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# Comparison of gene expression markers during zygotic and somatic embryogenesis in pine

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**Abstract** The relative expression of developmentally regulated genes was analyzed during zygotic embryo development in *Pinus taeda* and somatic embryo development/maturation in *P. taeda* and *Pinus oocarpa*. The following four embryo samples were studied: zygotic pro-embryos and somatic embryogenic suspensor masses; round/globular, early cotyledonary, and late cotyledonary. The relative transcript levels of six genes of interest: legumin-/vicilin-like, group 4 late embryogenesis abundant, homeodomain-leucine zipper I, 26S proteasome regulatory subunit S2, and clavata-like, associated with different aspects of embryo development, were analyzed by real-time PCR. In both pine species, the relative transcript levels for legumin-/vicilin-like storage proteins and the late embryogenesis abundant protein accumulated gradually through somatic embryo maturation, in contrast to zygotic embryos, where transcripts increased significantly to their highest levels at the late cotyledonary stage. The homeodomain-leucine zipper I relative transcript accumulation pattern differed between somatic and zygotic embryos. This difference was attributed to differences in cell-type compositions between the embryogenic suspensor masses and pro-embryos. Additionally, in *P. oocarpa*, the transcript levels of homeodomain-leucine zipper I remained high after the transfer of somatic embryos to maturation conditions, but declined during maturation in *P. taeda*. The highest 26S proteasome regulatory subunit S2 relative transcript levels in *P. taeda* were in round/globular somatic and zygotic embryos and in embryogenic suspensor masses and round/globular *P. oocarpa* somatic embryos. Finally, the relative transcripts levels for the clavata-like gene were more predominant in round/globular, early cotyledonary, and embryogenic suspensor masses in *P. taeda* and *P. oocarpa*, respectively. Somatic embryos exhibited relative gene expression patterns similar to their zygotic counterparts, although some differences were noted between zygotic and somatic embryos, as well as between the different pine somatic embryo systems.

**Keywords** *Pinus* · Storage protein · LEA · Clavata-like · RPN1 · HD-Zip I

Ulrika Egertsdotter and Barry S. Flinn contributed equally to project development, support, and supervision.

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## Introduction

In higher plants, zygotic embryo formation is divided into the following 3 phases: morphogenesis, maturation and desiccation (West and Harada 1993). These phases correspond to the definition of the shoot–root plant body pattern, the formation of storage organs (such as cotyledons), and the conversion to dormancy (Goldberg *et al*. 1994). Somatic embryogenesis is the process in which cells within the plant

are induced to form somatic embryos through the manipulation of culture medium components and plant growth regulators (PGRs; von Arnold *et al*. 2002) without the involvement of gamete fusion. Somatic embryos pass through similar stages of morphological development as they are exposed to culture conditions that attempt to mimic the growth conditions of their zygotic embryo counterparts (Ikeda and Kamada 2006). Thus, somatic embryogenesis has been used as a model system to study physiological, biochemical, and morphological pathways during zygotic embryogenesis (Zimmerman 1993) and to reveal novel pathways and gene interactions during embryo development (Stasolla *et al*. 2002; Quiroz-Figueroa *et al*. 2006).

Somatic embryogenesis in loblolly pine (*Pinus taeda*) was first reported more than 20 years ago (Gupta and Durzan 1987). Various improvements have been made to increase the number of "normal" plants produced, with a major emphasis placed on the improvement of initiation rates, especially in *P. taeda* (Pullman and Johnson 2002; Pullman *et al*. 2003a; Pullman *et al*. 2005; Pullman *et al*. 2006). However, many of the current protocols for somatic embryogenesis report low numbers of mature embryos per gram of fresh weight. In some cases, somatic embryos do not fully mature, resulting in slow germination and initial growth (Pullman *et al*. 2003a). With respect to Oocarpa pine (*Pinus oocarpa*), the most common pine species in the southern half of Mexico and Central America (Dvorak *et al*. 2009), somatic embryogenesis has only recently been reported (Lara-Chavez *et al*. 2011). Full seedling development was obtained from somatic embryos initiated from immature zygotic embryos; however, improvements are still needed to achieve a more reliable protocol across additional cell lines.

Currently, it is possible to distinguish embryogenic tissue from non-embryogenic tissue through visual observations and the use of histochemical stains and molecular markers. Genes associated with embryogenesis (SERK, LEC1, FUS3, and ABI3) are differentially expressed between embryogenic and non-embryogenic cultures (Yang and Zhang 2010). Following the induction and multiplication of embryogenic tissue, maturation of somatic embryos has been monitored using markers for storage protein gene expression and storage protein accumulation (Sterk and de Vries 1993). In addition, late embryogenesis abundant or LEA proteins are associated with the accumulation of abscisic acid (ABA) during maturation and embryo protection during desiccation (Stasolla *et al*. 2002). A better understanding of the underlying physiological differences between zygotic and somatic embryo development through the analysis of gene expression patterns has the potential to improve *in vitro* protocols (Bonga *et al*. 2010). Gene expression analyses can be a useful tool for monitoring embryo development from the acquisition of embryogenic competence through to maturation (Feher *et al*. 2003). Ideal "marker" genes should be universal, sensitive, detectable in small amounts of tissue, and reveal specific processes characteristic of each of the developmental stages or transitions.

Of the various PGRs, ABA regulates several essential processes during embryo maturation. More than 150 genes from a range of species were ABA-inducible (Giraudat *et al*. 1994). ABA is supplied to the pine (conifer) zygotic embryo through the megagametophyte (MG); a lack of this tissue during *in vitro* developmental conditions has made the application of ABA essential for the normal maturation of somatic embryos. The concentration of ABA supplied *in vitro* for the culture of somatic embryos in *Pinus* species varies from 15 μM in *Pinus elliottii* (Newton *et al*. 2005) to 120 μM in *Pinus pinaster* (Lelu-Walter *et al*. 2006). The aim of this study was to assess the relative expression of selected genes associated with embryogenesis in 4 samples of embryo development for both *P. taeda* and *P. oocarpa*. Analysis of gene expression could demonstrate the fidelity of the somatic embryo induction and development, identify changes that could be made to the culture medium and conditions to enhance the quality of the somatic embryos, and provide a developmental comparison between somatic embryos of two pine species. The following genes were analyzed for expression: legumin-like, vicilin-like (storage proteins), a group 4 LEA (late embryo development and desiccation tolerance), homeodomain-leucine zipper I (HD-Zip I) protein transcription factor, 26S proteasome regulatory subunit S2 (RPN1; ubiquitin-mediated proteolysis), and clavata-like protein (meristem development). This is the first report describing gene expression in somatic embryos of *P. oocarpa* and a comparison with a similar developmental series for somatic embryos of *P. taeda*.

## Materials and Methods

*Plant material. Collection of zygotic embryo samples*. Cones of *P. taeda* were collected weekly from the Reynolds Homestead (Critz, VA), from June 10 to September 15, 2009. Cones were opened and seeds were collected for isolation of zygotic embryos. MGs were excised from the seeds and zygotic embryos were dissected. Zygotic embryos were classified in the following four stages (Fig. 1*a–d*): (1) pro-embryo (PE), round/globular (RG), early cotyledonary (EC), and late cotyledonary (LC). Cones were collected from June 10, but discernable PE were not identified until July 14 and were then collected during the week of July 28. RG, EC, and LC samples were observed and collected from August 5, 12, and 25, respectively. Tissue was extracted from the cone collections until September 9, when the majority of the embryos were at the LC stage. Observations were made with a zoom stereomicroscope SZX7 (Olympus America Inc. Center Valley, PA), and collected samples were frozen in liquid nitrogen and kept at −80°C until RNA was extracted.

