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3 zygotic embryos of hybrid larch (*Larix x marschlinsii*)

4 **Authors:** Patrick von Aderkas^{1*}, Caroline Teyssier², Jean-Paul Charpentier², Markus
5 Gutmann³, Luc Pâques², Claire Le Metté², Kevin Ader², Philippe Label⁴, Lisheng Kong¹,
6 Marie-Anne Lelu-Walter²

7 **Affiliations:**

8 1 Centre for Forest Biology, Department of Biology, University of Victoria, 3800 Finnerty
9 Rd., Victoria BC, V8W 3N5, Canada, Fax: + 12507216611, E-mail: pvonader@uvic.ca

10 2 I.N.R.A., UR 0588 Research Unit on Breeding, Genetic and Physiology of Forest Trees,
11 2163 Avenue de la Pomme de pin CS 4001 Ardon F- 45075 Orléans Cedex 2 France

12 3 Fachgebiet Obstbau, Technische Universität München, Dürnast 2, D-85354 Freising,
13 Germany

14 4 INRA-Université Blaise Pascal, UMR_A 547 PIAF, Les Cézeaux, 24 Avenue des
15 Landais, 63177 Aubière cedex, France.

16 **Running title:** larch somatic and zygotic embryogenesis and the effect of light

17 **Email addresses**

18 Patrick von Aderkas – pvonader@uvic.ca

19

1 **ABSTRACT**

2 *Background and Aims* – In conifers, mature somatic embryos and zygotic embryos appear
3 to resemble one another physiologically and morphologically. Do zygotic embryos that
4 develop within light-opaque cones differ from somatic embryos developing in dark/light
5 conditions *in vitro*? Larch embryogenesis is well understood both *in situ* and *in vitro* and
6 provides a tractable system for answering this question.

7 *Methods* – Embryo features were quantified, such as cotyledon numbers, protein
8 concentration, and phenol chemistry. Developmental stages of embryos were embedded
9 and sectioned.

10 *Key Results* – Light, and to a lesser degree abscisic acid (ABA), influenced protein and
11 phenolic compound accumulation in somatic and zygotic embryos of larch (*Larix x*
12 *marschlinsii*). Dark-grown mature somatic embryos had more protein ($91.77 \pm 11.26 \mu\text{g}$
13 protein/mg fresh weight) than either dark-grown zygotic embryos ($62.40 \pm 5.58 \mu\text{g}$
14 protein/mg fresh weight) or light-grown somatic embryos ($58.15 \pm 10.02 \mu\text{g}$ protein/mg
15 fresh weight). Zygotic embryos never accumulated phenolic compounds at any stage,
16 whereas somatic embryos stored phenolic compounds in embryonal root caps and
17 suspensors. Light induced the production of quercetrin ($261.13 \pm 9.21 \mu\text{g/g DW}$) in somatic
18 embryos. Mature zygotic embryos that were removed from seed and placed on medium in
19 light rapidly accumulated phenolics in embryonal root cap and hypocotyl. Delaying
20 germination with ABA delayed phenolic compound accumulation, restricting it to the
21 embryonal root cap.

22 *Conclusions* – In larch embryos, light has a negative effect on protein accumulation, but a
23 positive effect on phenol accumulation. Light did not affect morphogenesis, e.g. cotyledon

1 number. Somatic embryos produced different amounts of phenolics, such as quercetrin,
2 depending on light conditions. In all embryo types and conditions, the greatest difference
3 was seen in the embryonal root cap.

4

5 **KEYWORDS:** *Larix x marschlinsii*, light response, phenolics, proteins, quercetrin, somatic
6 embryogenesis, starch, zygotic embryogenesis, cotyledon, embryonal root cap

7

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 found in mature zygotic embryos, e.g. stress tolerance, dormancy, desiccation tolerance. This is largely due to carefully designed maturation media that are supplemented with, among other compounds, appropriate plant growth regulators, such as 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 age of cultures (Klimaszewska *et al.*, 2009), type of osmoticant used (Klimaszewska *et al.*, 2000), and 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 1 – 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

1 induced changes in cold tolerance of somatic embryos have been noted (von Aderkas *et*
2 *al.*, 2007). A more spectacular example is brought on by temperature treatments applied
3 during both zygotic and somatic embryogenesis, the effects of which result in permanent
4 alteration of bud phenology of mature trees (Skroppa *et al.*, 2007). In spite of such
5 powerful effects, abiotic factors are not commonly studied experimentally *in vitro*. In
6 particular, the effect of light is often overlooked.

