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Effect of light conditions on anatomical and biochemical aspects of somatic and zygotic embryos of hybrid larch (*Larix × marschlinsii*)

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• **Background and Aims** In conifers, mature somatic embryos and zygotic embryos appear to resemble one another physiologically and morphologically. However, phenotypes of cloned conifer embryos can be strongly influenced by a number of *in vitro* factors and in some instances clonal variation can exceed that found in nature. This study examines whether zygotic embryos that develop within light-opaque cones differ from somatic embryos developing in dark/light conditions *in vitro*. Embryogenesis in larch is well understood both *in situ* and *in vitro* and thus provides a suitable system for addressing this question.
• **Methods** Features of somatic and zygotic embryos of hybrid larch, *Larix × marschlinsii*, were quantified, including cotyledon numbers, protein concentration and phenol chemistry. Somatic embryos were placed either in light or darkness for the entire maturation period. Embryos at different developmental stages were embedded and sectioned for histological analysis.
• **Key Results** Light, and to a lesser degree abscisic acid (ABA), influenced accumulation of protein and phenolic compounds in somatic and zygotic embryos. Dark-grown mature somatic embryos had more protein ($91{\cdot}77 \pm 11{\cdot}26\,\mu g$ protein mg^{-1} f.wt) than either dark-grown zygotic embryos ($62{\cdot}40 \pm 5{\cdot}58$) or light-grown somatic embryos ($58{\cdot}15 \pm 10{\cdot}02$). Zygotic embryos never accumulated phenolic compounds at any stage, whereas somatic embryos stored phenolic compounds in the embryonal root caps and suspensors. Light induced the production of quercetrin ($261{\cdot}13 \pm 9{\cdot}2\,\mu g\, g^{-1}$ d.wt) in somatic embryos. Mature zygotic embryos that were removed from seeds and placed on medium in light rapidly accumulated phenolics in the embryonal root cap and hypocotyl. Delaying germination with ABA delayed phenolic compound accumulation, restricting it to the embryonal root cap.
• **Conclusions** In larch embryos, light has a negative effect on protein accumulation, but a positive effect on phenol accumulation. Light did not affect morphogenesis, e.g. cotyledon number. Somatic embryos produced different amounts of phenolics, such as quercetrin, depending on light conditions. The greatest difference was seen in the embryonal root cap in all embryo types and conditions.

Key words: *Larix × marschlinsii*, larch, light response, phenolics, proteins, quercetrin, somatic embryogenesis, starch, zygotic embryogenesis, cotyledon, embryonal root cap.

INTRODUCTION

Embryogenesis is a complex sequence of events. As has been noted in angiosperms (Dodeman *et al.*, 1997), somatic and zygotic embryogenesis have cellular and genetic features in common during both histodifferentiation and the later acquisition of physiological traits associated with maturation. For pinaceous conifers, researchers have been fortunate in their ability to wrest control over somatic embryogenesis to the degree that today seedlings from this process are produced at industrial scale (see reviews by Nehra *et al.*, 2005; Lelu-Walter *et al.*, 2013). Such somatic embryos exhibit all of the same morphological characteristics and important physiological traits as those found in mature zygotic embryos, e.g. stress tolerance, dormancy and desiccation tolerance. This is largely due to carefully designed maturation media that are supplemented with, among other compounds, appropriate plant growth regulators, such as abscisic acid (ABA), and suitable osmoticants. This mixture regulates the transition to complete maturity. Plants derived from somatic embryos germinate and grow as well as their zygotic counterparts (Grossnickle and Major, 1994).

But how physiologically similar are zygotic and somatic embryogenesis? Phenotypes of cloned conifer embryos can be strongly influenced by a number of *in vitro* factors, such as culture age (Klimaszewska *et al.*, 2009), the type of osmoticant

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used (Klimaszewska *et al*., 2000) and the type and quantity of ABA (Kong and von Aderkas, 2007). In some instances clonal variation can exceed that found in nature. For example, cotyledon initiation appears to be much less regulated *in vitro*, with cotyledon number varying from one to 15 *in vitro*, depending on the amount of ABA in the medium (von Aderkas, 2002), whereas *in situ* cotyledon number is nearly always six. Phenotypic variation of physiology also occurs, which may have longer-lasting effects. Stage-specific induced changes in cold tolerance of somatic embryos have been noted (von Aderkas *et al*., 2007). A more spectacular example is brought on by temperature treatments applied during both zygotic and somatic embryogenesis, the effects of which result in permanent alteration of the bud phenology of mature trees (Skroppa *et al*., 2007). In spite of such powerful effects, abiotic factors are not commonly studied experimentally *in vitro*. In particular, the effect of light is often overlooked.

The morphological and physiological consequences of light for somatic embryo development have remained unstudied because, to some extent, light is not a factor in zygotic embryo development. Gymnosperm embryogenesis takes place in the dark interior of closed cones or, in the case of individual ovules such as those of yew, in low light conditions. Light is a factor that is studied post-germination, when the plant becomes autotrophic. The few studies on light's effect on embryogenesis are confined to angiosperms (Torne *et al*., 2001; Park *et al*., 2010). These studies were further limited to the initiation of embryogenesis. Various wavelengths and treatment combinations were studied. In comparison, conifer somatic embryogenesis from initiation to maturation is able to proceed in either light or darkness. Nevertheless, published maturation protocols often specify dark or light conditions for particular stages, giving the impression that these specifications are the fruit of experimental investigation. For conifers there are no such published studies. In their defence, researchers were practically motivated to produce high numbers of embryos, which was achieved in various laboratories in either light or dark conditions.

We were interested in whether light had any effect on somatic embryo anatomy or biochemistry. There are grounds for investigating the effects of light on maturation; e.g. *Lilium* somatic embryos grown in light were more numerous and larger than those grown in the dark (Lian *et al*., 2002).

