

Patterns of storage protein and triacylglycerol accumulation during loblolly pine somatic embryo maturation

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Received: 31 July 2006 / Accepted: 6 December 2006 / Published online: 16 January 2007
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Abstract Conifer somatic embryo germination and early seedling growth are fundamentally different than in their zygotic counterparts in that the living maternal megagametophyte tissue surrounding the embryo is absent. The megagametophyte contains the majority of the seed storage reserves in loblolly pine and the lack of the megagametophyte tissue poses a significant challenge to somatic embryo germination and growth. We investigated the differences in seed storage reserves between loblolly pine mature zygotic embryos and somatic embryos that were capable of germination and early seedling growth. Somatic embryos utilized in this study contained significantly lower levels of triacylglycerol and higher levels of storage proteins relative to zygotic embryos. A shift in the ratio of soluble

to insoluble protein present was also observed. Mature zygotic embryos had roughly a 3:2 ratio of soluble to insoluble protein whereas the somatic embryos contained over 5-fold more soluble protein compared to insoluble protein. This indicates that the somatic embryos are not only producing more protein overall, but that this protein is biased more heavily towards soluble protein, indicating possible differences in metabolic activity at the time of desiccation.

Keywords Embryogenesis · *Pinus taeda* · Seed storage reserves · Lipids · Zygotic embryo

Abbreviations

ABA Abscisic acid
PEG Polyethylene glycol
TAG Triacylglycerol

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Somatic embryogenesis holds enormous potential to capture the ability to produce and reproduce clonal superior trees in large scale. Many species, including both gymnosperms and angiosperms, have benefited from this technology (Stasolla et al. 2002). However, in order for somatic embryogenesis to reach its full potential for a given species, the somatic embryos produced must be not only viable but must exhibit adequate and predictable growth. Production of somatic embryos has been achieved for many conifer

species including white spruce (Attree et al. 1995), black spruce (Beardmore and Charest 1995), Douglas fir (Taber et al. 1998), and loblolly pine (Becwar et al. 1990). However, some species are recalcitrant to in vitro conditions (Stasolla et al. 2002) and even if germination does occur for these species, subsequent growth may not be sufficiently vigorous (Pullman et al. 2003a).

Loblolly pine somatic embryo production was first reported by Gupta and Durzan (1987). Although the efficiency of embryo production has since improved, loblolly pine somatic embryos still exhibit very low rates of germination and seedling conversion (Pullman et al. 2003b). Consequently, much effort has been spent optimizing conditions for the production and maturation of somatic embryos and plantlet regeneration. In contrast, comparatively little work has been done on the basic biology of the embryos produced.

Somatic embryos are fundamentally different from their zygotic counterparts. Conifer somatic embryos develop in culture without the tissues that surround a zygotic embryo in a seed, most importantly the megagametophyte. This absence may be problematic given that the majority of seed storage reserves are located in this tissue. In loblolly pine, approximately 80% of the total seed reserves are located in the megagametophyte (Stone and Gifford 1997; Stone and Gifford 1999). These reserves consist primarily of storage protein and triacylglycerol (TAG), which are converted to amino acids and sugars, respectively, by the developing seedling or plantlet to provide nitrogen and carbon prior to photoautotrophic independence. TAG comprises roughly 59% of the total storage reserves in a loblolly pine seed, with 80% of the TAG stored in the megagametophyte (Stone and Gifford 1999). However, TAG is also an important reserve in the embryo, as it composes up to 40% of the embryo's dry weight (Stone and Gifford 1999). Storage proteins are also predominantly stored in the megagametophyte (Stone and Gifford 1997). In mature desiccated seeds, the majority (72%) of total protein is buffer-insoluble and is dominated by specific seed storage proteins (King and Gifford 1997). As a part of this study we were interested in determining the quantity of protein in loblolly

pine somatic embryos and also the distribution between the buffer-soluble and -insoluble protein fractions.

Although storage reserves are present in somatic embryos the levels may not be sufficient to allow complete conversion of heterotrophic embryos to photoautotrophic seedlings to occur in the absence of supplemental nutrients. Thus, it has been suggested that increasing somatic embryo storage reserves might result in increased seedling production via improved conversion ratios (Attree et al. 1992, Mhaske et al. 1998). Here we present storage protein and TAG accumulation patterns throughout somatic embryo maturation for a single embryogenic line. We have quantified these major storage reserves and compared them to those found in mature zygotic embryos.