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

21 We were interested whether light had any effect on somatic embryo anatomy or
22 biochemistry. There are grounds for investigating light's effects on maturation, e.g. *Lilium*

1 somatic embryos grown in light were more numerous and larger than those grown in the
2 dark (Lian *et al.*, 2006).

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

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

MATERIALS AND METHODS

Plant material:

Experiments were conducted with one embryogenic line (69-18) of hybrid larch *Larix x marschlinisii* obtained in 1992 through secondary somatic embryogenesis (Lelu *et al.*, 1994b). Proliferation medium consisted in basal MSG medium (Becwar *et al.*, 1990) containing 1.45 gL⁻¹ glutamine (SIGMA) supplemented with 9 µM 2,4-dichlorophenoxyacetic acid, 2.3 µM 6-benzyladenine and 60 mM sucrose, solidified with 4 gL⁻¹ gellan gum (PhytigelTM, SIGMA). Embryonal mass (EM) was placed on proliferation medium for one week in darkness at 25 °C (Lelu *et al.*, 1994a). Immature cones of hybrid larch, obtained after controlled cross were collected in Orléans, France. Zygotic embryos dissected from the surrounding megagametophyte, were collected at different stages of development from early stage of late embryogeny (end of May) to 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 embryo, megagametophyte) 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 embryonal masses were incubated for one week on PGR-free medium supplemented with activated charcoal (10 gL⁻¹) and 100 mM sucrose. Petri dishes were placed under cool-white light (Philips) at a photon fluence 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

1 indolebutyric acid, 60 μM *cis-trans* (\pm) abscisic acid (ABA) for a period of seven weeks.
2 Light intensity for this 7-wk period was increased to 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Cotyledonary
3 somatic embryos were counted at the end of the culture period. In addition, embryogenic
4 potential was estimated, i.e. the number of somatic embryos per g fresh weight (FW). To
5 test the effect of light on somatic embryo maturation a set of embryonal masses (n=5) were
6 placed in either the light or dark for the entire maturation period. Experiments were
7 repeated three times. Cotyledons were counted from a minimum of 200 mature embryos
8 per treatment. Subsequently, somatic embryos were either fixed for later histological
9 investigation or they were frozen in liquid nitrogen for eventual biochemical analysis.

10 *Zygotic embryo germination:*

11 To test whether zygotic embryos produced phenolics in light prior to or during
12 germination, hybrid seed collected from trees in the breeding orchard located at INRA-
13 Orléans were dissected and embryos placed on MSG maturation medium supplemented
14 with 200 mM sucrose for 8 days. The control treatment – prevention of germination – was
15 MSG maturation medium supplemented with 200 mM sucrose and 60 μM ABA. Samples
16 (n = 33-48) were assessed for their colour at two-day intervals. A small number of
17 representative samples were fixed and included in the larger histological investigation
18 below.

19 *Histological analysis:*

20 Since the earliest stages of embryogenesis differ between zygotic and somatic (von
21 Aderkas et al., 1991), we focused on two more readily comparable stages that were later in
22 development, namely, 1. early embryos prior to histodifferentiation and 2. mature
23 embryos. Somatic embryos in dark and light treatments were morphologically very similar

1 to one another. Consequently, we have only shown sections of somatic embryos subjected
2 to the dark treatment.