A reason why conifer somatic embryos do not lend themselves to similar studies is the short lives of the cultures. Once a multiplying mass of early-stage embryos is induced, it will only be embryogenic for a short period before rapidly declining in its ability to produce mature embryos. Within a year or two, lines commonly lose their embryogenicity (Pullman and Bucalo, 2014). We were fortunate to discover a *Larix × marschlinsii* embryogenic line, 69-18, that exhibits an undiminished, virtually immortal ability to produce mature embryos over the decades (Lelu-Walter and Pâques, 2009). Since 69-18 is easy to propagate, it better lends itself to experimentation than all other lines. This clone has previously been used to explore aspects of embryogenesis, e.g. hormone physiology (Gutmann *et al*., 1996; von Aderkas *et al*., 2001). The attractiveness of using such a line is that it has a stable physiology, as opposed to other lines that are in a state of progressive diminution in their embryogenic capacity. By using such a genotype, it is possible to build up a more complex experimental study.

In the study presented here we tested the hypothesis that light makes a difference during the maturation of embryos. We compared somatic embryos matured in light with those matured in darkness. We also compared somatic embryos with zygotic embryos, which naturally develop in the dark, as well as with zygotic embryos that either germinated or were prevented from germination, to test whether the exogenously applied hormone ABA influences embryo colouration. We discovered that anatomical and biochemical differences in embryos of hybrid larch (*Larix × marschlinsii*) vary according to light conditions and the type of embryogenesis.

MATERIALS AND METHODS

Plant material

Experiments were conducted with one embryogenic line (69-18) of hybrid larch *Larix × marschlinsii* obtained in 1992 by secondary somatic embryogenesis (Lelu *et al*., 1994*b*). Proliferation medium consisted of basal MSG medium (Becwar *et al*., 1990) containing 1·45 g L^{-1} glutamine (Sigma) supplemented with 9 μM 2,4-dichlorophenoxyacetic acid, 2·3 μM 6-benzyladenine and 60 mM sucrose, solidified with 4 g L^{-1} gellan gum (Phytagel™, Sigma). Embryonal masses were placed on proliferation medium for 1 week in darkness at 25 °C (Lelu *et al*., 1994*a*). Immature cones of hybrid larch, obtained after controlled crossing, were collected in Orléans, France. Zygotic embryos dissected from the surrounding megagametophyte were collected at different stages of development from the early stage of late embryogeny (end of May) to the late stage of late embryogeny (middle of June) (terms according to von Aderkas *et al*., 1991). In addition, zygotic embryos were dissected from seeds stored at –20 °C. Samples (zygotic embryos and megagametophytes) were either frozen in liquid nitrogen for biochemical analysis or fixed for light microscopy.

Somatic embryo maturation

Somatic embryos were matured according to Lelu-Walter and Pâques (2009). Briefly, proliferating 1-week-old embryonic masses were incubated for 1 week on plant growth regulator (PGR)-free medium supplemented with activated charcoal (10 g L^{-1}) and 100 mM sucrose. Petri dishes were placed under cool white light (Philips) at a photon flux density of 10 μmol m^{-2} s^{-1} at 24/21 ± 1 °C under a photoperiod of 16 h light and 8 h dark. Next, embryonal masses were transferred to MSG medium supplemented with 200 mM sucrose, 1 μM indolebutyric acid and 60 μM *cis–trans* (±) ABA for a period of 7 weeks. Light intensity for this 7-week period was increased to 20 μmol m^{-2} s^{-1}. Cotyledonary somatic embryos were counted at the end of the culture period. We also estimated embryogenic potential, i.e. the number of somatic embryos per gram of fresh weight. To test the effect of light on somatic embryo maturation, a set of embryonal masses ($n = 5$) were placed in either the light or dark for the entire maturation period. Experiments were performed three times. Cotyledons were counted from a minimum of 200 mature embryos per treatment. Subsequently, somatic embryos were either fixed for later histological investigation or frozen in liquid nitrogen for eventual biochemical analysis.

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Zygotic embryo germination

To test whether zygotic embryos produced phenolics in light prior to or during germination, hybrid seed collected from trees in the breeding orchard located at INRA-Orléans were dissected and embryos placed on MSG maturation medium supplemented with 200 mM sucrose for 8 d. For the control treatment (prevention of germination) we used MSG maturation medium supplemented with 200 mM sucrose and 60 µM ABA. Samples ($n = 33$–48) were assessed for their colour at 2-d intervals. A small number of representative samples were fixed and included in the larger histological investigation described in the next section.

Histological analysis

Because the earliest stages of embryogenesis differ between zygotic and somatic (von Aderkas *et al*., 1991), we focused on two more readily comparable stages that occur later in development: (1) early embryos prior to histodifferentiation; and (2) mature embryos. Somatic embryos in the dark and light treatments were morphologically very similar to one another. Consequently, we have only shown sections of somatic embryos subjected to the dark treatment.

Somatic and zygotic embryos were prepared according to Gutmann *et al*. (1996). Briefly, samples were fixed with 2·5 % glutaraldehyde in 100 mM phosphate buffer at pH 7·5 for at least 12 h at room temperature. After two washes with buffer, the samples were dehydrated gradually in ethanol, infiltrated with glycol methacrylate (Historesin, Reichert-Jung) at room temperature for at least 2 d and finally polymerized. Longitudinal sections were cut on a Leitz 1400 microtome equipped with a tungsten carbide knife. Section thickness was 2 µm, except for the following two staining procedures, in which 5 µm sections were cut in order to gain sufficient staining intensity. Flavanols (i.e. catechins) were localized with the highly selective *p*-dimethylaminocinnamaldehyde (DMACA) reagent as described by Gutmann and Feucht (1991). Moreover, the deposition of proanthocyanidins (condensed tannins) was traced using a developed *in situ* hydrolysis procedure (Gutmann, 1993). Other staining methods used were described in detail by Gutmann (1995): toluidine blue O with sodium hypochlorite pretreatment (general tissue structure), safranin O/azure II with iodine/potassium iodide post-staining treatment (polyphenols, cell walls, starch) and safranin O with iodine/potassium iodide post-staining treatment (general tissue structure and starch). In addition, a rapid two-step method with Ponceau 2R and azure II was employed for the differential staining of storage protein and cell walls. The following steps were required: 5–10 min of staining with 0·5 % Ponceau 2R in 2 % acetic acid; a rinse with distilled water; 10 s of staining with 0·5 % azure II in distilled water; and a rinse with distilled water. Slides were dried with a brief blast of compressed air and allowed to dry in an oven for 10 min at 50 °C. Sections were then mounted in Canada balsam. Cytoplasmic and storage proteins stained red with Ponceau 2R (Gori, 1978) and cell walls stained blue.

