. Stock solutions of abscisic acid (α-cis-trans ABA; Sigma) were prepared with 0.1 M KOH. Stock solutions of both ABA and l-glutamine were filter-sterilized and added after autoclaving to the medium cooled to ~55 °C. All experiments were conducted at ~25 °C in the dark.

After 8 weeks of initial culture, calli originating from the cambial region of a shoot bud slice were recorded. The calli were then proliferated and cultured on maintenance medium (MM; Table 1), and subcultured every 2 or 4 weeks for further development. The putative embryogenic cultures were preliminarily identified by microscopic observation.

Immature seed culture

Twenty cones from each of the 15 genotypes were collected four times at an interval of 1 week starting from 19 June 2008. Immature cones were first cut longitudinally using an

Materials and methods

Apical bud culture

Fifteen 20-year-old lodgepole pine (P. contorta Dougl. ex Loud. Engelm) genotypes (Fig. 3a), which had previously been selected based on the level of resistance to MPB attack, from the British Columbia Ministry of Forests and Range seed orchard 307 (British Columbia, Canada), were collected bi-weekly from February to July in 2008 and 2009.

Apical shoot buds (Fig. 3b) were rinsed with 95% ethanol briefly to remove resin from the surface and then immersed in 30% H2O2 for 1 h. Afterward, the buds were soaked in 70% ethanol for 15 min and then rinsed three times with sterilized deionized water. The scales of the bud were then removed, and five transverse slices (0.5–1 mm thick) were taken sequentially from the tip of each bud. Approximately 25 slices were placed in Petri dishes (90 × 15 mm2) containing a Gupta and Durzan (DCR) medium (Gupta and Durzan 1985) supplemented with 3 g L−1 activated charcoal (AC; Sigma, St Louis, MO, USA) and 0.2 g L−1 polyvinylpyrrolidone (PVP) for pre-culture (AC medium, Table 1). The explants were cultured on either AC medium following alternative temperature treatment at 38°C for 4 h and then transferred to 4°C for 3 days or DCR induction medium (IM; Table 1) without pre-culture as control. All experiments were conducted with four to six replicate Petri dishes per treatment, arranged in a fully randomized design. After preculture, the explants were transferred to IM containing 2,4-Dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA) and 6-benzyladenine (BA), all obtained from Sigma. The pH of the media was adjusted to 5.7 with 1 M HCl after the addition of all ingredients, except gelling agent or filter-sterilized nutrients. Gellan gum (Phytage™; Sigma) was added prior to autoclaving at 121 °C for 20 min. Stock solutions of abscisic acid (α-cis-trans ABA; Sigma) were prepared with 0.1 M KOH. Stock solutions of both ABA and l-glutamine were filter-sterilized and added after autoclaving to the medium cooled to ~55 °C. All experiments were conducted at ~25 °C in the dark.

After 8 weeks of initial culture, calli originating from the cambial region of a shoot bud slice were recorded. The calli were then proliferated and cultured on maintenance medium (MM; Table 1), and subcultured every 2 or 4 weeks for further development. The putative embryogenic cultures were preliminarily identified by microscopic observation.

Immature seed culture

Twenty cones from each of the 15 genotypes were collected four times at an interval of 1 week starting from 19 June 2008. Immature cones were first cut longitudinally using an
electric bandsaw and then briefly dried in the laminar flow hood, and finally submerged in 95% ethanol for 10 min. The immature seeds were detached from the cone, and the seed coat and nucellus were removed with sterile forceps. Twenty megagametophytes containing immature ZEs were placed on each Petri dish. For initiation of embryonal mass (EM), three different induction media were tested (see Table 1). After 2 months of culture, the EM induction rate of each genotype and medium was quantified.

**Maturation and germination of seed-derived somatic embryos**

The lines of putative EM were maintained on MM (development medium (DM), Table 1) and subcultured bi-weekly. For maturation of somatic embryos, 100 mg of EM was cultured on maturation medium (MtM) containing 100 µM ABA (Table 1), using the filter paper method of Klimaszewska et al. (2001). All experiments were conducted at ≏25 °C in the dark. After 3 months of culture, fully developed, cotyledonary embryos were transferred to modified Litvay (MLV) [Litvay et al. 1985 modified as in Klimaszewska et al. (2001)], germination medium (GM; Table 1) without PGRs and maintained in the dark. Following 1 week on GM, the cultures were transferred to 40 µmol m⁻² s⁻¹ PPFD light originating from cool-white fluorescent lamps (at 23 °C).