3 Somatic and zygotic embryos were prepared according to Gutmann *et al.*, 1996.
4 Briefly, samples were fixed with 2.5 % glutaraldehyde in 100 mM phosphate buffer at pH
5 7.5 for at least 12 h at room temperature. After two washes with buffer, the samples were
6 dehydrated gradually in ethanol, infiltrated with glycol methacrylate (Historesin, Reichert-
7 Jung) at room temperature for at least 2 d and finally polymerized. Longitudinal sections
8 were cut on a Leitz 1400 microtome equipped with a tungsten carbide knife. Section
9 thickness was 2 μm , except for the following two staining procedures, where 5 μm
10 sections were cut in order to gain sufficient staining intensity. Flavanols (i.e. catechins)
11 were localized with the highly selective *p*-dimethylaminocinnamaldehyde (DMACA)
12 reagent as described by Gutmann and Feucht (1991). Moreover, the deposition of
13 proanthocyanidins ('condensed tannins') was traced using a developed *in situ* hydrolysis
14 procedure (Gutmann, 1993). Other staining methods used were described in detail by
15 Gutmann (1995): Toluidine blue O with sodium hypochlorite pretreatment (general tissue
16 structure), safranin O/azure II with iodine/potassium iodide post-staining treatment
17 (polyphenols, cell walls, starch), and safranin O with iodine/potassium iodide post-staining
18 treatment (general tissue structure and starch). In addition, a rapid two-step method with
19 Ponceau 2R and azure II was employed for a differential staining of storage protein and
20 cell walls. The following steps were required: 5-10 min staining with 0.5 % Ponceau 2R in
21 2 % acetic acid was followed by a rinse of distilled water, after which a 10 s stain with 0.5
22 % azure II in distilled water - followed by a distilled water rinse – completed the staining
23 procedure. Slides were dried with a brief blast of compressed air and allowed to dry in an

1 oven for 10 min at 50 °C. Sections were then mounted in Canada balsam. Cytoplasmic and
2 storage proteins stained red with Ponceau 2R (Gori, 1978): cell walls were blue.

3 *Material for biochemical analysis:*

4 Somatic embryos were sampled by stage of development following the protocol of
5 Guillaumot *et al.*, 2008. Samples were taken at time of transfer from charcoal medium to
6 maturation medium, as well as after further 1 and 7 weeks of culture. The 7-wk collection
7 included only mature cotyledonary embryos; any embryonal masses found in culture
8 dishes were excluded from analysis. To assay proteins, samples were weighed
9 immediately after harvest to determine FW. Five to seven samples ranging from 25 to 50
10 mg FW each were collected per developmental stage. To assay phenolic compounds,
11 samples were lyophilised and dry weight (DW) determined. Three samples (ranging from
12 20 to 49 mg DW) were collected per developmental stage.

13 *Total protein assay:*

14 Total protein extracts were prepared at least in quintuplet for each developmental
15 stage. Frozen embryos were homogenized with 0.5 mL of lysis buffer (10 % (v/v)
16 glycerol; 2 % (w/v) SDS; 5 % (v/v) β -mercapto-ethanol; 2 % (w/v) poly(vinyl)
17 polypyrrolidone; 50 mM Tris pH 6.8). Extracted samples were incubated for 5 min at 95
18 °C, and then centrifuged at 13500 rpm. Supernatant was transferred to tubes; pellets were
19 re-extracted with the same buffer minus both SDS and poly(vinyl) polypyrrolidone.
20 Supernatant was then pooled. Protein concentrations were determined using Bradford
21 assays in which BSA (Bovine Serum Albumin) was the standard.

1 *Protein separation:*

2 To determine subunit masses, denaturing gel electrophoresis (SDS-PAGE) was
3 performed according to standard protocols using 12-20 % polyacrylamide gradient gels
4 overlaid with a 4 % stacking gel. The gel was stained with Colloidal Coomassie Blue G-
5 250. Electrophoretic patterns were compared with protein markers, in particular,
6 phosphorylase-b (97 kD), serum albumin (66 kD), ovalbumin (45 kD), glyceraldehyde-3-
7 phosphate dehydrogenase (36 kD), trypsin inhibitor (20.1 kD) and lactalbumin (14.4 kD).

8 *Extraction of soluble polyphenols:*

9 Samples were ground in mortar using a glass rod in liquid nitrogen-filled 2 mL tube.
10 Soluble phenolic compounds were extracted twice from the dry powder in 2 mL
11 acetone/water (8:2, v/v) containing 10^{-4} M 6-methoxyflavone as internal standard. This
12 mixture was sonicated for 45 min then incubated while agitated for 1 h before being
13 centrifuged at 18000 g for 20 min. A 1 mL sample from the pooled supernatant was
14 removed and dried under vacuum using a Speed-Vac system (Savant Instrument, India).
15 The dry residue was diluted in 250 μ L of methanol. All steps were carried out at 4 °C.