Material for biochemical analysis

Somatic embryos were sampled by stage of development following the protocol of Guillaumot *et al*. (2008). Samples were taken at the time of transfer from charcoal medium to maturation medium, as well as after a further 1 and 7 weeks of culture. The 7-week collection included only mature cotyledonary embryos; any embryonal masses found in culture dishes were excluded from analysis. To assay proteins, samples were weighed immediately after harvest to determine fresh weight. Five to seven samples ranging from 25 to 50 mg fresh weight each were collected per developmental stage. To assay phenolic compounds, samples were lyophilized and dry weight was determined. Three samples (ranging from 20 to 49 mg dry weight) were collected per developmental stage.

Total protein assay

Total protein extracts were prepared at least in quintuplet for each developmental stage. Frozen embryos were homogenized with 0·5 mL of lysis buffer [10 % (v/v) glycerol; 2 % (w/v) SDS; 5 % (v/v) β-mercaptoethanol; 2 % (w/v) poly(vinyl) polypyrrolidone; 50 mM Tris, pH 6·8]. Extracted samples were incubated for 5 min at 95 °C and then centrifuged at 12 300 × *g*. Supernatant was transferred to tubes; pellets were re-extracted with the same buffer minus both SDS and poly(vinyl) polypyrrolidone. Supernatant was then pooled. Protein concentrations were determined using Bradford assays in which bovine serum albumin was the standard.

Protein separation

To determine subunit masses, denaturing gel electrophoresis (SDS–PAGE) was performed according to standard protocols using 12–20 % polyacrylamide gradient gels overlaid with a 4 % stacking gel. The gel was stained with colloidal Coomassie blue G-250. Electrophoretic patterns were compared with protein markers, in particular, phosphorylase b (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), trypsin inhibitor (20·1 kDa) and lactalbumin (14·4 kDa).

Extraction of soluble polyphenols

Samples were ground in a mortar using a glass rod in a 2-mL tube filled with liquid nitrogen. Soluble phenolic compounds were extracted twice from the dry powder in 2 mL of acetone/water (8:2, v/v) containing 10^{-4} M 6-methoxyflavone as internal standard. This mixture was sonicated for 45 min then incubated while being agitated for 1 h before being centrifuged at 18 000 *g* for 20 min. A 1 mL sample from the pooled supernatant was removed and dried under vacuum using a Speed-Vac system (Savant Instruments, India). The dry residue was diluted in 250 µL of methanol. All steps were carried out at 4 °C.

Total polyphenols quantification in embryo extract

Total polyphenols were estimated by the Folin–Ciocalteu method modified according to Boizot and Charpentier (2006). Phenolic extract (10–15 µL) was diluted in 85–90 µL of ultrapure water, 500 µL of Folin–Ciocalteu reagent diluted 10-fold

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in ultra-pure water and 400 µL of $NaCO_3$ 75 g L^{-1}. This mixture was incubated for 5 min at 40 °C. The absorbance was measured spectrophotometrically (735 nm); results were expressed in milligram equivalent of gallic acid per gram dry weight. Calibration was carried out using gallic acid methanol solutions (0–20 µg mL^{-1}).

Chromatographic separation of phenolic compounds

Chromatographic analysis of phenolic compounds was performed according to a published method (Faivre-Rampant *et al*., 2002). Briefly, after centrifugation at 10 000 *g* for 3 min, a 15-µL aliquot of the phenolic extract was separated, characterized and quantified by HPLC on a 32 Karat system (Beckman Coulter, France) using a 250 × 4 mm Licrosphere 100RP-18e column (5 µm) (Merck, Germany) stabilized at 40 °C; flow rate was 1 mL min^{-1}. The following linear elution six-step gradient was used: (1) initial conditions, 15 % solvent B (methanol/acetonitrile, 50:50 v/v) in solvent A (1 % acetic acid in ultra-pure water); (2) 0–20 min, 15–40 % solvent B; (3) 20–25 min, 40–60 % solvent B; (4) 25–30 min, 60–100 % solvent B; (5) 30–35 min, 100 % solvent B; (6) 35–38 min, 100–15 % solvent B. Compounds were characterized by their retention time and UV absorption spectrum (diode array 230–430 nm). Quercitrin was identified by co-chromatography with a standard (Extrasynthese, France). It was determined quantitatively at 340 nm with external calibration (solutions of quercitrin in methanol; five points from 0·3 to 6·7 µg) and the results were expressed in micrograms per gram dry weight. Other phenolic compounds were determined quantitatively at 340 and 280 nm and expressed in milligram equivalents of 6-methoxyflavone per gram dry weight.

Statistical analysis

One-way analysis of variance was performed with R (R Development Core Team, 2008). Multiple mean comparisons with confidence intervals for general linear hypotheses in parametric models were obtained by the use of the Multcomp R library (Hothorn *et al*., 2008).