Loblolly pine somatic embryo production and desiccation were induced from embryogenic tissue through alteration of ABA and PEG levels. The basal media consisted of (concentrations in parentheses are reported in units of mg l⁻¹ unless otherwise noted): MgSO₄·7 H₂O (925), CaCl₂·2 H₂O (211), KNO₃ (950), KH₂PO₄ (170), ZnSO₄·7 H₂O (43), H₃BO₃ (31), MnSO₄·H₂O (21), KI (4.15), Na₂MoO₄·2 H₂O (1.3), CuSO₄·5 H₂O (0.5), CoCl₂·6 H₂O (0.13), FeSO₄·7 H₂O (27.8), Na₂EDTA (37.2), myo-Inositol (100); Nicotinic acid (0.5), Pyridoxine-HCl (0.1), Thiamine-HCl (0.1), 2.5% (w/v) Maltose, Casamino acids (3000), L-Glutamine (2500), 0.6 % (w/v) Phytigel, and was modified by the addition of ABA and PEG to the levels indicated in Table 1. Media pH was adjusted to 5.7. Casamino acids, glutamine and ABA were filter sterilized prior to addition to cooled medium. The length of each maturation stage is also indicated in Table 1. Cultures were

Table 1 Somatic embryo stages and maturation conditions

Stage	Weeks	[PEG] (%)	[ABA] (μM)
1	0	-	-
2	1–3	5	100
3	4–6	10	150
4	7–9	13	200
5	10–12	16	250
6	13	Air dried	

maintained in the dark at 22°C. For the final desiccation step the embryos were removed from the media surface and allowed to air dry. Using this protocol conversion of embryos to viable plantlets occurs routinely at rates greater than 80%.

Developing somatic embryos (Fig. 1) were grouped into stages indicated in Table 1. Triacylglycerol was extracted from 100–500 mg (fresh weight) of embryo tissue using the method of Stone and Gifford (1999), and quantified according to Feirer et al (1989) using triolein (Sigma-Aldrich, Oakville, ON, Canada) as a standard. We observed that TAG levels increased throughout maturation with the greatest increase occurring between stages 3 and 4 (Fig. 2a). After desiccation (Stage 6), the final TAG content of the somatic embryos was approximately 60 $\mu\text{g mg dry weight}^{-1}$ (Fig. 2b). This was 24% of the TAG content of mature zygotic embryos. Because of potential differences in Stage 6 somatic embryo and mature zygotic embryo hydration state, comparison of TAG (Fig. 2b) and protein (Fig. 3b) levels between these embryos was based on dry weight. In other species, somatic embryos containing significantly more TAG have been achieved using similar maturation protocols. For example, white spruce (*Picea glauca*) somatic embryos containing 5–9 fold more TAG than their zygotic counterparts have been produced by this method (Attree et al. 1992), however, using a similar maturation protocol, loblolly pine somatic embryos did not achieve a dramatic increase in TAG levels.

Storage proteins were also quantified during embryo maturation. We isolated total protein

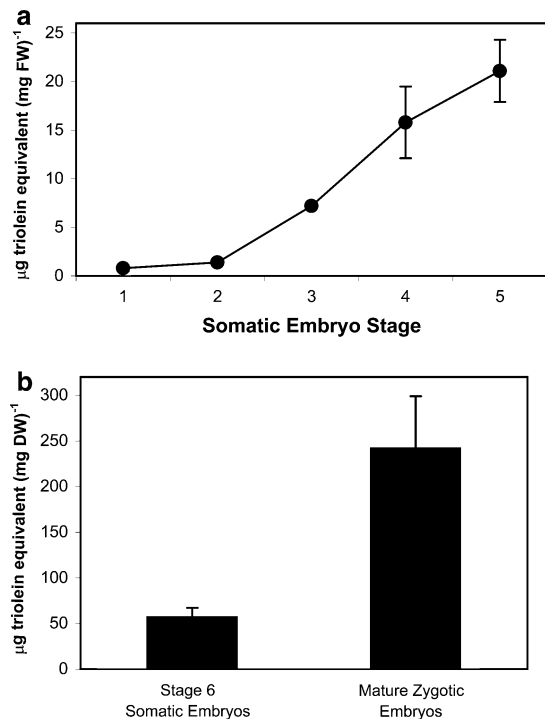


Fig. 2 (a) Triacylglycerol content during somatic embryo maturation. (b) Final triacylglycerol level comparison of mature and desiccated somatic embryos (Stage 6) and mature zygotic embryos on a dry weight basis. Data are the mean of at least three independent replicates. Error bars represent standard deviation