16 *Total polyphenols quantification in embryo extract:*

17 Total polyphenols were estimated by Folin-Ciocalteu method modified according to
18 Boizot and Charpentier (2006). Phenolic extract (10 - 15 μ L) was diluted in 85 to 90 μ L of
19 ultra-pure water, 500 μ L of Folin-Ciocalteu reagent diluted 10 times in ultra-pure water,
20 and 400 μ L of NaCO_3 75 g L^{-1} . This mixture was incubated for 5 min at 40 °C. The
21 absorbance was measured spectrophotometrically (735 nm); results were expressed in mg
22 equivalent of gallic acid per g DW. Calibration was carried out using gallic acid methanol
23 solutions (0–20 μ g mL^{-1}).

1 *Chromatographic separation of phenolic compounds:*

2 Chromatographic analysis of phenolic compounds was performed according to a
3 previously published method (Faivre-Rampant *et al.*, 2002). Briefly, after centrifugation at
4 10,000 g for 3 min, a 15 μ L aliquot of the phenolic extract was separated, characterized
5 and quantified by HPLC on a 32 Karat system (Beckman Coulter, France) using a 250 x 4
6 mm Licrosphere 100RP-18e column (5 μ m) (Merck, Germany) stabilised at 40 °C; flow
7 rate was 1 mL min⁻¹. The following linear elution six-step gradient was used: i. initial
8 conditions – 15 % solvent B (methanol/acetonitrile, 50:50 v/v) in solvent A (1% acetic
9 acid in ultra-pure water); ii. 0-20 min - 15% to 40% B; iii. 20–25 min 40% to 60% B; iv.
10 25–30 min - 60% to 100% B; v. 30–35 min 100% B; vi. 35-38 min: 100% to 15% B.
11 Compounds were characterised by their retention time and UV absorption spectrum (diode
12 array: 230-430 nm). Quercitrin (QUER) was identified by co-chromatography with a
13 standard (Extrasynthese, France). Its quantitative determination was performed at 340 nm
14 with an external calibration (QUER methanol solutions; 5 points from 0.3 to 6.7 μ g) and
15 the results are expressed in μ g g⁻¹ DW. Quantitative determination of other phenolic
16 compounds was performed at 340 nm and 280 nm and was expressed in mg equivalent of
17 6-methoxyflavone per gram DW.

18 *Statistical analysis:*

19 One-way analysis of variance was performed with R (R Development Core Team,
20 2008). Multiple mean comparisons with confidence intervals for general linear hypotheses
21 in parametric models were obtained by the use of Multcomp R library (Hothorn *et al.*,
22 2008).

23

RESULTS

Effect of light on number of mature somatic embryos:

There was no significant effect of light on the numbers of embryos that are able to mature. Light treatments have an average of 384 ± 40 somatic embryos g^{-1} FW compared to 316 ± 53 somatic embryos g^{-1} FW in the dark ($p=0.03197$). 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 $p=0.05$ level. Light treatments had an average of 6.21 ± 1.06 cotyledons/embryo in the light compared to 6.31 ± 0.86 cotyledons/embryo in the dark. A slightly significant batch difference was noted ($p=0.0466$). 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 $60 \mu\text{M}$ ABA that prevents germination, embryos developed a slight red, i.e. reddish in Table 1, colouration in their embryonal root caps by six days (Table 1; Fig. 1C), by which time cotyledons and hypocotyls were beginning to turn green.

Phenolic analysis:

Phenolics were absent in zygotic embryos, but found in somatic embryos. The concentrations in somatic embryos varied over the course of development. At maturity, light-treated somatic embryos in the light treatment had a significantly higher concentration of phenolics than embryos in the dark treatment. In somatic embryos, one

1 flavonoid, quercetrin, was only found in light-treated somatic embryos (Table 2). We did
2 not measure quercetrin in zygotic embryos.

3 *Protein analysis:*

4 Dark-treated immature embryos had equivalent quantity of protein than light-
5 treated ones (Table 2). By the last stage of development (7 wk), dark-treated mature
6 somatic embryos had approximately 50 % more protein than light-treated somatic embryos
7 or zygotic embryos. The difference in total proteins did not correspond to a qualitative
8 difference between the types of mature embryo since they showed the same protein
9 profiles (Fig. 2). The major bands corresponding to the storage proteins observed in the
10 megagametophyte were absent in both types of early somatic embryos.