RESULTS

Effect of light on number of mature somatic embryos

There was no significant effect of light on the numbers of embryos that were able to mature. For light treatments, there was an average of 384 ± 40 somatic embryos g^{-1} fresh weight compared with 316 ± 53 in the dark ($P = 0{\cdot}03197$). There was no difference between batches, i.e. no block effects.

Effect of light on cotyledon number and colouration

There was no significant effect of light on the number of cotyledons initiated at the $P = 0{\cdot}05$ level. For light treatments, there was an average of 6·21 ± 1·06 cotyledons/embryo in the light compared with 6·31 ± 0·86 in the dark. A slightly significant batch difference was noted ($P = 0{\cdot}0466$). There were no block effects attributable to the Petri dish in which an embryo occurred.

Somatic embryos matured in the dark remained yellowish, whereas those matured in the light had red embryonal root caps (Fig. 1A, B). Comparably mature zygotic embryos that were dissected from seed were yellow. If placed in light on medium supplemented with 60 µM ABA, which prevents germination, embryos developed a slight red colouration ('Reddish' in Table 1) in their embryonal root caps by 6 d (Table 1; Fig. 1C), by which time cotyledons and hypocotyls were beginning to turn green.

Phenolic analysis

Phenolics were absent in zygotic embryos but present in somatic embryos. The concentrations in somatic embryos varied over the course of development. At maturity, somatic embryos in the light treatment had a significantly higher concentration of phenolics than embryos in the dark treatment. In somatic embryos, one flavonoid, quercetrin, was only found in light-treated somatic embryos (Table 2). We did not measure quercetrin in zygotic embryos.

Protein analysis

Dark- and light-treated immature embryos had equivalent quantities of protein (Table 2). By the last stage of development (7 week), dark-treated mature somatic embryos had ~50 % more protein than light-treated somatic embryos or zygotic embryos. The difference in total protein did not correspond to a qualitative difference between the types of mature embryos since they showed the same protein profile (Fig. 2). The major bands corresponding to the storage proteins observed in the megagametophyte were absent in both types of early somatic embryo.

Anatomy of early embryos prior to histodifferentiation

Embryonal tube cells were formed by a rib meristem that was found below the cells of the embryonal mass (Figs 3A and 4A). The embryonal mass was actively growing, as indicated by the numerous mitotic figures (Fig. 3A). Starch was found in embryonal tube cells as well as in cells of the rib meristem. No protein bodies were seen in either zygotic or somatic embryos. In contrast to zygotic embryos, which always lacked phenols, somatic embryos were rich in phenols, particularly in suspensors. Phenolic substances were deposited in vesicles and vacuoles (Fig. 4A).

Anatomy of mature embryos

Mature zygotic and somatic embryos had fully developed cotyledons, ground tissues and organs (Figs 3B and 4B). However, the different types of embryogenesis resulted in differences in proportions, somatic embryos being both shorter and squatter than zygotic ones. Starch was found in all tissues, e.g. embryonal root cap (Figs 3C and 4C) and hypocotyl (Fig. 3D). In larch seed, numerous protein bodies were found in both megagametophte and embryo (Figs 3E and 4D). Embryos had protein bodies throughout their cotyledons and hypocotyls.

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Fig. 1 (A–D) Somatic and zygotic embryo colouration in hybrid larch, *Larix* × *marschlinsii*. (A) Somatic embryos matured in light had red embryonal root caps. (B) Somatic embryos matured in the dark had no colouration. (C) Zygotic embryos that were dissected from the ovule and placed on medium with 60 µM ABA to prevent germination developed red colouration in the embryonal root cap. (D) Zygotic embryos placed on ABA-free medium germinated producing an elongated red hypocotyl and root. Remnants of the very red embryonal root cap are seen at the base of the hypocotyl. Scale bars = 1 mm.

Table 1. *Colour in dissected and mature zygotic embryos of hybrid larch (*Larix × marschlinsii*) cultured in light on medium supplemented or not with ABA (60 µM)*

ABA (µM)	Time (d)	Number	Colourless	Reddish	Red	Germinated
0	1	48	–	48	–	–
60	1	45	43	2	–	–
0	2	44	–	–	44	–
60	2	41	–	41	–	–
0	4	40	–	–	40	40
60	4	37	–	37	–	–
0	6	37	–	–	37	37
60	6	33	–	33	–	–

Between cell layers in the hypocotyl ground tissue, idioblastic cells grew that did not have any storage products (Figs 3B and 4B, E). Phenolic compounds were only found in light- and dark-treated somatic embryos. These compounds were mainly restricted to the embryonal root cap (Fig. 4C). Two types of phenolic compounds, proanthocyanidins (Fig. 4F) and catechins (Fig. 4G), were restricted to the periphery of the root cap. Embryos had abundant protein bodies in their hypocotyl, cotyledon and embryonal root caps, in particular in the central zone or column. Protein bodies were not found in idioblastic cells, suspensors, procambial tissues, shoot apical meristems, root apical meristems or the pericolumn of the embryonal root cap.

Zygotic embryos germinating in light

When embryos were removed from seeds and placed on ABA-free medium in light, they germinated quickly (Fig. 1D), and within a day had begun depositing phenolic compounds. Embryos placed on ABA-supplemented medium did not germinate, and only began to deposit phenolic compounds after 4 d in the light (Table 1). In both treatments phenolic deposition

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TABLE 2. *Storage protein and phenolic compound concentrations in somatic embryos (SE) matured in light or darkness, and mature zygotic embryo (ZE) and megagametophyte of* Larix × marschlinsii

Samples	Culture conditions	Protein (μg protein mg^{-1} f.wt)[1]	Phenolic compounds	
			(mg eq. gallic acid g^{-1} d.wt)[2]	quercetrin (μg g^{-1} d.wt)[2]
SE 1w charcoal	Light	14·66 ± 6·77[a]	27·58 ± 9·19[b]	n.d.
SE 1w ABA		24·37 ± 6·51[a]	15·57 ± 6·42[a]	n.d.
SE 7w ABA		58·15 ± 10·02[b]	26·54 ± 9·88[b]	261·13 ± 9·2[3]
SE 1w charcoal	Dark	15·66 ± 4·37[a]	19·32 ± 5·42[a]	n.d.
SE 1w ABA		21·74 ± 5·39[a]	20·31 ± 2·15[a]	n.d.
SE 7w ABA		91·77 ± 11·26[c]	14·81 ± 3·50[a]	n.d.
ZE		62·40 ± 5·58	n.d.	n.d.
Megagametophyte		132·12 ± 15·79	n.d.	n.d.