**Non-embryogenic culture**

Non-embryogenic (callus) (NE) cultures were induced from young needles of 1-month-old seedlings of lodgepole pine. The segments of needles (~1 cm in length) were cultured on IM, the same medium as used to culture shoot bud explants, and maintained under the same conditions as described earlier for embryogenic cell lines. The seedling calli (NE) were used in this study as a negative control for gene expression analysis. For maintenance, the calli were subcultured every 2 weeks.

**RNA preparation and cDNA synthesis**

Mature bud-derived cell lines and all other tissues analyzed were collected in duplicate, and immediately frozen in liquid nitrogen. Approximately 0.5 g of fresh frozen tissue from each sample was ground in a mortar and pestle with liquid nitrogen. Total RNA was extracted according to the method of Azevedo et al. (2003). To remove residual genomic DNA, 10 µg of RNA was treated with TURBO-DNase™ (Ambion, Austin, TX, USA). cDNA was generated from 1 µg of DNase-treated RNA using the Superscript II RT system (Invitrogen, CA, USA) according to the manufacturer’s protocol. Each reaction was run in duplicate, generating two independent cDNA samples for each RNA sample.

**Cloning of PcWOX2 and PcHAP3A**

In an attempt to isolate the putative WOX2-ortholog from *P. contorta* EM culture, primers (forward: 5'-ATGGCCG

---

**Table 1. Media composition for pretreatment and culture of *P. contorta* shoot bud explants and immature embryos.**

<table>
<thead>
<tr>
<th>Explant</th>
<th>Media name</th>
<th>Culture purpose</th>
<th>Medium recipe</th>
<th>PGR (µM) L-glutamine</th>
<th>Casein hydrolysate</th>
<th>Myo-inositol</th>
<th>PVP</th>
<th>Maltose (mM)</th>
<th>Sucrose (mM)</th>
<th>Gellan gum (%)</th>
<th>Maltose (g l⁻¹)</th>
<th>Mycoidine (g l⁻¹)</th>
<th>Hygromycin (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot buds</td>
<td>IM</td>
<td>Pre-culture</td>
<td>2-4-D NAA BA ABA</td>
<td>20</td>
<td>2.2</td>
<td>1.1</td>
<td>0.5</td>
<td>1.0</td>
<td>0.5</td>
<td>0.1</td>
<td>60</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Mature bud</td>
<td>MM</td>
<td>SE induction</td>
<td>2-4-D NAA BA ABA</td>
<td>2.0</td>
<td>2.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.2</td>
<td>120</td>
<td>0.6</td>
</tr>
<tr>
<td>Immature ZEs</td>
<td>MM</td>
<td>SE induction</td>
<td>2-4-D NAA BA ABA</td>
<td>2.0</td>
<td>2.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.2</td>
<td>120</td>
<td>0.6</td>
</tr>
<tr>
<td>Immature ZEs</td>
<td>MM</td>
<td>SE induction</td>
<td>2-4-D NAA BA ABA</td>
<td>2.0</td>
<td>2.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.2</td>
<td>120</td>
<td>0.6</td>
</tr>
<tr>
<td>Immature ZEs</td>
<td>MM</td>
<td>SE induction</td>
<td>2-4-D NAA BA ABA</td>
<td>2.0</td>
<td>2.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.2</td>
<td>120</td>
<td>0.6</td>
</tr>
<tr>
<td>Immature ZEs</td>
<td>MM</td>
<td>SE induction</td>
<td>2-4-D NAA BA ABA</td>
<td>2.0</td>
<td>2.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.2</td>
<td>120</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*Activated charcoal 3 g l⁻¹ was added only into the AC medium for preculture and 4 mM CaCl₂ was added for entire shoot bud cultures.*