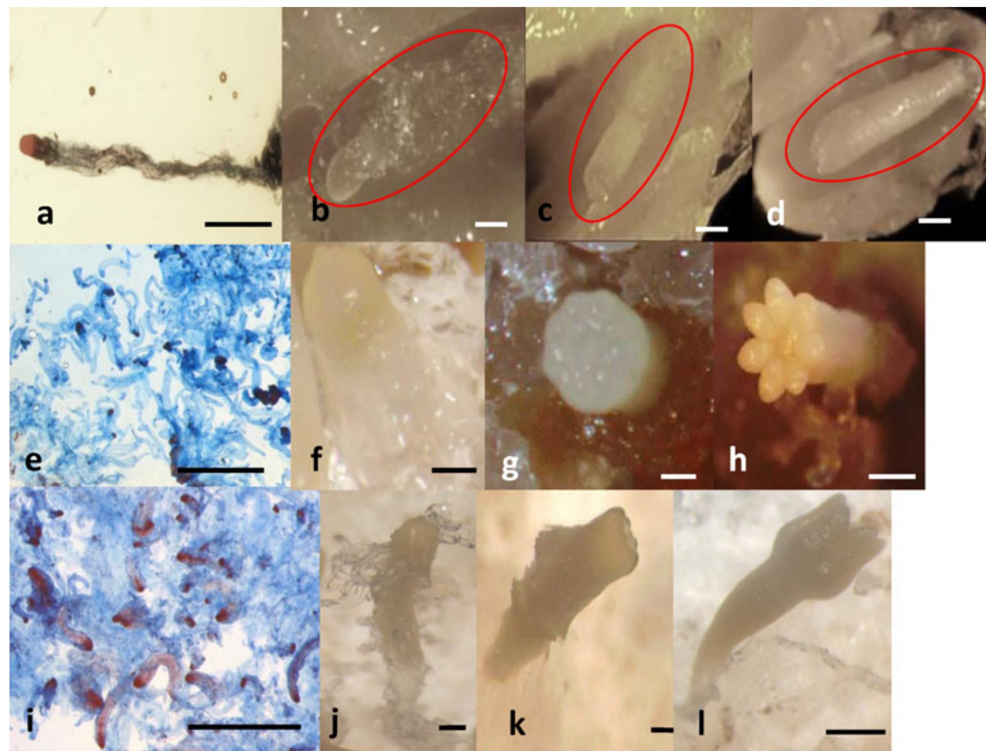


**Figure 1.** Developmental stages of zygotic embryos of *P. taeda* (*a–d*) and somatic embryos for *P. taeda* (*e–h*) and *P. oocarpa* (*i–l*): (*a*) pro-embryo; (*e*, *i*) embryogenic suspensor mass (ESM) after double staining; (*b*, *f*, *j*) round/globular (RG) stage; (*c*, *g*, *k*) early cotyledonary (EC) stage; and (*d*, *h*, *l*) late cotyledonary (LC) stage. (*Bar* in *e*, *h*, *i*, *l*=1 mm; *Bar* in *a*, *b*, *c*, *d*, *f*, *g*, *j*, *k*=0.1 mm). *Circle* denotes zygotic embryo within megagametophyte (*b–d*).

*Collection of somatic embryo samples*. Embryogenic *P. taeda* (courtesy of Dr. Scott Merkle, University of Georgia, Athens, GA) and *P. oocarpa* cell cultures (Lara-Chavez *et al*. 2011) were used to produce the somatic embryos. Initial embryogenic suspensor masses (Fig. 1*e*, *i*) were confirmed by histological methods using double staining with acetocarmine and Evan's blue (Lara-Chavez *et al*. 2011). These cultures produced morphologically correct embryos through all stages, eventually yielding LC embryos capable of germination (Fig. 1*e–l*). Classification of somatic embryo samples in both pine species was based on morphological similarities with their counterpart zygotic embryos of *P. taeda*, following a similar characterization for somatic embryos of *Picea glauca* (Dong *et al*. 1997). Briefly, the samples were characterized as follows: (1) ESM (similar to zygotic PEs) collected from cultures on maintenance medium consisting of small cytoplasmic cells and long vacuolated suspensor-like cells; (2) RG, in which the heads of the embryos were opaque and round-shaped; (3) EC embryos, characterized by small cotyledon primordia below the circumference of a prominent central meristem; and (4) LC embryos, fully mature in appearance with well-formed, elongated cotyledons and still hydrated. The last three samples were collected after cultures were transferred to maturation medium. For maturation, ESM cultures of both species were placed on pre-maturation medium (multiplication medium lacking PGRs) for 1 wk. For *P. taeda*, the cultures were resuspended with PGR-free maintenance medium at concentration of 20 % (*w*/*v*); 2 ml was spread per plate containing maturation medium, and the remaining liquid medium was removed from each plate. For *P. oocarpa*, small clumps of ESM were placed on the maturation medium. For both pine species, the maturation medium consisted of 927 basal medium (Pullman *et al*. 2003b) with 6 % (*w*/*v*) maltose, 40 μM ABA, and 0.3 % (*w*/*v*) Phytagel (Phytotechnology Labs, Shawnee Mission, KS) instead of Gelrite. Maturation duration was 8 and 15 wk for *P. taeda* and *P. oocarpa*, respectively. Collected samples were frozen in liquid nitrogen and kept at −80°C until RNA extraction.

*RNA extraction and complementary deoxyribonucleic acid (cDNA) synthesis*. Total RNA was extracted from zygotic and somatic embryo samples using the Concert™ Plant RNA Reagent (Invitrogen, Carlsbad, CA,). To eliminate any residual genomic DNA present in the samples, *P. taeda* RNA samples were treated with RQ1 RNase-Free DNase (Promega, Madison, WI) and *P. oocarpa* RNA samples with DNA-free kit (Ambion, Austin, TX) according to the manufacturer's protocols. The quantity of isolated RNA both before and after DNase treatment was measured using a Nanodrop-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Total RNA was quantified and the integrity verified by agarose gel electrophoresis. Because of the small amount of tissue collected from PE of *P. taeda* zygotic embryos, the amount of RNA was too limiting to proceed through the entire range of experiments. Thus, all of the *P. taeda* zygotic embryo RNA samples were subjected to *in vitro* amplification using the T7 RNA Polymerase amplification method (van Gelder *et al*. 1990). Therefore, total RNA from each sample underwent

one round of amplification using the MessageAmp II aRNA kit (#AM1751, Ambion), according to the manufacturer's protocol, using an *in vitro* transcription time of 14 h. Subsequently, RNA of each sample was used for cDNA synthesis using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA) for *P. taeda* somatic embryo samples; and SuperScript III, First-Strand Synthesis System for RT-PCR (Invitrogen) for *P. taeda* zygotic embryo samples and all *P. oocarpa* samples, using random hexamer and oligo (dT) primers, respectively. Subsequently, cDNA was verified *via* PCR using the two endogenous control primers (Table 1).

*Relative real-time PCR.* The relative transcript levels of the six genes of interest (Table 1) were analyzed by real-time PCR. Publicly accessible databases were searched to identify *Pinus* nucleotide sequences for primer design. If *Pinus* sequences were not available, closely related *Picea* sequences were used for primer design. Primers were initially tested for their ability to generate the correct-sized PCR products in both *Pinus* species (Supplemental Fig. 1), and no other PCR products. The reactions were carried out using a Bio-Rad iQ5 Multicolor Real Time Detection system under the same conditions used by Ratnaparkhe *et al.* (2009). Briefly, reactions were carried out in 20 μl, containing 10 μl iQ SYBR green Supermix (Bio-Rad), 1 μl each of forward and reverse primer (each primer a 10-μM stock), 2 μl cDNA template, and 6 μl nuclease-free water. Tubulin and ubiquitin-conjugating enzyme 1 (based on *Picea abies* UBC1 [Palovaara and Hakman 2008]) genes (Table 1) was used for normalization. To analyze gene expression within each embryo developmental series in *P. taeda* and *P. oocarpa*, the relative transcript levels were normalized using UBC1, and the transcript levels were compared relative to LC for somatic and zygotic samples; LC samples showed more similarities between the LC somatic embryos with the LC zygotic embryos. To perform the analysis of *P. taeda* gene expression comparison between individual somatic and zygotic embryo samples, the relative transcript levels were normalized using tubulin and compared relative to the zygotic samples. Each sample was subjected to three technical replicates, and the relative transcript level expression was analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Mean values are shown with standard errors (SE). Statistical comparisons were evaluated by the *t* test using the ΔCt values (Yuan *et al.* 2006). Differences of $P<0.05$ were regarded as significant. The JMP 8.0 (SAS, Inc., Cary, NC) was used for statistical analysis.