[1]Values are mean ± s.d. ($n = 5$ for all, except $n = 7$ for SE 1w charcoal). Significantly different means are indicated by different letters ($P = 0{\cdot}05$).
[2]Values are mean ± s.d. ($n = 4$ for all, except $n = 5$ for SE 7w ABA light and $n = 3$ for SE 7w ABA dark). Significantly different means are indicated by different letters ($p = 0{\cdot}1$).
[3]Significance test not applicable.
d. wt, dry weight; f.wt, fresh weight; n.d., not detectable.

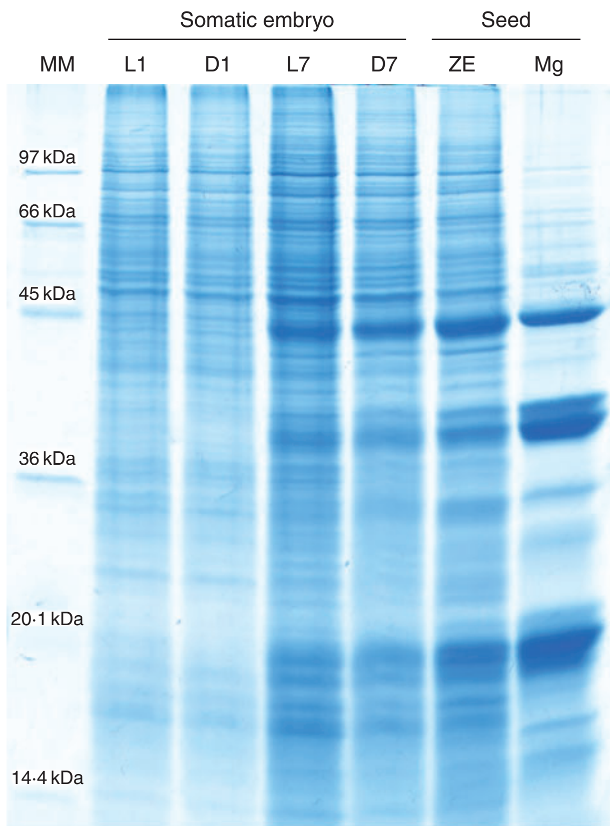

FIG. 2. SDS–PAGE total protein profile comparison in seed and somatic embryo of hybrid larch. Lanes L1 and D1, light- and dark-treated early somatic embryos at 1 week on ABA medium; L7 and D7, light- and dark-treated mature somatic embryos at 7 weeks on ABA medium; ZE, mature zygotic embryos; Mg, megagametophyte; MM, molecular markers (kDa).

occurred in outer cells of the embryonal root cap, especially in the junction zone where the root cap and hypocotyl meet (Fig. 5A, B, Table 1). Germinated embryos also accumulated phenolics in their hypocotyl (Fig. 1D). ABA delayed the development of red colour, as embryos not exposed to ABA were much deeper red than those that had been exposed to 60 μM ABA.

DISCUSSION

Protein accumulation and phenolic compound production are both influenced by light during embryogenesis. Zygotic embryos that develop within megagametophytes in near complete darkness from ovules centrally located in closed cones do not produce phenolic compounds. By comparison, somatic embryos produce phenolic compounds abundantly in light, as well as in the dark. Light also affects protein accumulation, which is greater in dark-grown embryos (zygotic or somatic) than in light-grown somatic embryos. These findings contribute new information to our understanding of the influence of light during conifer embryogenesis.

There are probably other effects of light to be discovered, because the *in vitro* effects of light have been relatively little studied. Recent reviews of somatic embryogenesis make no mention of the effect of light (Nehra *et al*., 2005; Elhiti and Stasolla, 2011). In angiosperms, the effect of light on somatic embryo growth has been investigated with respect to quality and light treatments, e.g. alternating red and far-red light (Park *et al*., 2010). In conifers, experiments on the effect of light have not been carried out during embryogenesis, but only on germinants and seedlings derived from somatic embryos. Kvaalen and Appelgren (1999) studied the effect of red light on the germination of somatic embryos. Högberg and co-workers (2001) showed that exposing emblings to continuous light was detrimental to their growth. Our results on the effect of light on the differential accumulation of storage products are important because they clearly pinpoint peculiarities unique to somatic embryogenesis.

The influence of light can be general or specific within the embryo itself. As we have shown, protein body formation occurs throughout the embryo, but phenolic compounds accumulate most readily in somatic embryos, mainly in suspensor and embryonal root cap.