11 *Anatomy of early embryos prior to histodifferentiation:*

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

19 *Anatomy of mature embryos:*

20 Mature zygotic and somatic embryos had fully developed cotyledons, ground tissues
21 and organs (Figs. 3B, 4B). However, the types of embryogenesis resulted in differences in
22 proportions, with somatic embryos being both shorter and squatter than zygotic ones.
23 Starch was found in all tissues, e.g. embryonal root cap (Figs. 3C, 4C) and hypocotyl (Fig.

1 3D). In larch seed, numerous protein bodies were found in both megagametophyte and
2 embryo (Figs. 3E, 4D). Embryos had protein bodies throughout their cotyledons and
3 hypocotyls. Between cell layers in the hypocotyl ground tissue, idioblastic cells grew that
4 did not have any storage products (3B, 4B, 4E). Phenolic compounds were only found in
5 both light- and dark-treated somatic embryos. These compounds were mainly restricted to
6 the embryonal root cap (Fig. 4C). Two types of phenolic compounds, proanthocyanidins
7 (Fig. 4F) and catechins (Fig. 4G), were restricted to the periphery of the root cap. Embryos
8 had abundant protein bodies in their hypocotyl, cotyledon and embryonal root caps, in
9 particular, in the central zone or column. Protein bodies were not found in idioblastic cells,
10 suspensors, procambial tissues, shoot apical meristems, root apical meristems, as well as
11 the pericolumn of the embryonal root cap.

12 *Zygotic embryos germinating in light:*

13 When embryos were removed from seed and placed on ABA-free medium in light,
14 they germinated quickly (Fig. 1D), and within a day had begun depositing phenolic
15 compounds. Embryos placed on ABA-supplemented medium did not germinate, and only
16 began to deposit phenolic compounds after four days in the light (Table 1). In both
17 treatments phenolic deposition occurred in outer cells of the embryonal root cap,
18 especially in the junction zone where root cap and hypocotyl meet (Figs. 5A, 5B, Table 1).
19 Germinated embryos also accumulated phenolics in their hypocotyl (Fig. 1D). ABA
20 delayed the development of red colour, as embryos not exposed to ABA were much deeper
21 red than those that had been exposed to 60 μ M ABA.

DISCUSSION

1
2 Protein accumulation and phenolic compound production are both influenced
3 during embryogenesis by light. Zygotic embryos that develop within megagametophytes in
4 near complete darkness of ovules centrally located in closed cones do not produce
5 phenolic compounds. By comparison, somatic embryos abundantly produce phenolic
6 compounds in light, as well as in the dark. Light also affects protein accumulation, which
7 is greater in dark-grown embryos, i.e. zygotic or somatic, than in light-grown somatic
8 embryos. These findings contribute new information to our understanding of the influence
9 of light during conifer embryogenesis.

10 There are probably other effects of light to be discovered, because in vitro effects of
11 light have been relatively unstudied. Recent reviews of somatic embryogenesis make no
12 mention of the effect of light (Nehra *et al.*, 2005; Elhiti and Stasolla, 2011). In
13 angiosperms, the effect of light on somatic embryo growth has been investigated with
14 respect to quality and light treatments, e.g. alternating red and far-red light (Park *et al.*,
15 2010). In conifers, experiments on the effect of light have not been carried out during
16 embryogenesis, but only on somatic-embryo-derived germinants and seedlings. Kvaalen
17 and Appelgren (1999) studied the effect of red light on germination of somatic embryos.
18 Högberg and coworkers (2001) showed that exposing emblings to continuous light was
19 detrimental to their growth. Our results on the effect of light on differential accumulation
20 of storage products are important, because they clearly pinpoint peculiarities unique to
21 somatic embryogenesis.

22 The influence of light can be general or specific within the embryo itself. As we
23 have shown, protein body formation occurs throughout the embryo, but phenolic

1 compounds accumulate most readily in somatic embryos mainly in the both parts of the
2 embryo, i.e. suspensor and embryonal root cap.