## Results

*Gene expression in P. taeda—relative transcript level changes during zygotic and somatic embryo development.* Two endogenous controls were tested in this study, and UBC1 showed less variation than tubulin within each developmental series for zygotic and somatic embryos. Thus, UBC1 was used for the normalization of the data.

*Storage proteins (legumin-like and vicilin-like).* During both somatic and zygotic *P. taeda* embryo development, legumin-like relative transcript levels followed the same pattern in which the relative transcript levels were minimal in the PE/ESM, increased during the RG stage, decreased slightly in the EC stage and significantly increased to a maximum level during the LC stage (Fig. 2*a*). A similar

**Table 1.** Description of genes and primer sets used for real time PCR, with identifiers current as of June 13, 2011

| Gene | Accession number | Description | Primer pair (forward/reverse, 5′→3′) | Product size (bp) |
|---|---|---|---|---|
| Endogenous controls | | | | |
| Tubulin | Spruce X57980.1 (GENBANK) | Essential for the structure and kinetics of the cytoskeleton | ACAGCTGATCTCGGGCAAAGAAGA/ TGCAGTTATCGGCTAGCTTCCTCA | 119 |
| UBC1 | Spruce AY639585 (GENBANK) | Ubiquitin-conjugating enzyme 1 | GGAACAGTGGAGTCCTGCTTT/ CCTTGCGGTGGACTCATATT | 148 |
| Genes in study | | | | |
| Legumin-like | Pine TC185485 (TIGR GI) | Storage protein | CAGCGCTTCTGTTGCTCTAC/ CTCCGACATGCTGATGATCT | 133 |
| Vicilin-like | Pine TC181320 (TIGR GI) | Storage protein | AGCTTACGTGCACCAGAATG/ AGACTGGTAATGCGAAGGCT | 129 |
| LEA | Pine TC183914 (TIGR GI) | Late Embryogenesis Abundant | AGATCCTGGGTCAGTGAAGG/ GTAAGGCCAATCTCCCATGT | 138 |
| Clavata-like | Spruce DQ530597 (GENBANK) | Meristem development | TCTTCGTGGATGCTGACTTC/ TCCGTCGCTCACAATAACAT | 96 |
| HD-Zip I | Spruce DQ201170 (GENBANK) | Homeodomain-leucine zipper-like | GCCATGATCGAACGTATGAG/ TCATGAGAAGCTGCAAGTCC | 109 |
| RPN1 | Pine TC174280 (TIGR GI) | Similar to 26S proteasome regulatory subunit S2 | CAATGCTGGATTTGGTCAAG/ TCCCAAAGGAGAATCAAACC | 144 |

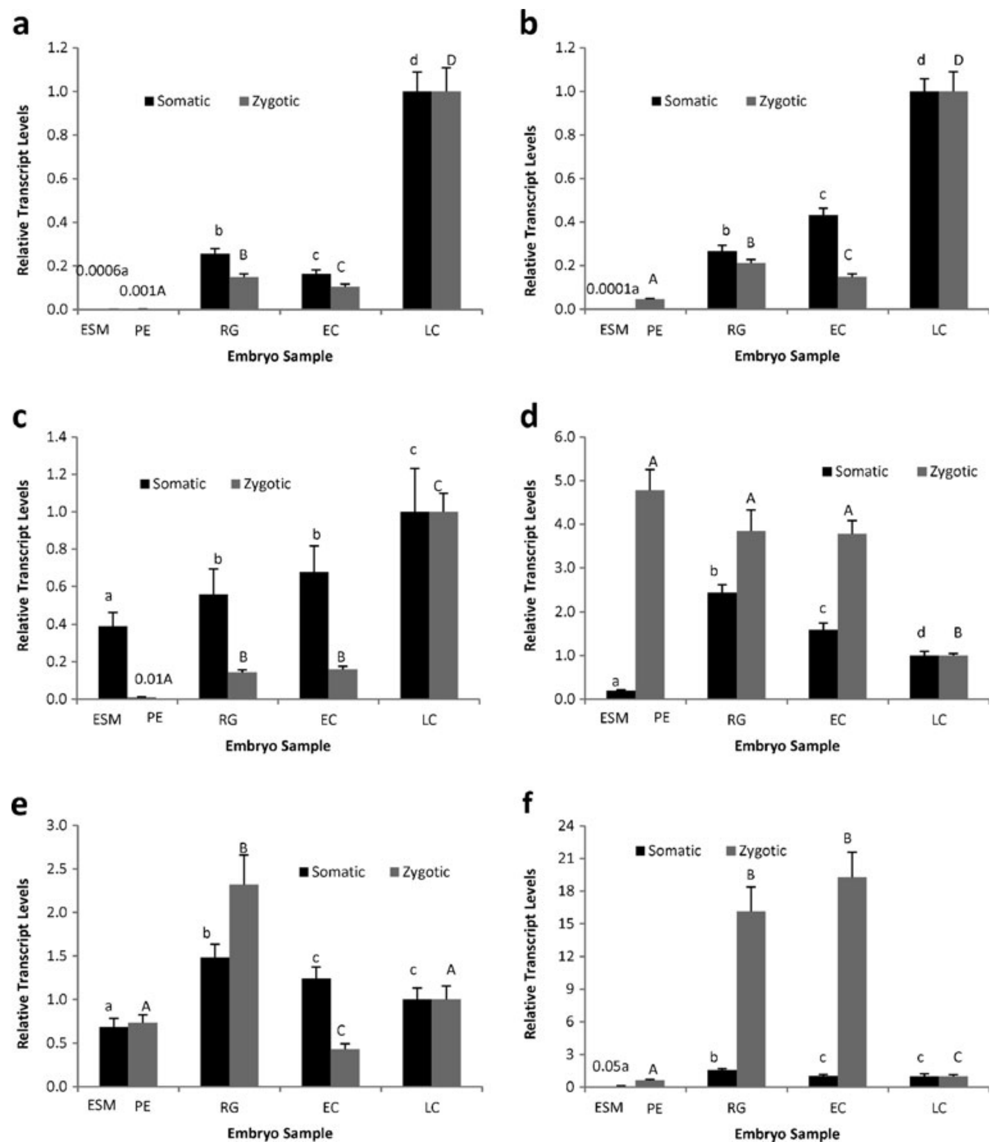


**Figure 2.** Relative transcript levels for target genes at four stages of *P. taeda* somatic and zygotic embryos, using ubiquitin-conjugating enzyme 1 (UBC1) as endogenous control. Genes under study: (*a*) legumin-like, (*b*) vicilin-like, (*c*) LEA, (*d*) HD-Zip I, (*e*) 26S proteasome subunit S2 (RPN1), and (*f*) clavata-like. *ESM* embryogenic suspensor masses, *PE* pro-embryo, *RG* round/globular, *EC* early cotyledonary, *LC* late cotyledonary. Values were normalized to the value of LC set at 1. Each *value* represents a mean+SE of 3 technical replicates. *Bars with different letters* are statistically different ($P<0.05$), with *lower-case letters* used to represent the comparison among somatic samples and *upper-case letters* for zygotic samples.

vicilin-like expression pattern was observed during both zygotic and somatic embryo development (Fig. 2*b*), except in the somatic embryos, the relative transcript levels gradually increased from ESM to LC. The maximum vicilin-like relative transcript levels were also observed at the LC stage, as noted for the other storage protein gene transcript.