That the embryonal root cap of somatic embryos in our study of light effects should exhibit marked differences in

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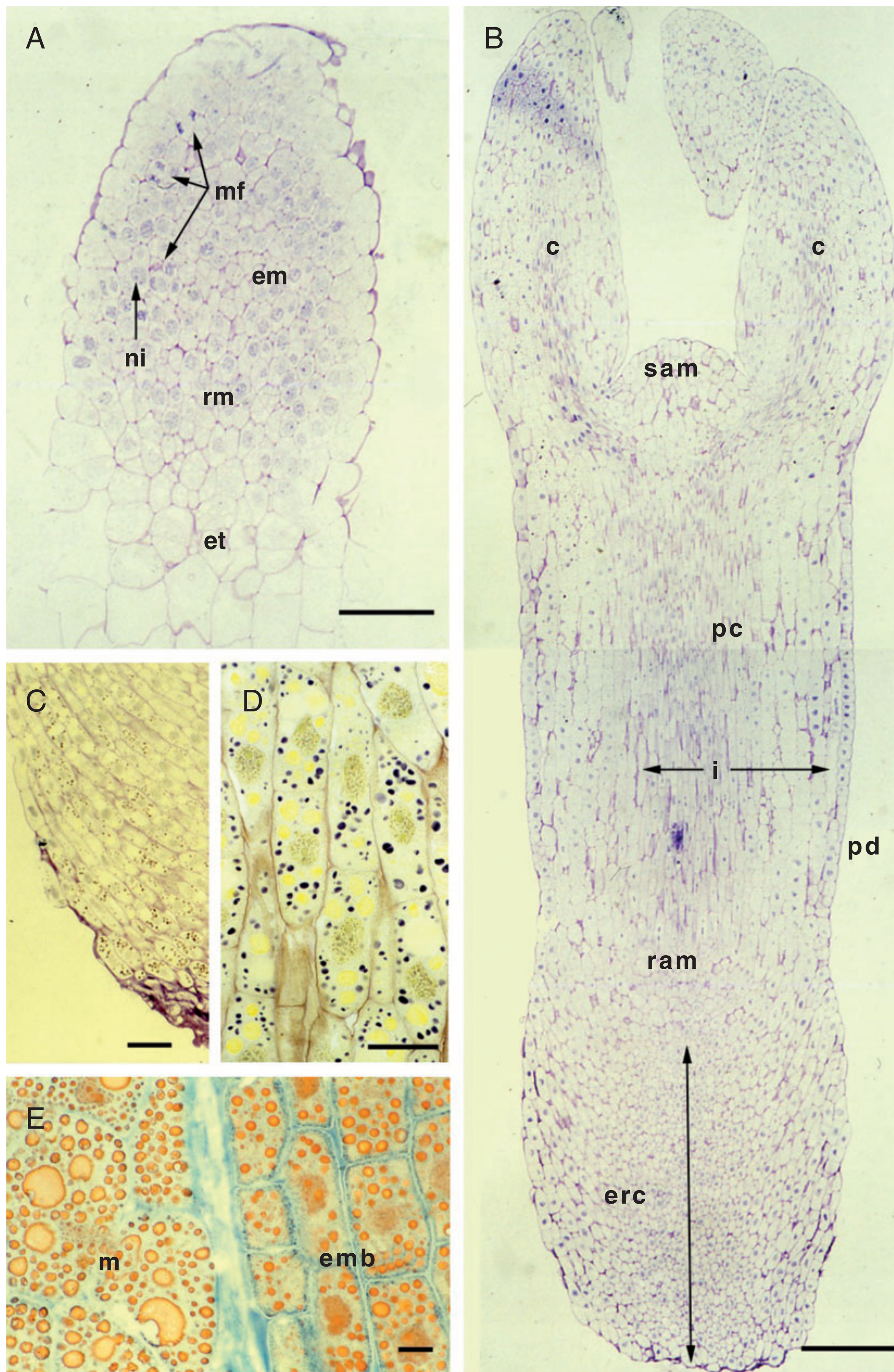

FIG. 3. Zygotic embryogenesis. (A) Early embryo prior to histodifferentiation, showing a rib meristem (rm) and formed embryonal tube (et) cells. Embryonal mass cells (em) are distinguished by their prominent nucleoli (ni) and active division. Numerous mitotic figures (mf) can be seen. Toluidine blue O stain. Scale bar = 100 µm. (B) Longitudinal section of mature zygotic embryo with cotyledons (c), shoot apical meristem (sam), root apical meristem (ram) and embryonal root cap (erc). Ground tissues include procambium (pc), protoderm (pd) and two idioblasts (i, arrows). Toluidine blue O stain. Scale bar = 200 µm. (C) Pericolumn region of embryonal root cap with abundant starch. Lugol stain. Scale bar = 100 µm. (D) Hypocotyl cells stained for starch. Lugol stain. Scale bar = 50 µm. (E) Embryo (emb) beside megagametophyte (m) stained for proteins (Ponceau S) and cell walls (azure II). Megagametophyte cells have larger and more numerous protein bodies than embryo cells. Scale bar = 10 µm.

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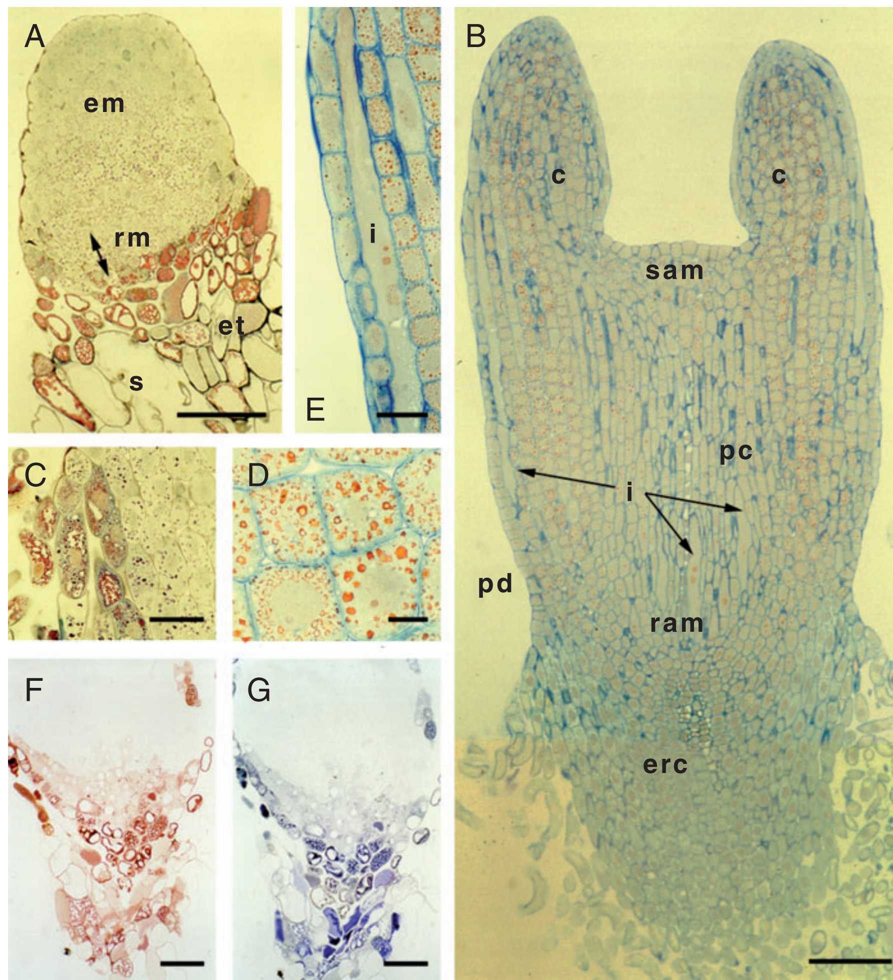