---

**Table 1. Media composition for pretreatment and culture of *P. contorta* shoot bud explants and immature embryos.**

<table>
<thead>
<tr>
<th>Explant</th>
<th>Media name</th>
<th>Culture purpose</th>
<th>Medium recipe</th>
<th>PGR (µM) L-glutamine</th>
<th>Casein hydrolysate</th>
<th>Myo-inositol</th>
<th>PVP</th>
<th>Maltose (mM)</th>
<th>Sucrose (mM)</th>
<th>Gellan gum (%)</th>
<th>Maltose (g l⁻¹)</th>
<th>Mycoidine (g l⁻¹)</th>
<th>Hygromycin (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot buds</td>
<td>IM</td>
<td>Pre-culture</td>
<td>2-4-D NAA BA ABA</td>
<td>20</td>
<td>2.2</td>
<td>1.1</td>
<td>0.5</td>
<td>1.0</td>
<td>0.5</td>
<td>0.1</td>
<td>60</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Mature bud</td>
<td>MM</td>
<td>SE induction</td>
<td>2-4-D NAA BA ABA</td>
<td>2.0</td>
<td>2.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.2</td>
<td>120</td>
<td>0.6</td>
</tr>
<tr>
<td>Immature ZEs</td>
<td>MM</td>
<td>SE induction</td>
<td>2-4-D NAA BA ABA</td>
<td>2.0</td>
<td>2.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.2</td>
<td>120</td>
<td>0.6</td>
</tr>
<tr>
<td>Immature ZEs</td>
<td>MM</td>
<td>SE induction</td>
<td>2-4-D NAA BA ABA</td>
<td>2.0</td>
<td>2.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.2</td>
<td>120</td>
<td>0.6</td>
</tr>
<tr>
<td>Immature ZEs</td>
<td>MM</td>
<td>SE induction</td>
<td>2-4-D NAA BA ABA</td>
<td>2.0</td>
<td>2.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.2</td>
<td>120</td>
<td>0.6</td>
</tr>
<tr>
<td>Immature ZEs</td>
<td>MM</td>
<td>SE induction</td>
<td>2-4-D NAA BA ABA</td>
<td>2.0</td>
<td>2.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.2</td>
<td>120</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*Activated charcoal 3 g l⁻¹ was added only into the AC medium for preculture and 4 mM CaCl₂ was added for entire shoot bud cultures.*
AGGGTCAATCCACCATGA-3'; reverse: 3'-CTACTT GCCAGGATGCTGAGGGATA-5') were designed based on homologous sequences publicly available, including PaWOX2 (P. abies WOX2, Acc. AM286747) and PtWOX2 (Pinus taeda WOX2, Acc. DR693345; Cairney et al. 2006). For the HAP3A ortholog, primers (forward: 5'-TTGTAGGT ATGATGTCCGAAGTTGG-3' and reverse: 3'-CCATCAGTCT ATTCTACAAGTITTA-5') were designed from homologous sequence regions of Picea glauca HAP3A (Acc. DR548381) and a cDNA expressed sequence tag (EST) of P. taeda HAP3A (Acc. DT627043).

Gene alignment was performed with the BioEdit program. Following PCR amplification from P. contorta cDNAs, the resulting amplicons were cloned into the TOPO-TA cloning vector (Invitrogen) and sequence was confirmed.

Absolute real-time qRT–PCR

Critical threshold (ct) values for PcWOX2 and PcHAP3A were quantified in triplicate with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) on an Mx3000P Real-Time PCR System (Stratagene, CA, USA). The forward and reverse primers for RT–PCR analysis were dtWX2-F (5'-CCACAGCAGCGATCCACAACGACCC-3') and dtWX2-R (3'-AGCGATGCCGGACGGATGCAATGGGG-5') for PcWOX2, and dtHAP3-F (5'-GCTGTGAGAGAGCAAGATAGGTTCA-3') and dtHAP3-R (3'-CACTGGTGATGAAGCTTATGTACTC-5') for PcHAP3A. Negative (distilled water) and no-template (total RNA) controls were included in each run. Thermocycler conditions for all PCR reactions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 1 min and 72 °C for 30 s. Absolute quantification of PcWOX2 and PcHAP3A copy number in each cDNA sample was determined using a standard curve and normalized per microgram of total RNA. The standard curve was generated with purified PCR product-obtained gene-specific primers for PcWOX2 and PcHAP3A that were serially diluted from 10^1 to 10^6 copies. The corresponding copy number was calculated as previously described (Whelan et al. 2003).

Statistics

Statistically significant differences were evaluated by one-way analysis of variance, followed by post hoc Tukey’s HSD (honestly significant differences) test comparisons, using STATISTICA v7.1 (StatSoft, Inc.). Differences of P < 0.05 were regarded as significant.