*Late embryogenesis abundant (LEA)*. Relative transcript levels for the group 4 LEA gene were detected at 1/3 maximum level in the ESM, gradually increased during *P. taeda* somatic embryo maturation and peaked at the LC stage (Fig. 2*c*). In contrast, the relative transcript levels in *P. taeda* zygotic embryos accumulated later at the RG stage (about 1/6 the maximum level), showed little difference at the EC stage and then rapidly increased to maximum levels at the LC stage (Fig. 2*c*). The temporal accumulation of LEA transcripts in somatic embryos was somewhat different from that observed in *P. taeda* zygotic embryos, with a more gradual accumulation representing a 2.5-fold change in abundance over the somatic embryo development processes, and a more controlled accumulation in zygotic embryos, representing an approximate 100-fold change over the zygotic embryo developmental series. The highest levels of expression were observed in LC embryos in both somatic and zygotic systems.

*Homeodomain-leucine zipper I (HD-Zip I)*. The expression of HD-Zip I in both *P. taeda* somatic and zygotic embryos decreased during overall embryo development and maturation from peak levels at the RG stage (somatic embryos) or in PEs (zygotic embryos; Fig. 2*d*) through to LC stage. There was some difference between the earliest somatic and zygotic embryo stages, in which the highest relative expression level was found at the zygotic embryo PE stage, while the somatic embryo ESM showed the lowest relative expression level for somatic embryo development, before increasing to peak levels at the RG stage and subsequently declining.

*26S Proteasome subunit S2 (RPN1)*. The highest relative expression for RPN1 was detected at the RG stage in both zygotic and somatic embryo developmental samples. In the somatic embryo system, RPN1 transcripts maintained a high level with a slight decline at the LC stage (Fig. 2*e*). In zygotic embryos, the relative expression declined more drastically at the subsequent stage (EC) and then slightly increased at the LC stage.

*Clavata-like*. The relative expression profiles of the clavata-like gene exhibited differences between *P. taeda* somatic and zygotic embryos when comparing the level of transcript change during embryo maturation (Fig. 2*f*). The overall variation in transcript abundance was similar in both cases when comparing lowest and highest abundance (approximately 31-fold and 32-fold in somatic and zygotic embryos, respectively). During somatic embryogenesis, the relative expression levels went from their lowest levels at the ESM stage to peak levels at the RG stage but did not vary much during the developmental period between the RG and LC stages. There was a slight decline from the maximum abundance at the RG stage to a similar level in both EC and LC stages. Zygotic embryos also exhibited their lowest clavata-like transcript levels at the PE stage, followed by a significant increase in transcript level at the RG stage, which increased slightly by the EC stage and then declined significantly at the LC stage. The decline between the EC and LC stages represented an approximate 19-fold change, much greater than observed for the somatic embryo developmental series.

*Gene expression in P. taeda—comparison of relative transcript levels between specific somatic and zygotic embryo developmental stages*. The previously described work assessed the relative transcript level changes within somatic or zygotic embryo samples during the developmental series relative to the level in their respective LC stage embryos. These changes did not provide an assessment of the relative transcript level differences between the somatic and zygotic embryos themselves at different stages of development. To provide this assessment, relative transcript levels were compared at each individual embryo stage between zygotic and somatic embryo samples of *P. taeda*. Samples were normalized using the housekeeping gene tubulin chosen because it exhibited less variation between the individual somatic and zygotic embryo samples than UBC1.

The comparison between *P. taeda* embryo samples at the PE and ESM stages showed that with most of the genes, with the exception of legumin-like and RPN1, the zygotic PE had higher expression levels compared to the somatic ESM (Fig. 3*a*). The greatest variation in expression level was observed for the vicilin-like transcript (ESM was 333-fold lower than PE). The least variation was observed for the clavata-like transcript (ESM was 5.3-fold lower than PE). The relative expression level of legumin-like and RPN1 in ESM was 1.3- and 2.2-fold higher than in the PE, respectively.

For the remaining three embryo samples (RG, EC, and LC), the somatic embryo samples exhibited higher relative expression levels than the similar-stage zygotic embryo samples for all genes except for LEA (Fig. 3*b–g*). The expression levels of vicilin-like, clavata-like, HD-Zip I, legumin-like, and RPN1 genes were approximately 5.7-, 2.0-, 1.4-, 30.0-, and 12.0-fold higher in somatic RG sample than at the same zygotic stage (Fig. 3*b*, *c*). During the EC stage, the levels of vicilin-like, clavata-like, HD-Zip I, legumin-like, and RPN1 transcripts were approximately 30.0-, 2.0-, 9.0-, 49.0-, and 90.0-fold greater, respectively, in the somatic embryos compared with the zygotic embryos (Fig. 3*d*, *e*). This trend continued through to the LC sample, in which the relative transcript levels for vicilin-like, clavata-like, HD-Zip I, legumin-like, and RPN1 were 38-, 55-, 40-, 100-, and 45-fold greater, respectively, in the somatic LC sample (Fig. 3*f*, *g*).

Thus, for almost all of the marker genes used in this study, the differences in relative expression level between somatic and zygotic embryos increased during maturation. As an example, for the legumin-like transcripts, the somatic embryo RG relative transcript level was 30-fold higher compared to the zygotic embryo RG, and this difference increased to 50- and 100-fold at the EC and LC samples, respectively. These higher transcript levels in the somatic embryo samples compared to their counterpart zygotic samples meant that the transcripts could be detected more readily in the somatic embryos.

*Gene expression in P. taeda and P. oocarpa—comparison of relative transcript level changes during somatic embryo development. Storage proteins (legumin-like and vicilin-like)*. During *P. oocarpa* somatic embryo development, both legumin-like (Fig. 4*a*) and vicilin-like (Fig. 4*b*) relative transcript levels were low in the ESM and RG stages, peaked at the EC stage and declined at the LC stage. Furthermore, the levels of legumin-like transcripts declined to near minimal levels at the LC stage. These results were in distinct contrast to those observed during *P. taeda* somatic embryogenesis, in which both storage protein transcripts increased from minimal levels at the ESM stage to peak levels at the LC stage (Fig. 2*a*, *b*). Hence, the decline in relative transcript abundance during the LC stage, especially for the legumin-like transcripts, was a very distinct difference during *P. oocarpa* somatic embryogenesis.