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

21 Protein accumulation is more complicated in conifer somatic embryogenesis. In
22 *Pinus pinaster*, supplementation of media with different maltose and polyethylene glycol
23 levels influenced both starch and protein body size and number (Tereso *et al.*, 2007)

1 between treatments and in comparison with zygotic embryogenesis. Although we found
2 measurable differences in protein storage, we did not see differences in the size of protein
3 bodies, nor in the protein profiles. In conifers, improvements of maturation protocols
4 resulted in somatic embryos accumulating similar amounts of storage products to those
5 found in mature zygotic embryos including *Pinus sylvestris*, *Pinus pinaster* and *Larix x*
6 *eurolepis* (Lelu-Walter *et al.*, 2008; Morel *et al.*, 2014; Teyssier *et al.*, 2014). The protein
7 profiles were also similar in both somatic and zygotic embryos. By comparison,
8 megagametophytes had much larger protein bodies than either type of embryo. Somatic
9 embryos of loblolly pine not only produce more protein overall than zygotic embryos, but
10 they differ in protein metabolism, e.g. the ratios of insoluble to soluble proteins differ
11 greatly between the types of embryos (Brownfield *et al.*, 2007). By comparison, zygotic
12 and somatic embryogenesis in palms differ not only in the amount of protein but in the
13 kinds of proteins that accumulate (Aberlenc-Bertossi *et al.*, 2008). Studies comparing
14 zygotic and somatic embryogenesis record so many differences (Jones and Rost, 1989;
15 Alemanno *et al.*, 1997; Kärkonen, 2000) that it is fair to conclude that they always differ.
16 The differences in protein content of light- and dark-grown somatic embryos may have an
17 effect on subsequent germination performance, but this requires further experimentation.

18 Some of the differences in the physiological responses between zygotic embryos and
19 embryos of conifers are due to hormones, in particular ABA and related compounds.
20 Somatic embryos are able to produce endogenous ABA (Kong and Yeung, 1995; Kong
21 and von Aderkas, 2007), but at insufficient levels to induce organ development. To
22 mature, developing embryos require a healthy dose of exogenously applied ABA. ABA
23 controls not only differentiation of organs, but acquisition of physiological traits

1 characteristic of mature somatic embryos, such as desiccation tolerance (Attree et al.,
2 1995). ABA concentrations are not the same in zygotic and somatic embryos. Somatic
3 embryos are grown on media with very high concentrations of ABA. As a result, somatic
4 embryos have internal concentrations of ABA that are orders of magnitude higher than
5 zygotic embryos (von Aderkas *et al.*, 2001). This may be peculiar to conifer somatic
6 embryogenic systems. In the angiosperm *Nothofagus*, exogenously applied ABA has no
7 such effect. Endogenous ABA concentrations even in the presence of exogenously applied
8 ABA are lower than ABA concentrations found in zygotic embryos (Riquelme *et al.*,
9 2009). Coming back to conifers, in *Larix* somatic embryos phenolic compound
10 metabolism is influenced by ABA. A previous study (Gutmann et al., 1996) showed that in
11 the absence of ABA, embryos at all stages of embryogenesis were red in colour. Mature
12 embryos accumulated phenolic compounds in surface cells of cotyledon, hypocotyl and
13 embryonal root cap. In the study reported here, mature zygotic embryos germinated in
14 light accumulated phenolic compounds rapidly, but mature embryos placed on a medium
15 supplemented with a concentration of ABA sufficient to inhibit germination accumulated
16 these phenolics much more slowly. These various effects of exogenous ABA allow us to
17 conclude that ABA regulates more than histodifferentiation and acquisition of late embryo
18 physiological characteristics such as protein storage and desiccation tolerance; ABA
19 appears to control aspects of phenol metabolism. This regulation is not simply on or off,
20 but involves some interaction between light and ABA. Quercetrin is a case in point. It is
21 only produced when ABA is supplied during maturation of somatic embryos that have
22 been grown in light. Quercetrin is not found in embryos grown on ABA in the dark. ABA
23 is also known to have long-term effects. In *Picea abies*, overexposure to ABA during

1 maturation of somatic embryos, i.e. maturation on ABA for overly long periods, is
2 responsible for poor seedling growth (Högberg *et al.*, 2001).

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

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

20 **FUNDING INFORMATION**

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FIGURE LEGENDS

1
2 Figure 1A–D. Somatic and zygotic embryo colouration. 1A – Hybrid larch, *Larix x*
3 *marschlinsii*, somatic embryos matured in light had red embryonal root caps. 1B – Those
4 matured in the dark had no colouration. 1C – Zygotic embryos that were dissected from
5 the ovule and placed on medium with 60 μ M ABA to prevent germination developed red
6 colouration in the embryonal root cap. 1D – Zygotic embryos placed on ABA-free
7 medium germinated producing an elongated red hypocotyl and root. Remnants of the very
8 red embryonal root cap are seen at the base of the hypocotyl. bar = 1 mm

9 Figure 2. SDS-PAGE total protein profile comparison in seed and somatic embryo of hybrid
10 larch. L1 and D1: light- and dark-treated early somatic embryos at 1 week on ABA
11 medium; L7 and D7: light- and dark-treated mature somatic embryos at 7 weeks on ABA
12 medium; ZE: mature zygotic embryos; Mg: megagametophyte. Protein size (kD) is
13 indicated to the left of the gel.