Results

Shoot bud culture

In both 2008 and 2009, the lodgepole pine shoot buds initiated growth in March and rapidly elongated until 17 April. Most of the bud scales opened during the same period (Figure 1a). The highest number of explants producing EM-like callus was obtained between 27 March and 2 April (Figure 1b), the survival rate of explants from later collections decreased and EM-like callus could not be recovered. Genotypic specificity was also observed, as genotypes 1506 and 1537 produced EM-like callus from >10% of all explants with preculture treatment (Figure 1b). The same two genotypes consistently showed a similar response throughout the experiment. In contrast, two genotypes (1520 and 1530) were strongly recalcitrant and did not produce EM-like callus at any sampling time (Figure 1b). Bud growth ceased in the middle of May, and needles flushed. More than 50% of the explants taken from apical buds collected from these latter time points became necrotic, whereas the remainder of the explants produced only brownish callus (data not shown). In three of the four genotypes, the survival rate of the explants originating from 27 March collection decreased slightly; however, preculture at alternative temperatures (38 °C for 4 h and 4 °C for 3 days) on the AC medium increased the induction of EM-like callus in line 1539 relative to control (Figure 2).

Throughout the entire collection series, <2% of the shoot bud explants produced callus from the cambial region (Figure 3c), regardless of genotype. The calli that did develop grew slowly compared with those originating from tissues other than the cambium and displayed a characteristic transparent and white morphology (Figure 3i). Staining the isolated calli with 2% aceto-carmine clearly showed the presence of embryogenic cells that were small and dense with
cytoplasm (Figure 3d–f). It was difficult to differentiate the origin (cambial or not) of the calli induced from whole explant tissues. Those calli initially grew quickly, but after 6 months on MM began to grow slowly and eventually turned brown and subsequently necrotized (Figure 3j and k).

The calli originating from the cambial region were carefully separated from mother explants after 2 months of culture and were transferred to fresh IM. After 2 months, the proliferating calli were transferred to MM for further development and proliferation, and were sub-cultured every 2–4 weeks depending on their growth. When the calli were substantially proliferated, sub-samples were observed under the microscope to investigate the developmental stage of the EM-like aggregates. On MM, some calli clearly revealed the presence of early-stage EM, which consisted of dividing cells and elongated suspensor-like cells (Figure 3g and h).

From the apical bud-derived EM-like callus, eight putatively embryogenic lines were maintained from 2 (1506 and 1537) of the 15 genotypes for a complete year. These cell lines were used for both routine evaluations of cell types and divisions, and as a source of material for embryogenesis marker evaluation. Despite the abundance of embryogenic cells and early EMs, the cultures were developmentally arrested even after culturing on spent medium or on the DM, which contained half the amount of PGRs compared with MM. Those cultures also failed to produce somatic embryos on MtM (Table 1) containing ABA (data not shown).

**Immature seed culture**

The developmental stage of the immature ZEs was late cleavage polyembryony to early dominance at the time of the first cone collection (19 June 2008). Extrusion, which results from the expansion of zygotic tissue and can lead to SE initiation, was visible on both standard (mLV-S) and low PGR (mLV-L) media for some megagametophytes after 2 weeks of culture (Figure 4a). However, most megagametophytes produced callus from whole explants after 4–5 weeks of culture and resulted in a mixture of various types of callus (data not shown). Approximately 50% of the calli turned brown after 8 weeks of culture.

Megagametophytes from one genotype (1532) from the first collection formed nodule-like callus, unlike callus induced from other collections (Figure 4b). With aceto-carmine staining, it was apparent that the nodules consisted of compact embryogenic cells (data not shown). However, the nodules failed to develop to EM on IM or MM, and only proliferated consistently as a nodule-type callus. In contrast, on DM with half the concentration of PGRs (Table 1), nodules formed EM that showed a clear demarcation between embryo and suspensors (Figure 4c), and the EM (iZE-1532) culture proliferated and was successfully maintained on DM (Figure 4d and e). Nodule-derived EM produced morphologically normal cotyledonal somatic embryos on MtM containing 120 µM ABA (Figure 4f) that germinated on GM after withdrawal of ABA (Figure 4g and h). The calli produced from mature ZEs (third or fourth collection) also contained a few EM-like cells in the cell aggregates; however, these cells failed to develop further and did not produce cotyledonal somatic embryos on MtM.

**Morphological characterization of the lines**

In this study, we observed three main types of cell from bud explants and immature ZEs: (i) bud-derived EM-like, (ii) immature ZE-derived EM (iZE-1532) and (iii) NE from seedling needles. Morphologically, the first type of callus was translucent and yellowish-white in color, whereas the second was translucent and white. In addition, both calli failed to turn green after transfer to a light environment. In contrast, the third NE callus was opaque and brownish-yellow in color when grown in the dark, and turned green in the light. Bud explant-derived calli did not develop mature somatic embryos on MtM containing ABA, whereas iZE-1532 produced mature somatic embryos and plants.