*Late embryogenesis abundant (LEA)*. During *P. oocarpa* somatic embryo development, relative transcript levels for LEA increased from minimal levels at the ESM stage to the RG stage, remained relatively steady at the EC stage and increased to peak levels at the LC stage (Fig. 4*c*). This

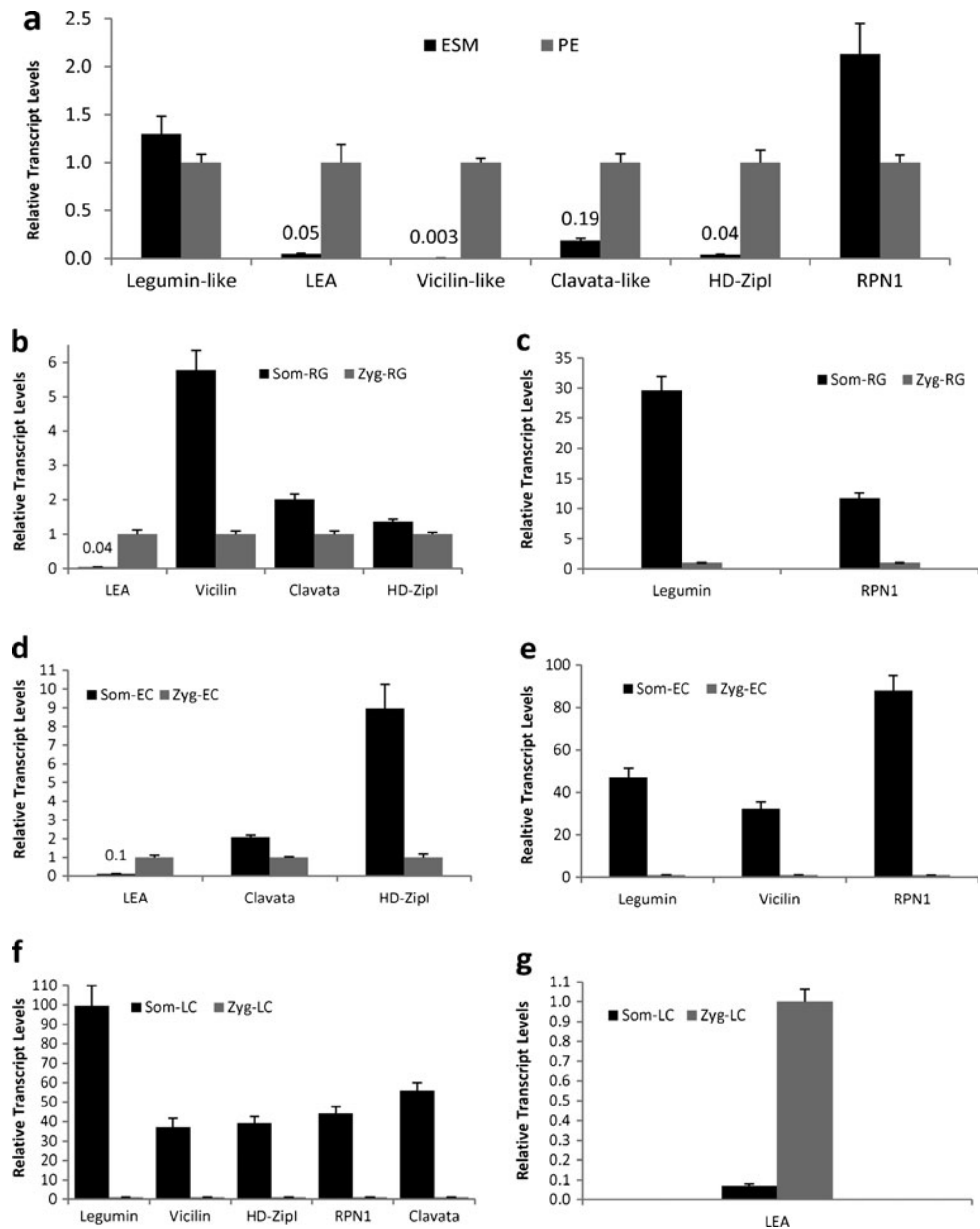


**Figure 3.** Comparison of relative target gene transcript levels between somatic and zygotic embryo stages of *P. taeda*: (*a*) somatic embryogenic suspensor masses (ESM) and zygotic pro-embryos (PE); (*b*, *c*) round/globular (Som-RG) and zygotic round/globular (Zyg-RG); (*d*, *e*) early cotyledonary (Som-EC) and zygotic early cotyledonary (Zyg-EC); (*f*, *g*) late cotyledonary (Som-LC) and zygotic late cotyledonary (Zyg-LC). Tubulin was used as endogenous control, and values were normalized to the value of their respective zygotic sample set at 1. Each value represents a mean +SE of 3 technical replicates. All means are statistically different ($P<0.05$) except between ESM and PE for legumin-like.

developmental expression pattern was very similar to that observed during *P. taeda* somatic embryogenesis (Fig. 2*c*). Similar to *P. taeda*, the *P. oocarpa* ESM stage also showed 1/3 the maximum levels of LEA, which occurred at the LC stage.

*Homeodomain-leucine zipper I (HD-Zip I)*. The lowest relative transcript level for HD-Zip I occurred at the ESM stage, increased by the RG stage and remained relatively constant during the subsequent somatic embryo EC and LC stages of maturation (Fig. 4*d*). This general trend was similar to that observed during *P. taeda* somatic embryogenesis (Fig. 2*d*), where the lowest relative transcript levels were observed at the ESM stage and increased once the embryos were transferred to maturation medium. However, a gradual decline in HD-Zip I transcripts during the *P. oocarpa* cotyledonary stage progression was not observed as it was for *P. taeda*.

*26S Proteasome subunit S2 (RPN1)*. Similar high relative transcript levels for RPN1 were detected at both the ESM and EC stages (Fig. 4*e*) during *P. oocarpa* somatic embryo development. There was a significant decline in these levels between these two stages, with the RG stage exhibiting the lowest relative levels of RPN1 during the stages assessed. The expression levels also decreased drastically at the LC stage, such that the overall pattern of RPN1 transcript accumulation appeared biphasic. These results were in major contrast to those observed for *P. taeda* somatic embryos, where the overall variation in transcript levels during the developmental period was not as great, and with a single