14 Figures 3A–E. Zygotic embryogenesis. 3A – early embryo prior to histodifferentiation; a rib
15 meristem (rm) and formed embryonal tube (et) cells. Embryonal mass cells (em) are
16 distinguished by their prominent nucleoli (ni) and active division. Numerous mitotic
17 figures (mf) can be seen (Toluidine Blue O stain). bar = 100 μ m. 3B – longisection of
18 mature zygotic embryo with cotyledons (c), shoot apical meristem (sam), root apical
19 meristem (ram), and embryonal root cap (erc). Ground tissues including procambium (pc),
20 protoderm (pd) and two idioblasts (i - arrows) (Toluidine Blue O stain). bar = 200 μ m. 3C
21 – pericolumn region of embryonal root cap with abundant starch (Lugol stain). bar = 100
22 μ m. 3D – hypocotyl cells stained for starch (Lugol stain). bar = 50 μ m. 3E – embryo (emb)
23 beside megagametophyte (m) stained for proteins (Ponceau S) and cell walls (Azur B):

1 megagametophyte cells have larger and more numerous protein bodies than embryo cells.
2 bar = 10 μm .

3 Figures 4A-F. Somatic embryogenesis in dark. 4A – early embryo prior to
4 histodifferentiation; embryonal mass (em) was subtended by a rib meristem (rm). The
5 suspensor (s) was composed of embryonal tube cells (et) in which abundant phenolic
6 compounds were deposited either in small vesicles or along the inner margins of large
7 vacuoles (Safranin O and Fast Green). bar = 100 μm . 4B – longisection of mature somatic
8 embryo with cotyledons (c), root (ram) and shoot (sam) apical meristems, an embryonal
9 root cap (erc) and ground tissues, such as procambium (pc) and protoderm (pd). Three
10 idioblasts (i) are indicated with arrows. (Ponceau S and Azur Blue). bar = 200 μm . 4C –
11 starch grains in the hypocotyl/embryonal root cap junction: root cap cells are rich in
12 phenols which stain yellow to orange with a combined Safranin/Lugol stain. bar = 50 μm .
13 4D - protein bodies are abundant throughout all tissues, e.g. hypocotyl cells (Ponceau S
14 and Azur Blue). bar = 10 μm . 4E – idioblast (i) stained with Ponceau S and Azur Blue. bar
15 = 30 μm . 4F – proanthocyanidins stained red after *in situ* hot sulfuric acid treatment are
16 restricted to the periphery of the embryonal root cap. bar = 50 μm . 4G – catechins stained
17 by DMACA. bar = 50 μm .

18 Figures 5. Germinating zygotic embryos in light on medium with or without 60 μM ABA. 5A
19 – Junction zone of hypocotyl with embryonal root cap of embryo germinating on medium
20 without ABA after two days of control treatment (0 μM ABA). Phenolic deposits are
21 indicated with arrows. Section stained with Toluidine Blue O. bar = 50 μm . 5B – Junction
22 zone of hypocotyl with embryonal root cap of embryo germinating on medium with 60

- 1 μM ABA after six days of treatment. Phenolic deposits are indicated with arrows. Section
- 2 stained with Toluidine Blue O. bar = 50 μm .

Table 1: Colour in dissected and mature zygotic embryos of hybrid larch (*Larix x marschlinsii*) cultured in light on medium supplemented with and without ABA (60 μ M)

ABA (μ M)	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	-	-

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 x marschlinsii*.