The different cell lines were clearly distinguishable by microscopic observation (Figure 5). Morphologically, the suspensor-like cells in the bud-derived tissue were thicker and shorter (Figure 5a) than those of true suspensors (Figure 5b). Furthermore, bud explant-derived cells were rich in starch granules, unlike true suspensors (Figure 5a). In contrast, the iZE-1532 EM was composed of compact

---

**Figure 2**. Effects of preculture with alternative temperature on survival and EM-like callus induction from shoot bud explants collected on 27 March.
cells that were small and had condensed cytoplasm. Furthermore, the whole part of the cells stained strongly with aceto-carmine, whereas the cells derived from the bud explants and megagametophyte tissue were pale in color, but were distinguishable from NE (Figure 5c), by vigorously dividing cell centers and elongated suspensor-like cells.

**PcWOX2 and PcHAP3A cloning and their expression**

As putative markers of embryogenesis, two transcription factors, *PcWOX2* (Acc. HM852975) and *PcHAP3A* (Acc. HM852976), were cloned from lodgepole pine EM (iZE-1532) and employed with absolute RT–PCR as an assessment tool for the pre-selection of EM tissue. *PcWOX2* was expressed in the bud-derived lines, whereas no expression was detected in the callus (NE) derived from a seedling needle (Figure 6a). In contrast, *PcHAP3A* was detected in all tissues, including NE and EM, with high copy number. The expression of *PcHAP3A* in the EM (iZE-1532) was 3–12 times higher than that in bud cell lines and just six times higher than that in NE (Figure 6b).

Among the different tissues, expression of *PcWOX2* was highest in the EM, whereas that of *PcHAP3A* was highest in the nodule-type callus and, with both genes, expression was dramatically decreased beyond the mature embryo stage (Figure 7a and b).

**Discussion**

In an attempt to establish in vitro culture from mature trees, the source of explants has been considered the most important factor, because their responses are strongly dependent on age, position, timing and genetic traits (see reviews by McCown 2000, von Aderkas and Bonga 2000). Previously, the genotype effect on embryogenicity has been established in many species (Al-Khayri and Al-Bahran 2004, Niskanen et al. 2004). In our study, 2 (1506 and 1537) of the 15 *P. contorta* genotypes examined were consistently responsive, and 2 others (1520 and 1530) were recalcitrant. These observations were unrelated to the level of MPB resistance. Interestingly, the responsive genotypes in the shoot bud cultures did not produce SE from immature ZE culture. These observations were unrelated to the level of MPB resistance. Interestingly, the responsive genotypes in the shoot bud cultures did not produce SE from immature ZE culture. These findings are in contrast to those reported for immature ZE culture of Scots pine, where the ability to initiate SE was mostly inherited from the maternal parent (Niskanen et al. 2004). We speculate that some of the genetic traits expressed during the vegetative growth phase (i.e., secondary metabolites such as resin, sensitivity to wounding, etc.) may influence the overall response of embryogenicity of
individual genotypes. Alternatively, the vegetative explants derived from the mature tree express the genetic characteristics of the mother tree only, whereas immature ZE contains the genetic coding for both maternal and paternal genetic traits, and as such embryogenicity of the genotypes may be different in ZEs compared with explants from mother trees.

Even if a genotype is responsive, the establishment of SE in gymnosperms is notoriously difficult if the age of the explant is beyond that of a mature ZE. Therefore, there is a pressing need to assess alternative tissues or cells that may prove less recalcitrant (Bonga and von Aderkas 1992, Bonga et al. 2010). The cambial layer, the lateral meristem and pluripotent stem cells are all potential alternative tissues. In an attempt to initiate totipotent embryogenic cells from the cambial zone, herein we applied an alternative temperature regime as a stress factor and demonstrated that such an approach can indeed induce EM-like tissue in an adult tree. However, these cells still fail to develop into mature somatic embryos. More intriguing is what the molecular and physiological mechanisms are that permit the establishment of embryogenic cells from somatic cells of adult trees (Bonga et al. 2010). von Aderkas and Bonga (2000) speculated that DNA methylation may somehow be involved in changing somatic cells of an adult tree and initiation of an embryogenesis-competent cell and that methylation may itself be governed by auxin levels, which results in cell division and dedifferentiation.
Previously, it has been shown in carrot that in order for organized embryo development to occur, the tissues require an oxidizing environment for cell proliferation, and that supplementation of media with 2,4-D facilitates such an environment by lowering the amount of endogenous antioxidants, i.e., glutathione, ascorbic acid, vitamins, etc. (Nissen and Minocha 1993, von Aderkas and Bonga 2000). In this experiment, EM-like lines were initiated from shoot buds by applying high concentrations of 2,4-D; however, those cells did not develop into organized somatic embryos even though the exogenous 2,4-D was removed from the media. Three possible reasons may explain these contradictory findings in gymnosperms when compared with the carrot model system: (i) the cellular/tissue composition may be responsible for failure of further development of the embryogenic lines, as the shoot bud tissue-derived EM-like lines were heterogeneous, consisting also of callus cells, and the latter contain high levels of lethal phenolics and oxidative compounds that can inherently increase the endogenous antioxidant level in culture and possibly arrest further development of the EM; (ii) the cells exposed to high levels of 2,4-D simply mimic embryogenic cell behavior and are not truly converted to embryogenic cells; and (iii) shoot bud tissue-derived EM-like lines may simply lose their embryogenic capacity because of the required extensive maintenance period.