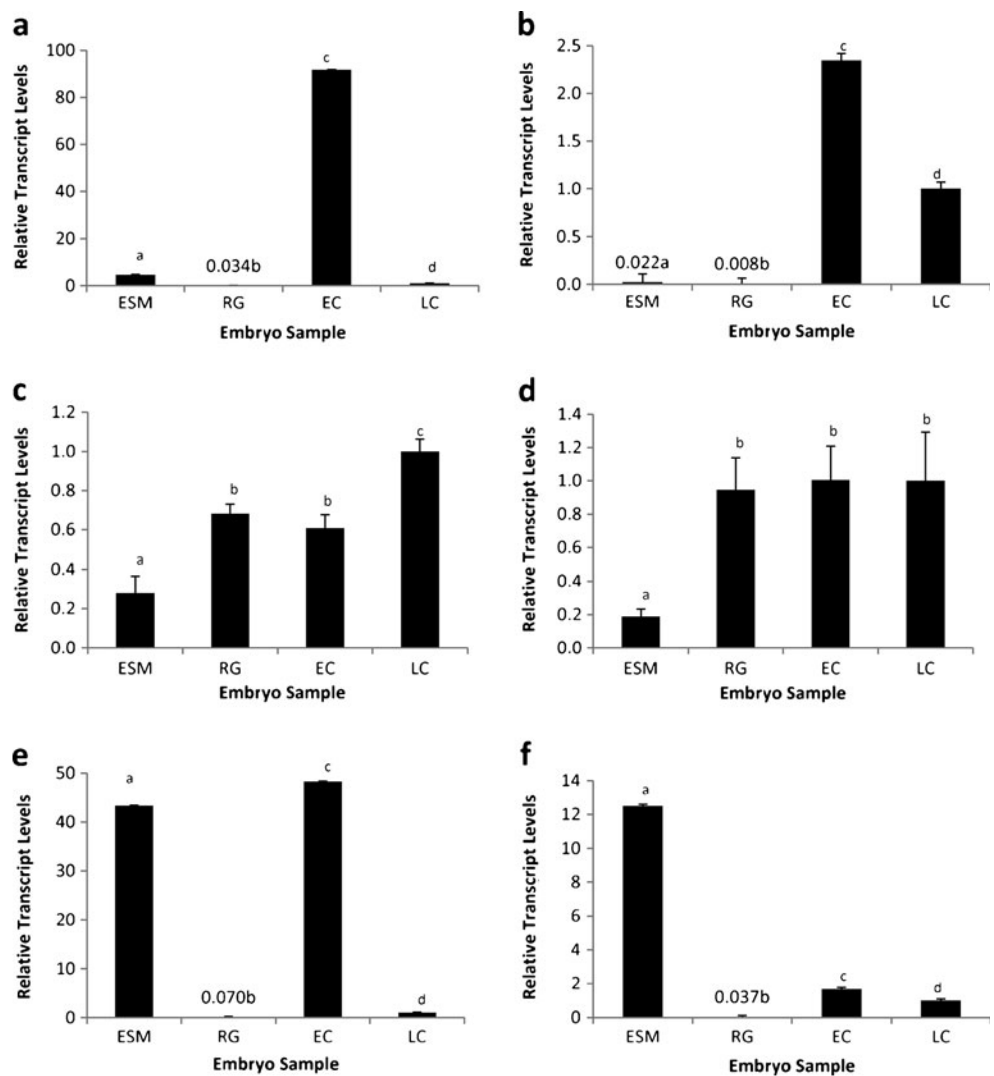


**Figure 4.** Relative transcript levels for target genes at four stages of *P. oocarpa* somatic embryo development using ubiquitin-conjugating enzyme 1 (UBC1) as endogenous control. Genes under study: (*a*) legumin-like, (*b*) vicilin-like, (*c*) LEA, (*d*) HD-Zip I, (*e*) 26S proteasome subunit S2 (RPN1), and (*f*) clavata-like. *ESM* embryogenic suspensor masses, *RG* round/globular, *EC* early cotyledonary, *LC* late cotyledonary. Values were normalized to the value of LC set at 1. Each value represents a mean+SE of 3 technical replicates. *Bars with different letters* are statistically different ($P<0.05$).

peak of accumulation at the RG stage (Fig. 2*e*), followed by a gradual decline in transcript during continued maturation.

*Meristem development (clavata-like)*. The relative expression of the clavata-like gene in *P. oocarpa* somatic embryos peaked at the early ESM stage, decreased drastically to minimal levels at the RG stage, slightly increased at the EC stage and declined slightly at the LC stage (Fig. 4*f*). Again, this pattern of transcript accumulation was very different from that observed during *P. taeda* somatic embryo development, where there was little variation in transcript levels between the RG, EC, and LC stages, with the highest transcript levels at the RG stage and the lowest levels at the ESM stage (Fig. 2*f*).

## Discussion

To assess the developmental similarity of somatic embryos of two pine species matured under the same conditions, as well as the similarity with zygotic embryos, we characterized the relative expression of six developmentally regulated genes during zygotic embryo development in *P. taeda* and somatic embryo development/maturation in *P. taeda* and *P. oocarpa*. While somatic embryos were similar in relative gene expression patterns to their zygotic counterparts, some variation was encountered between zygotic and somatic embryos, and also between the two pine somatic embryo systems (Fig. 5).

*Relative gene expression during P. taeda zygotic and somatic embryo developmental series.* We targeted genes for two of the storage proteins classes, and our results with *P. taeda* showed that during somatic and zygotic embryo development, both legumin-like and vicilin-like relative transcript levels followed the same pattern: the transcripts were minimal in the PE/ESM stages, increasing during subsequent development, with the maximum relative expression of both storage protein genes at the LC stage. These results mirrored storage protein accumulation in somatic embryos of *P. taeda* (Brownfield *et al*. 2007) and *Pinus strobus* (Klimaszewska

*et al*. 2004), as well as transcript and storage protein accumulation in somatic and zygotic embryos of *P. glauca* (Flinn *et al*. 1993). We observed, major transcript accumulation during the cotyledonary phase in both somatic and zygotic embryos, and *P. taeda* somatic embryos showed a slightly more rapid increase in vicilin-like transcripts compared to zygotic embryos. Lippert *et al*. (2005) similarly noted that vicilin-like protein was detected early in somatic embryos after 7 d on maturation medium, and it accumulated gradually during embryo maturation. While storage protein transcript patterns were not assessed, Brownfield *et al*. (2007) did note that somatic embryos of *P. taeda* exhibited a shift in the ratio of their soluble to insoluble protein components, indicating some possible differences in somatic embryo metabolic activity. In some plants, the accumulation of storage proteins is not observed during somatic embryo development, and this feature has been used to distinguish "normal" somatic embryos (Stasolla and Yeung 2003). Thus, a high-quality somatic embryo includes the accumulation of storage proteins analogous to those of zygotic embryos (Merkle *et al*. 1995), and represents an excellent marker to check somatic embryo quality and fidelity.

In this study, *P. taeda* somatic embryos matured on a medium containing 40 μM ABA and 6 % (*w*/*v*) maltose appeared similar to zygotic embryos, with no precocious germination. As stated previously, the relative transcript levels of two storage proteins (legumin-like and vicilin-like) in these somatic embryo developmental samples followed a similar expression pattern than the counterpart zygotic samples. Thus, the actual maturation conditions used for *P. taeda* somatic embryo development were sufficient to stimulate similar expression patterns of the storage proteins. In *P. glauca*, a positive relationship was found between storage accumulation and ABA concentration, where low concentrations (10 μM) in the medium promoted precocious embryo germination, while higher concentration (40 μM) yielded normal embryos which contained significant amounts of storage protein (Roberts *et al*. 1990) and also promoted storage protein synthesis to similar levels found in their zygotic counterparts (Flinn *et al*. 1991). In the case of our group 4 LEA gene marker, the maximum transcript levels during both zygotic and somatic embryo development occurred at the LC stage, although expression was detected in pre-cotyledonary stages of both embryo systems. However, *P. taeda* somatic embryos exhibited a more prolonged and gradual accumulation of these transcripts than corresponding zygotic embryos. The continual exposure of our somatic embryos to ABA during maturation most likely accounted for the earlier and more gradual accumulation of LEA transcripts. In somatic embryos, a strong promotion of *QrEM* (LEA gene in *Quercus robus*) transcript accumulation was observed after induction of maturation on 6 % (*w*/*v*) sorbitol (no ABA) for 5 wk, then increased after partial desiccation (somatic embryos at 25 % loss of moisture) and declined when embryos lost more than 30 % of their moisture (Šunderlíková *et al*. 2009). In *P. glauca*, LEA gene transcripts accumulate after somatic embryo transfer to ABA (Dong and Dunstan 1996) and in the presence of polyethylene glycol (PEG) during the late stages of embryo maturation, which may enhance somatic embryo desiccation tolerance and thus improve postembryonic growth (Stasolla *et al*. 2003). These reports indicate the significance of ABA and/or osmotic agents in the stimulation of LEA gene expression. Hence, it is not surprising that modifications of the levels of these compounds in a tissue culture medium could influence in the LEA relative transcript levels. Our somatic embryo culture system maintained a prolonged culture on a constant level of 40 μM ABA, and we are unsure if this has any negative effects on the cultures. Thus, a modification that could be made during *in vitro* conditions would be to gradually increase the concentration of ABA or osmoticum during somatic embryo maturation and assess further impacts on embryo quality.

The LEA gene transcripts in zygotic *P. taeda* embryos accumulated later at the RG stage, showed little difference at the EC stage and then rapidly increased to maximum levels at the LC stage. Somewhat similar to our results, Leal and Misra (1993) noted LEA gene transcript accumulation in developing *P. glauca* zygotic embryos just prior to the LC phase, which reached maximal level in the fully mature embryo and was stable in dry seeds. Additionally, the isolation and characterization of *QrEm* (LEA gene in *Q. robus*) indicated that expression of this gene first occurred in the zygotic embryo EC stage, with gradual accumulation throughout mid-maturation and a subsequent decline toward the end of seed development (Šunderlíková *et al*. 2009).