FIG. 4. Somatic embryogenesis in the dark. (A) Early embryo prior to histodifferentiation; the embryonal mass (em) was subtended by a rib meristem (rm). The suspensor (s) was composed of embryonal tube cells (et) in which abundant phenolic compounds were deposited either in small vesicles or along the inner margins of large vacuoles. Safranin O and fast green stain. Scale bar = 100 µm. (B) Longitudinal section of mature somatic embryo with cotyledons (c), root (ram) and shoot (sam) apical meristems, an embryonal root cap (erc) and ground tissues, such as procambium (pc) and protoderm (pd). Three idioblasts (i) are indicated by arrows. Ponceau S and azure II stain. Scale bar = 200 µm. (C) Starch grains in the hypocotyl/embryonal root cap junction. Root cap cells are rich in phenols, which stain yellow to orange with a combined safranin/Lugol stain. Scale bar = 50 µm. (D) Protein bodies are abundant throughout all tissues, e.g. hypocotyl cells. Ponceau S and azure II stain. Scale bar = 10 µm. (E) Idioblast (i) stained with Ponceau S and azure II. Scale bar = 30 µm. (F) Proanthocyanidins stained red after *in situ* hot sulphuric acid treatment are restricted to the periphery of the embryonal root cap. Scale bar = 50 µm. (G) Catechins stained with DMACA. Scale bar = 50 µm.

accumulation of proteins and phenols, such as quercetrin, is not surprising, as previous studies of this organ have shown that it will show differential accumulation of protein as well as altered ABA metabolism in response to alteration of exogenous ABA application during embryo maturation (von Aderkas *et al*., 2002). The role of the embryonal root cap is generally underappreciated. In part it is the name that deceives: the embryonal root cap is less an organ protecting a developing root than it is a major storage organ. It can make up to 50 % of a zygotic embryo's mass [see illustrations in reviews by Singh (1978) and von Guttenberg (1961)]. The high accumulation of protein in dark-treated embryos in our experiment, which was greater even than in zygotic embryos, points to the significance of this organ in providing nutritional storage support for developing somatic embryos and seedlings. The embryonal root cap's storage capacity is an important reason why somatic embryos germinate readily and establish well as seedlings. Somatic embryos are able to perform as well as zygotic embryos even though somatic embryos lack the surrounding storage product-rich megagametophyte with which zygotic embryos are endowed. Although protein accumulation in somatic embryos is generally considered to be under the control of ABA (Roberts, 1991), light also affected protein accumulation in larch somatic embryos matured on ABA.

Protein accumulation is more complicated in conifer somatic embryogenesis. In *Pinus pinaster*, supplementation of the medium with different maltose and polyethylene glycol levels influenced both starch and protein body size and number (Tereso *et al*., 2007) between treatments and in comparison with zygotic embryogenesis. Although we found measurable differences in protein storage, we did not see differences in the size of protein bodies or in the protein profiles. In conifers, improvements in maturation protocols resulted in somatic embryos accumulating amounts of storage products similar to

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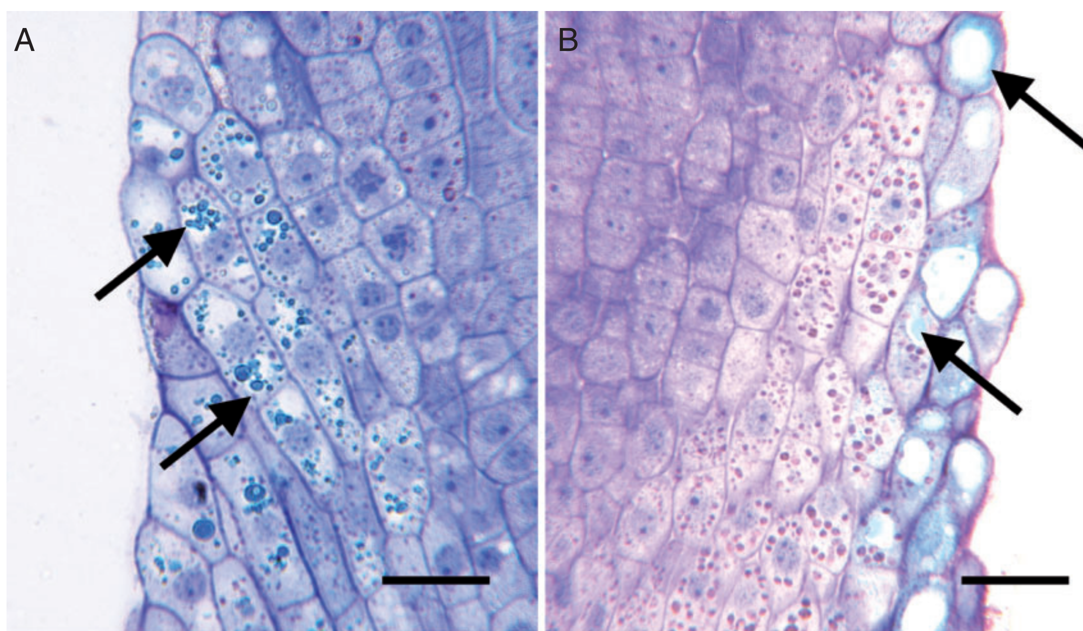