As far as we are aware there are no other reports that show somatic cells mimicking embryogenic cell behavior as a consequence of application of exogenous growth regulators. Microscopic observation of the lodgepole pine cultures, however, clearly shows EM-like cells and suspensors in lines derived from tissue of mature trees, and indeed resembled the general features of the EM. However, there are slight differences in morphology as they contain more starch and less cytoplasm than the true, seed-derived EM, and as a result support the idea of cellular mimicry.

Although eight mature tree bud-derived cell lines of lodgepole pine were preliminarily identified as embryogenic by microscopic observation, true evidence of embryogenicity is still lacking. Therefore, molecular or biochemical markers are required. Such markers would offer a mechanism to assess whether bud-derived cells are truly embryogenic or just mimic cell division and/or to discriminate cell lines that contain more EM than others. Here, the expression of two transcription factors, WOX2 and HAP3A, were investigated as potential molecular markers. In gymnosperms, no WUS homolog was found (Palovaara and Hakman 2008) and as such it is believed that the WOX and WUS genes have not diverged in some species (Kiselev et al. 2009). An interrogation of the WOX family members in gymnosperms obtained from the public databases identified that PcWOX2 has 80% similarity to P. abies PaWOX2 and is almost identical to other pine species sequence such as PsWOX2 of Pinus sylvestris (99% homology) and PtWOX2 of P. taeda (100% homology). As a marker in lodgepole pine, PcWOX2 was shown to be strongly expressed in seed-derived EM (IZE-1532, positive control) and in all the selected cell lines from shoot bud cultures even though the transcript abundance was 100–150 times lower than the corresponding positive control. In contrast, no expression was
detected in callus. These findings are consistent with previous findings (Malik et al. 2007, Palovaara and Hakman 2008, Palovaara et al. 2010) which suggested that WOX2 may be a potential marker to predict the embryogenic potential of spruce and Brassica cultivars.

In contrast, PcHAP3A was expressed in all cell lines derived from bud cultures, even in callus. The expression of PcHAP3A was higher in nodule-type tissue than in EM, which is more developed and organized when compared with callus. On the other hand, WOX2 was comparatively higher in EM than in nodule-type tissue. Interestingly, this contrasts with observations in P. glauca where CHAP3A (conifer LEC1 gene) was expressed only in the EM, not in callus (Klimaszewska et al. 2009) even though PcHAP3A shows 90.2% amino acid homology with CHAP3A of P. glauca. It can be surmised that CHAP3A may therefore have different functions in conifers.

According to the observations, we postulate that PcHAP3A is expressed mainly in the callus and may be involved in cell division. Therefore, PcHAP3A is unable to differentiate between embryogenesis and NE tissue (callus), as both are actively dividing tissues. The findings from this study, based on the molecular assessment, suggest that the cell lines derived from bud cultures were truly embryogenic, not just cells that imitate embryogenic cultures (EC) in morphology. Moreover, these experimental observations also suggest that PcWOX2 could be used as an early genetic marker to discriminate EC from callus. In summary, we suggest that the use of cell stress, in combination with other culture conditions, has the potential to induce SE in culture of mature gymnosperms, which are notoriously recalcitrant. Understanding the underlying molecular and biochemical mechanisms that underpin these processes will aid in our understanding of the reprogramming process(es) that occur during SE.

Acknowledgments

The authors thank Dr Alvin Yanchuk and Nicholas Ukrainetz for sample ID and collection.

Funding

This work was financially supported by the British Columbia Ministry of Forests and Range, the Alberta Sustainable Resource Development and the National Research Foundation of Korea.

References