The LEA proteins are more abundant during late embryogenesis than during mid-embryogenesis (Galau *et al*. 1986), their genes are expressed at the LC stage during zygotic embryogenesis, and they are considered a marker gene for the quality of the mature somatic embryo before undergoing germination (Zimmerman 1993). Although these genes were first identified from developing seeds, many LEA-like genes are induced by ABA or environmental stresses (Galau *et al*. 1986). In this study, we used a representative of the group 4 LEA genes for expression profiling during development. The group 4 LEA proteins are expressed during different developmental stages and plant organs in response to water deficit, and their deficiency also correlates with a reduced seed production, supporting a role in fruit and/or seed development (Olvera-Carrillo *et al*. 2010).

The genes described above are usually associated with the later stages of zygotic embryo maturation, so we also assessed the expression of a homeobox gene, HD-Zip I, a class of transcription factors in plants involved in the activation of other genes controlling tissue patterning (Chugh and Khurana 2002). We observed that HD-Zip I transcripts increased in somatic embryos following ESM transfer to

maturation media containing ABA, but peaked at the RG stage and then declined. This was somewhat different from the zygotic embryos, which exhibited the highest relative expression of HD-Zip I in PEs and remained high until decreasing at the LC stage. Hence, there was a more prolonged expression pattern in the zygotic embryos than in our somatic embryos. HD-Zip I has been implicated in ABA responses in plants, and in water and light stress responses (Elhiti and Stasolla 2009). In our study, *P. taeda* HD-Zip I expression was somewhat similar to that previously reported for *P. glauca* (Tahir *et al*. 2008), who noted that *PgHZ1* increased after 7 d on maintenance medium but remained high afterward during the maturation stage. However, no comparison of *PgHZ1* expression during zygotic embryogenesis was addressed in that study. A phenotypic analysis of plants with higher levels of HD-Zip I gene expression showed that elevated levels could be related to cotyledon formation (Hanson *et al*. 2001) and that the expression was responsive to ABA and water deficit stress (Henriksson *et al*. 2005). While *P. taeda* zygotic and somatic embryos exhibited slightly different developmental transcript accumulation patterns, the key developmental stage difference between the somatic and zygotic embryos was the *P. taeda* PE/ESM sample; nevertheless, both zygotic and somatic embryos exhibited a progressive decrease from RG to LC during subsequent embryo maturation. While the mode of action of HD-Zips is poorly understood, these genes appear to be important regulators of plant development and differentiation in response to environmental factors, and in embryo-associated ABA responses (Deng *et al*. 2006). Here, we found that HD-Zip I was expressed during pine embryogenesis, perhaps increasing the sensitivity to ABA and promoting embryogenesis *in vitro* as previously suggested (Tahir *et al*. 2008).

In the case of the 26S Proteasome subunit S2 (RPN1), our results showed that expression of the *P. taeda* RPN1 gene was associated with the RG embryo stage transition of both somatic and zygotic embryos, with transcript levels increasing between PE/ESM and RG stage, and then declining with progression into later development. It is possible that the RG stage indicates a major morphological and physiological point, and thus the degradation of proteasome-targeted proteins is increased, with RPN1 participating in the target specificity and overall proteasome regulation at this crucial stage (Brukhin *et al*. 2005). Inactivation of subunit RPN1 arrests embryogenesis at the globular stage in *Arabidopsis*, suggesting that it is a key gene involved in controlling early embryo development and progress into maturation. Plant 26S subunit gene expression is regulated by growth regulator levels, which vary during plant development (Santner and Estelle 2010); thus, different embryo developmental stages (including somatic embryos) could require different proteasome activity levels controlled by endogenous growth regulators (Stasolla *et al*. 2003).

The final marker gene we considered, the clavata-like gene, was expressed during both somatic and zygotic embryo development. However, in *P. taeda*, differences were observed in the expression patterns between embryo samples, where more constant relative expression was observed during somatic embryogenesis. In contrast, zygotic embryos exhibited a peak level of expression during the RG and EC samples. The significance of these differences between zygotic and somatic embryos is unknown, as our somatic embryos exhibited SAM domes and cotyledonary development; however, we did not assess the SAM structure microscopically. The clavata-like gene regulates the activity of the shoot apical meristem, which is formed and develops during embryogenesis, and is required to produce new cells and tissues during post-germination growth (Tahir and Stasolla 2006). Previous studies have shown that SAM formation in somatic embryos of *P. glauca* (Tahir and Stasolla 2006) and *P. pinaster* (Tereso *et al*. 2007) followed the same pattern as their counterpart zygotic embryos. It is possible that the differences in relative expression levels observed in this study during somatic embryo development might be reflected in modified apical growth during subsequent germinative growth; this remains to be determined. If so, additional modifications of our current maturation medium, such as the addition of reduced glutathione (GSH; Belmonte *et al*. 2005) and PEG (Stasolla *et al*. 2003), may result in somatic embryo expression profiles more similar to zygotic embryo samples (Stasolla and Yeung 2003), and reflect on subsequent *ex vitro* performance.

*Comparison between samples of somatic and zygotic embryos of P. taeda.* When the transcript levels of our marker genes were compared between similar zygotic and somatic embryo stages through normalization against the housekeeping control, we observed that in almost every case post-PE/ESM stage, transcript levels were higher in the somatic embryos than corresponding stage zygotic embryos, with the exception of the LEA transcripts, which were always higher in the zygotic embryos. Also, in the PE/ESM stage embryos, transcript levels were higher in the zygotic embryos, except for the legumin-like and RPN1 transcripts. In assessing these results, it is worth remembering that the total tissue of the *P. taeda* ESM was used for RNA extractions, and the ESM tissue is characterized by the continued multiplication of cell masses composed of aggregates of small, rapidly dividing cells and vacuolated non-dividing cells (Palovaara and Hakman 2008). These small, immature embryos highly resemble the early stages of zygotic embryos, designated in this study as PE, which were collected from seeds. Therefore, the transcript levels

measured in the embryogenic cells of the ESM were diluted by the many surrounding accessory cells associated with the ESM mass, which could account for the lower relative transcripts observed for most of the genes in the ESM. Once the ESM tissues were transferred to maturation media, distinct individual somatic embryos were able to be identified and sampled for the later developmental stages; these later developmental sampled stages resembled their zygotic counterparts. During maturation from the RG stage and onward, differences in both somatic and zygotic embryos could be observed, but the basic developmental strategies are similar (Yeung 1995). It is possible that the direct exposure of the somatic embryos to 6 % (*w*/*v*) maltose and 40 μM ABA *in vitro* was sufficient to enhance gene transcript levels, as exposure to ABA and/or osmoticum can enhance embryo-associated gene expression in conifer somatic embryos (Flinn *et al*. 1993; Leal *et al*. 1995; Stasolla *et al*. 2003). It was interesting that the expression levels of the LEA gene were found to be higher in all of the zygotic embryo stages relative to the somatic embryo stages. The expression period exhibited less variation over the somatic embryo developmental series than the zygotic developmental series, which we attributed to the continual somatic embryo exposure to 40 μM ABA. This suggests that additional endogenous control mechanisms, apart from just applied ABA, are associated with overall LEA gene expression levels. Perhaps this may relate to differences in the embryogenic environments, such as other factors normally produced within the seed megagametophyte tissues, or to differences in osmotic conditions within the zygotic and somatic embryos.