from 100–500 mg (fresh weight) of embryo tissue using the method of Stone and Gifford (1997). The major loblolly pine seed storage proteins are a 47 kDa globulin and a crystalloid-like 37.5 and 22.5 kDa disulphide-bridged storage protein which is glutelin-like in solubility (Groome et al. 1991). The glutelin-like proteins are insoluble in 50 mM sodium phosphate buffer (pH 7.5), and for

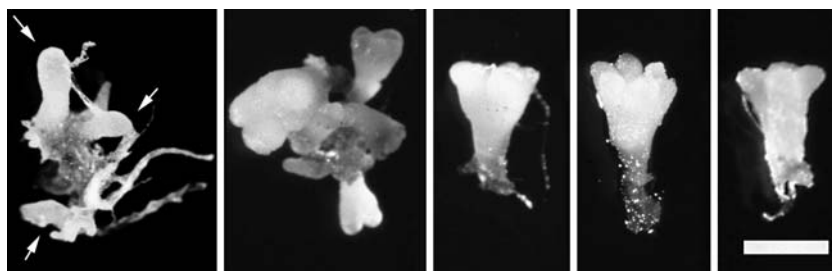


Fig. 1 Representative images of somatic embryo maturation. Stages 2–6 are depicted from left to right. Arrows in Stage 2 panel indicate developing embryos. Only those stages with readily identifiable embryos depicted. Scale bar

1 mm. Image adjustments for figure preparation were performed with Adobe Photoshop 9.0 (Adobe Systems Incorporated, San Jose, CA)

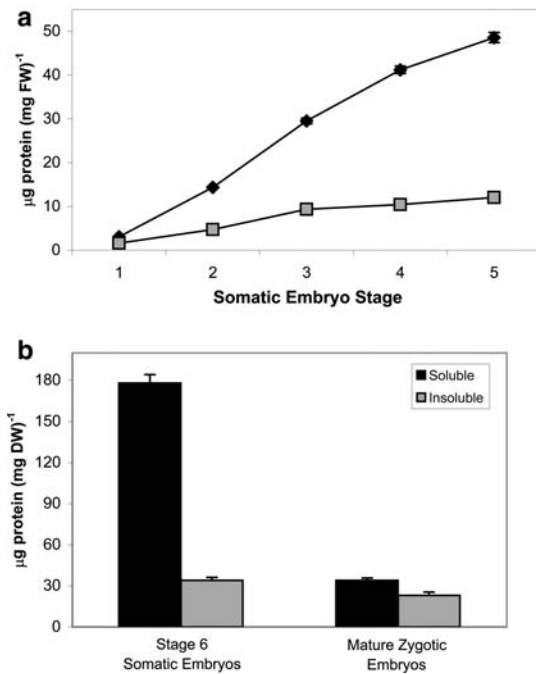
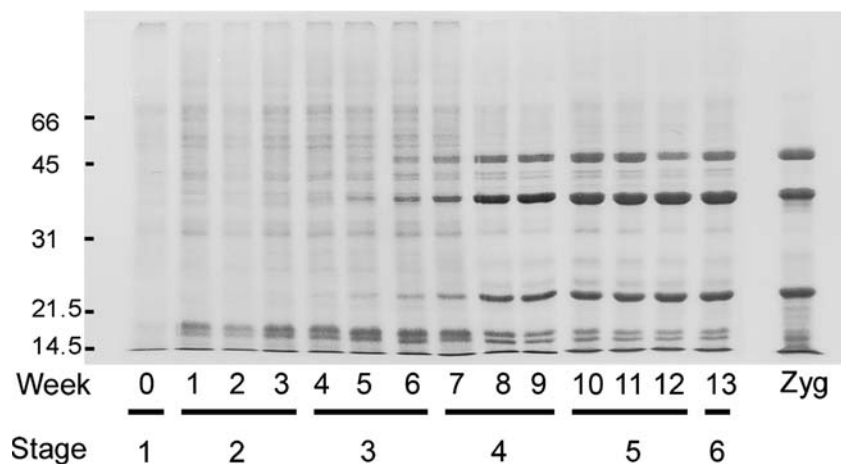