FIG. 5. Germinating zygotic embryos in light on medium with or without 60 μM ABA. (A) Junction zone of hypocotyl with embryonal root cap of embryo germinating on medium without ABA after 2 d of control treatment (0 μM ABA). Phenolic deposits are indicated by arrows. Toluidine blue O stain. Scale bar = 50 μm. (B) Junction zone of hypocotyl with embryonal root cap of embryo germinating on medium with 60 μm ABA after 6 d of treatment. Phenolic deposits are indicated by arrows. Toluidine blue O stain. Scale bar = 50 μm.

those found in mature zygotic embryos, including *Pinus sylvestris*, *P. pinaster* and *Larix* × *eurolepis* (Lelu-Walter *et al*., 2008; Morel *et al*., 2014; Teyssier *et al*., 2014). The protein profiles were also similar in somatic and zygotic embryos. By comparison, megagametophytes had much larger protein bodies than either type of embryo. Somatic embryos of loblolly pine not only produce more protein overall than zygotic embryos, but they differ in protein metabolism, e.g. the ratio of insoluble to soluble proteins differs greatly between the types of embryo (Brownfield *et al*., 2007). By comparison, zygotic and somatic embryogenesis in palms differs not only in the amount of protein but in the kinds of protein that accumulate (Aberlenc-Bertossi *et al*., 2008). Studies comparing zygotic and somatic embryogenesis record so many differences (Jones and Rost, 1989; Alemanno *et al*., 1997; Kärkonen, 2000) that it is fair to conclude that they always differ. The differences in protein content between light- and dark-grown somatic embryos may have an effect on subsequent germination performance, but this requires further experimentation.

Some of the differences in the physiological responses between zygotic embryos and embryos of conifers are due to hormones, in particular ABA and related compounds. Somatic embryos are able to produce endogenous ABA (Kong and Yeung, 1995; Kong and von Aderkas, 2007), but at insufficient levels to induce organ development. To mature, developing embryos require a large dose of exogenously applied ABA. ABA controls not only the differentiation of organs, but also the acquisition of physiological traits characteristic of mature somatic embryos, such as desiccation tolerance (Attree *et al*., 1995). ABA concentrations are not the same in zygotic and somatic embryos. Somatic embryos are grown on media with very high concentrations of ABA. As a result, somatic embryos have internal concentrations of ABA that are orders of magnitude higher than those of zygotic embryos (von Aderkas *et al*., 2001). This may be peculiar to conifer somatic embryogenic systems. In the angiosperm *Nothofagus*, exogenously applied ABA has no such effect. Endogenous ABA concentrations even in the presence of exogenously applied ABA are lower than ABA concentrations found in zygotic embryos (Riquelme *et al*., 2011). Coming back to conifers, in *Larix* somatic embryos the metabolism of phenolic compounds is influenced by ABA. A previous study (Gutmann *et al*., 1996) showed that, in the absence of ABA, embryos at all stages of embryogenesis were red in colour. Mature embryos accumulated phenolic compounds in surface cells of the cotyledon, hypocotyl and embryonal root cap. In the study reported here, mature zygotic embryos that germinated in light accumulated phenolic compounds rapidly, but mature embryos placed on a medium supplemented with a concentration of ABA sufficient to inhibit germination accumulated these phenolics much more slowly. These various effects of exogenous ABA allow us to conclude that ABA regulates more than histodifferentiation and the acquisition of late embryo physiological characteristics such as protein storage and desiccation tolerance; ABA appears to control aspects of phenol metabolism. This regulation is not simply switching on or off, but involves some interaction between light and ABA. Quercetrin is a case in point. It is only produced when ABA is supplied during maturation of somatic embryos that have been grown in light. Quercetrin is not found in embryos grown on ABA in the dark. ABA is also known to have long-term effects. In *Picea abies*, overexposure to ABA during maturation of somatic embryos, i.e. maturation on ABA for overly long periods, is responsible for poor seedling growth (Högberg *et al*., 2001).

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Our study also shows that light leaves some important embryogenic processes unaltered. These include the acquisition of form and the accumulation of starch. In our study, zygotic and somatic embryos had a full set of organs, including cotyledons, hypocotyl and embryonal root cap. In the case of cotyledons, six cotyledons per embryo developed in all embryos, which was identical to previously published values for larch zygotic embryos (Butts and Buchholz, 1940; von Aderkas, 2002), but higher than studies in which ABA was omitted (Harrison and von Aderkas, 2004) or in which ABA had been replaced with a cytokinin, 6-benzylaminopurine (von Aderkas, 2002).

The importance of this study lies in a nuanced aspect of embryogenesis. Embryos that develop in the dark, as is the case for zygotic embryos inside an ovule, are not exposed to light, which would appear to eliminate light as a factor in development. However, once embryogenesis is made to take place in the light, as is the case with *in vitro* somatic embryo development, the effects of light are noticeable, particularly in the embryonal root cap. Here, phenolic compound metabolism is promoted compared with other embryo parts. That some of these effects are partially due to ABA, a growth regulator that is present in excessive amounts, is a novel finding. This paper offers more support for a new interpretation of the embryonal root cap's role in nutrition and embryo development.

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