*Gene expression during somatic embryogenesis of P. oocarpa and comparison with P. taeda.* We previously described the significance of the marker genes used in this study and the overall gene expression similarities observed between the *P. taeda* zygotic and somatic embryo series, suggesting that the maturation protocol used was adequate to produce quality embryos. Our *P. oocarpa* somatic embryos, which were matured using the same media composition, exhibited a certain degree of similarity with the *P. taeda* somatic embryos in gene expression for some of the target genes. Overall, the legumin- and vicilin-like storage proteins exhibited low transcript levels at the ESM and RG stages, with major increases during the cotyledonary stage of development. However, the *P. oocarpa* somatic embryos, contrasting with the *P. taeda* somatic embryos, exhibited a significant decline in both storage protein transcripts at the later cotyledonary stage. Apart from storage protein transcripts, LEA gene transcripts exhibited a similar developmental expression profile between the somatic embryos of the two pine species, with the lowest levels at the ESM stage, increased levels at the RG and EC stages, and maximal levels at the LC stage. The HD-Zip I transcripts also displayed some similarity in developmental expression between *P. oocarpa* and *P. taeda*, with their lowest relative levels at the ESM stage followed by peak relative levels at the RG stage. However, while the *P. oocarpa* HD-Zip I transcript levels remained high, they subsequently declined during the *P. taeda* maturation series. Hence, it appeared that for these 4 marker genes, the variation in developmental expression patterns between the two pine somatic embryo series occurred during the later stages (cotyledonary) of maturation. In contrast to the above four genes, the RPN1 and clavata-like gene expression profiles were very dissimilar between the two pine somatic embryo systems. Both genes exhibited maximal or near maximal transcript abundance at the *P. oocarpa* ESM stage followed by a significant decline at the RG stage; but there was a minimal abundance in *P. taeda* at the ESM stage, followed by an increase at the RG stage. Taken together, all of these results suggest that under our current maturation protocol, the *P. taeda* somatic embryos may be of a higher quality than those of *P. oocarpa*, and that the *P. oocarpa* somatic embryo system requires further optimization.

Given that both pine species were matured using the same media, what might account for the observed differences? One initial difference between the two pine systems was that the ESM tissue was dispersed and plated out for *P. taeda*, while the *P. oocarpa* ESM was cultured as tissue clumps. Hence, the developing embryos for *P. taeda* would be closer to the nutritional medium, while the *P. oocarpa* embryos would most likely be exposed to nutritional/hormonal/oxygen gradients occurring within the clumps during development, which may impact on overall development and associated gene expression profiles. Others have reported that dispersion of suspended pine embryogenic tissue substantially enhances embryogenic tissue growth over the use of small tissue clumps (Aronen *et al*. 2009; Carneros *et al*. 2009). Additionally, the *P. oocarpa* embryos were matured for a longer period of time than the *P. taeda* embryos, which could impact on later stage developmental gene expression profiles. A similar observation relating to maturation time was made in which somatic embryos of *P. strobus* that matured for 9 wk accumulated storage proteins, but extension of the maturation period to 12 wk resulted in a significant decline in storage proteins, even on optimal maturation media (Klimaszewska *et al*. 2004). These observations may reflect depletions of tissue culture media components that have occurred during the culture period, or in the initiation of precocious germination. Storage protein transcript decreases have been reported in conifer somatic embryos during precocious germination *in vitro* (Flinn *et al*. 1993). Our *P. oocarpa* somatic embryos were not undergoing visual precocious germination, but it is possible that

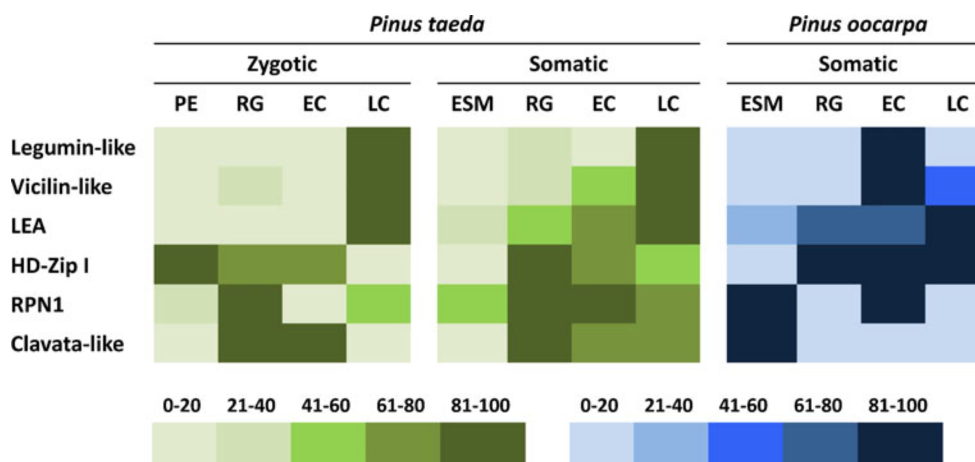


**Figure 5.** Summary of relative expression of different target genes in four developmental stages of zygotic embryos of *P. taeda* and four developmental stages of somatic embryos of *P. taeda* and *P. oocarpa*. *Heat map shading* indicates relative values of gene expression (*darker color* represents higher expression), expressed as percentage of maximum gene expression.

molecular processes associated with precocious germination had been initiated.

The maturation series reported here represented two different pine species. Therefore, the variations observed in overall gene expression profiles may reflect genotype effects. Genotypic variation has frequently been reported to help explain differences in somatic embryogenesis induction and development capacity within a species (Niskanen *et al.* 2004; Carneros *et al.* 2009). In this regard, a description of gene expression variation between normally maturing embryogenic cultures of *P. abies* was reported by Stasolla *et al.* (2004). In that report, similar transcription level patterns occurred for many genes, but some differential gene expression was observed, even though the embryogenic cultures developed normally through similar stages.

The present results describe the expression of 6 developmentally regulated genes during similar morphological stages of zygotic embryo development in *P. taeda* and somatic embryo development/maturation in *P. taeda* and *P. oocarpa*. This study also represents, to the best of our knowledge, the first analysis of somatic embryos of *P. oocarpa* and the molecular comparison of somatic embryo developmental series of 2 pine species matured under the same conditions. Our analyses indicated that all six marker genes were expressed during zygotic and somatic embryogenesis. Zygotic and somatic embryos of *P. taeda* exhibited similar developmental gene expression profiles, although some differences were observed. Gene expression levels tended to be higher in somatic embryos, in which transcripts were compared at similar zygotic embryo stages. In contrast, while somatic embryos of *P. oocarpa* exhibited some degree of similarity in developmental gene expression profiles with the *P. taeda* somatic embryos, there were also significant differences. In our study, the lower relative transcript levels observed with our *P. oocarpa* somatic embryos could explain their low germination success during somatic embryogenesis. Furthermore, they point to areas where modifications could be made to *in vitro* protocols using these and other markers.

The results from this study are in agreement with those obtained using other methods, such as microscopy of SAM development, biochemical quantification, and characterization of storage proteins and gene expression studies indicating general somatic embryo fidelity. Our study used a very small set of molecular markers for genes associated with key aspects of the embryo developmental process. The development of additional markers that could differentiate the quality of embryos from different cell lines and under different degrees of embryo maturation would facilitate the screening or identification of those somatic embryos cell lines that more closely resemble their zygotic counterparts. Theoretically, this could increase the potential for conversion of somatic embryos into plantlets by more closely following the program of zygotic embryo development. Metabolite analyses could be used to further characterize differences in the regenerative capacity of *P. taeda* embryogenic cultures (Robinson *et al.* 2009) and to differentiate between embryogenic and non-embryogenic cells in pine species (Park *et al.* 2011). From the latter study, approximately 50 compounds displayed significant differences between embryogenic and non-embryogenic cell lines (Park *et al.* 2011), while a model of 47 metabolites could predict the regenerative capacity of somatic embryogenic masses in *P. taeda* cell lines (Robinson *et al.* 2009). A metabolomics study at different embryo stages could provide new insights into the development and quality of somatic embryo during maturation. As suggested by Robinson *et al.* (2009), the identification of metabolite sets could aid in the monitoring of the physiological status of the embryo cultures from embryogenic suspensor masses through maturation/development stages. This may help to identify amendments needed in the culture conditions to improve the production of “normal” embryos.

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