Fig. 3 (a) Buffer-soluble (black symbols) and -insoluble (grey symbols) protein levels during somatic embryo maturation. (b) Comparison of buffer-soluble (black bars) and -insoluble (grey bars) protein levels in mature, desiccated somatic embryos and zygotic embryos. Data are the mean of at least three independent replicates. Error bars represent standard deviation

consistency we follow the nomenclature of Groome et al. (1991) and Stone and Gifford (1997) and refer to the two separate pools as buffer-soluble and buffer-insoluble. Protein concentration was determined using the method of

Lowry et al. (1951) using bovine serum albumin as the standard. For protein electrophoresis, samples (8 µg per lane) were loaded onto 12% SDS-PAGE gels. Electrophoresis of protein samples was carried out as described previously (Stone and Gifford, 1997). We observed an increase in protein content throughout somatic embryo development (Fig. 3). Soluble and insoluble protein distribution patterns showed variation in the timing and level of accumulation during development. The insoluble protein fraction accumulated to its final levels relatively early, yet storage protein appeared to continue to accumulate beyond Stage 4 (Figs. 3a, 4). In contrast, soluble protein accumulated throughout development and reached the maximum level at the end of development. When protein levels in mature, desiccated somatic embryos were compared to those of mature zygotic embryos, we found that the somatic embryos contained higher amounts of both soluble and insoluble proteins on a dry weight basis (Fig. 3b). Somatic embryos contained 1.5-fold more insoluble protein and 5-fold more soluble protein. A shift in the ratios of the proteins produced was also observed. In mature zygotic embryos there were similar amounts of insoluble and soluble proteins present with a soluble to insoluble ratio of approximately 1.5:1. In contrast, mature somatic embryos contained over 5-fold the amount of soluble protein compared to insoluble protein. Thus, the somatic embryos were not only producing more protein overall, but the protein production was biased more heavily towards soluble proteins.

Fig. 4 Representative SDS-PAGE insoluble protein profile of somatic embryo during maturation. Protein was isolated each week and SDS-PAGE gels were run under reducing conditions. Embryo stages as indicated in Table 1; Zyg = mature zygotic embryo sample

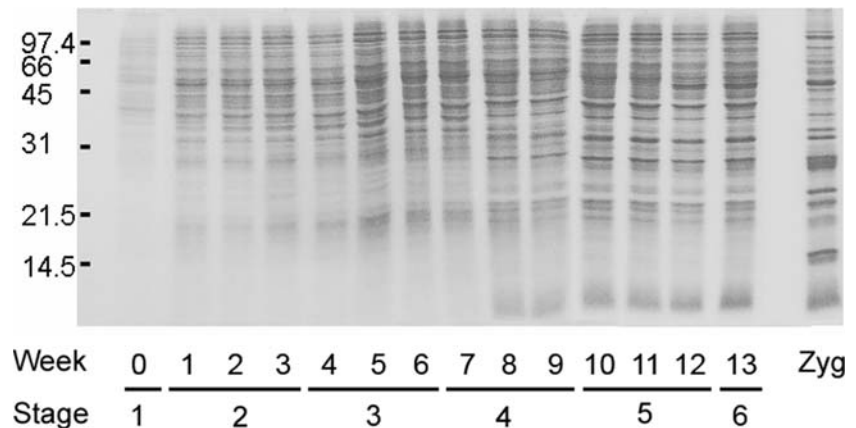


Increases in soluble protein in the embryos could be due to accumulation of specific storage proteins or may indicate a general increase in metabolic proteins (enzymes) accompanying active cellular metabolism. To identify differences in storage protein accumulation between zygotic and somatic embryos, we generated separate electrophoretic profiles for insoluble and soluble protein extracts (Figs. 4, 5). The profile of buffer-insoluble proteins extracted from loblolly pine somatic embryos closely resembled that of mature zygotic embryos (Fig. 4) and was consistent with previous reports of loblolly pine zygotic embryos (Groome et al. 1991), and with *Pinus strobus* somatic embryos (Klimaszewska et al. 2004). The polypeptides that migrated at 22.5, 37.5 and 47.0 kDa (Fig. 4) corresponded to embryonic seed storage proteins (Groome et al. 1991; Stone and Gifford 1997). Although minor differences in the buffer-insoluble protein profiles of mature somatic and zygotic embryos were apparent, the overall electrophoretic patterns were very similar. In contrast, when we compared electrophoretic patterns for buffer-soluble proteins, we noted the absence of any detectable 14 and 14.5kDa soluble seed storage proteins in the samples derived from somatic embryos, although these proteins were clearly present in mature zygotic embryo samples (Fig. 5). However, absence of these 14 and 14.5kDa polypeptides in somatic embryos does not account for the high ratio of soluble proteins we observed in somatic embryos, and the electrophoretic patterns were otherwise generally similar. Thus, the high

relative abundance of soluble protein we detected in somatic embryos was not due to unusual patterns of seed storage protein accumulation, and no single class of proteins was responsible for the high ratio of soluble protein observed in mature somatic embryos as compared to mature zygotic embryos. It is possible that our observation of an increased ratio of soluble protein in somatic embryos instead reflected differences in partitioning of C and N during embryo development, independent of the megagametophyte.

Within a mature loblolly pine seed the embryo contains only 20% of the total TAG and 14% of total seed protein (Stone and Gifford 1997; 1999). In the megagametophyte, 80% of total protein is buffer-insoluble (King and Gifford 1997) and insoluble proteins comprise the majority of the storage protein reserves for a developing seedling. Since a somatic embryo does not have these reserves present, and the reserves it does possess are quantitatively different, it may be more difficult for somatic embryos to survive and produce viable seedlings in the absence of nutrients supplied to the germinating embryos. Accumulation of a high amount of soluble protein in desiccated somatic embryos may indicate that they continue in a more metabolically active state throughout drying. In addition, there is less TAG available to the somatic embryo. TAG makes up 59% of the total storage reserves in the mature loblolly pine seed (Stone and Gifford 1999) yet the mature somatic embryos contain less TAG but more protein than their zygotic counterparts. Combined, these data show that the storage

Fig. 5 Representative SDS-PAGE soluble protein profile over somatic embryo maturation time course. Protein was isolated each week and SDS-PAGE gels were run under reducing conditions. Protein size in kD indicated to the left of the gel. Embryo stages as indicated in Table 1; Zyg = mature zygotic embryo sample



reserves present in the somatic embryos of this study are biased towards increased nitrogen at the expense of carbon stores. This difference not only in total carbon and nitrogen stores, but also in the ratio between them may make post-germinative regulation of storage reserves problematic for the developing plantlet. Methods for somatic embryo production and embryo development are evaluated upon the ability to provide viable plantlets and it is important to note that germination of somatic embryos on artificial medium provides much of the nutrition otherwise provided by the megagametophyte. Increases in protein content at the expense of storage lipids in viable somatic embryos may indicate that during early seedling growth the plantlets are better able to obtain C than N from their environment, which may select for accumulation of protein at the expense of TAG using the protocol described. However, we point out that in any in vitro system this will be tempered by specific media formulations and our results may be indicative of the C and N sources (maltose, casamino acids, glutamine and KNO_3) provided to somatic embryos during embryo development.

Attempts have been made to make somatic embryos (or their culture environment) more similar to zygotic embryos. Pullman and Buchanan (2003) analyzed megagametophyte and zygotic embryo tissue during development to determine the elemental composition. This provided information for developing culture conditions more similar to the environment of a developing zygotic embryo. Similarly, Pullman et al. (2003b) analyzed loblolly pine somatic and zygotic embryos to determine differences in metal composition and subsequently modified the media to produce somatic embryos that had a gene expression pattern more similar to zygotic embryos. The megagametophyte is a living, metabolically active tissue that interacts with the germinating embryo and young seedling. It is not surprising that the somatic embryo line we investigated differed in seed reserve composition; since the environment during somatic embryo development and germination is fundamentally different, we would be extremely surprised to find them exactly the same as their zygotic counterparts. Isolated loblolly pine

zygotic embryos can produce viable seedlings under culture conditions and initial seedling growth mimics that of intact seeds, but after several days embryos cultured with intact megagametophytes show increased seedling weight, length and cotyledon development (Todd and Gifford 2002, 2003). Comparison of similarities between somatic and zygotic embryos and seedlings will continue to generate insight into conifer seedling physiology as developmental limitations of the seedling or embryo are identified, distinguishing them from those which are allowed a certain degree of flexibility.

Acknowledgements This work was supported by funds from NSERC awarded to DJG. This work is in memory of Dr. David Gifford who passed away prior to publication.

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