Samples	Culture Condition	Protein		Phenolic compounds	
		($\mu\text{g prot mg}^{-1}\text{FW}$) ⁱ		(mg eq.gallic acid.g ⁻¹ DW) ⁱⁱ	quercetrin ($\mu\text{g.g}^{-1}\text{DW}$) ⁱⁱ
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 ± 9.21 ⁱⁱⁱ
SE 1w charcoal	Darkness	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.

i- values are mean ± SD (n=5 for all, except n=7 for SE 1w charcoal). Significantly different means are indicated by different letters ($p=0.05$).

ii- values are mean ± SD (n=4 for all, except n=5 for SE 7w ABA light and n=3 for SE 7w ABA darkness). Significantly different means are indicated by different letters ($p=0.1$).

iii- significance test not applicable.

nd: not detectable.

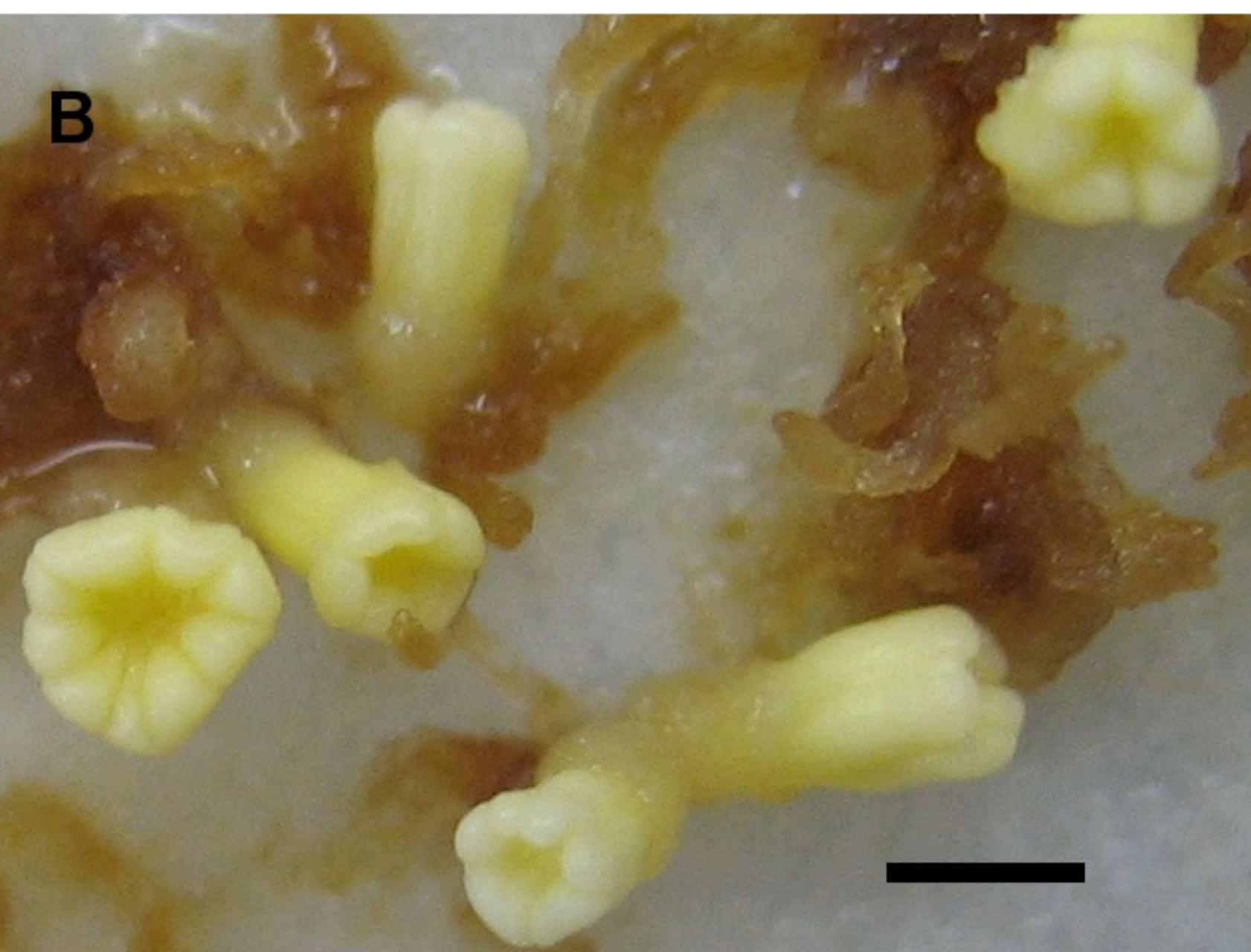


Figure 1

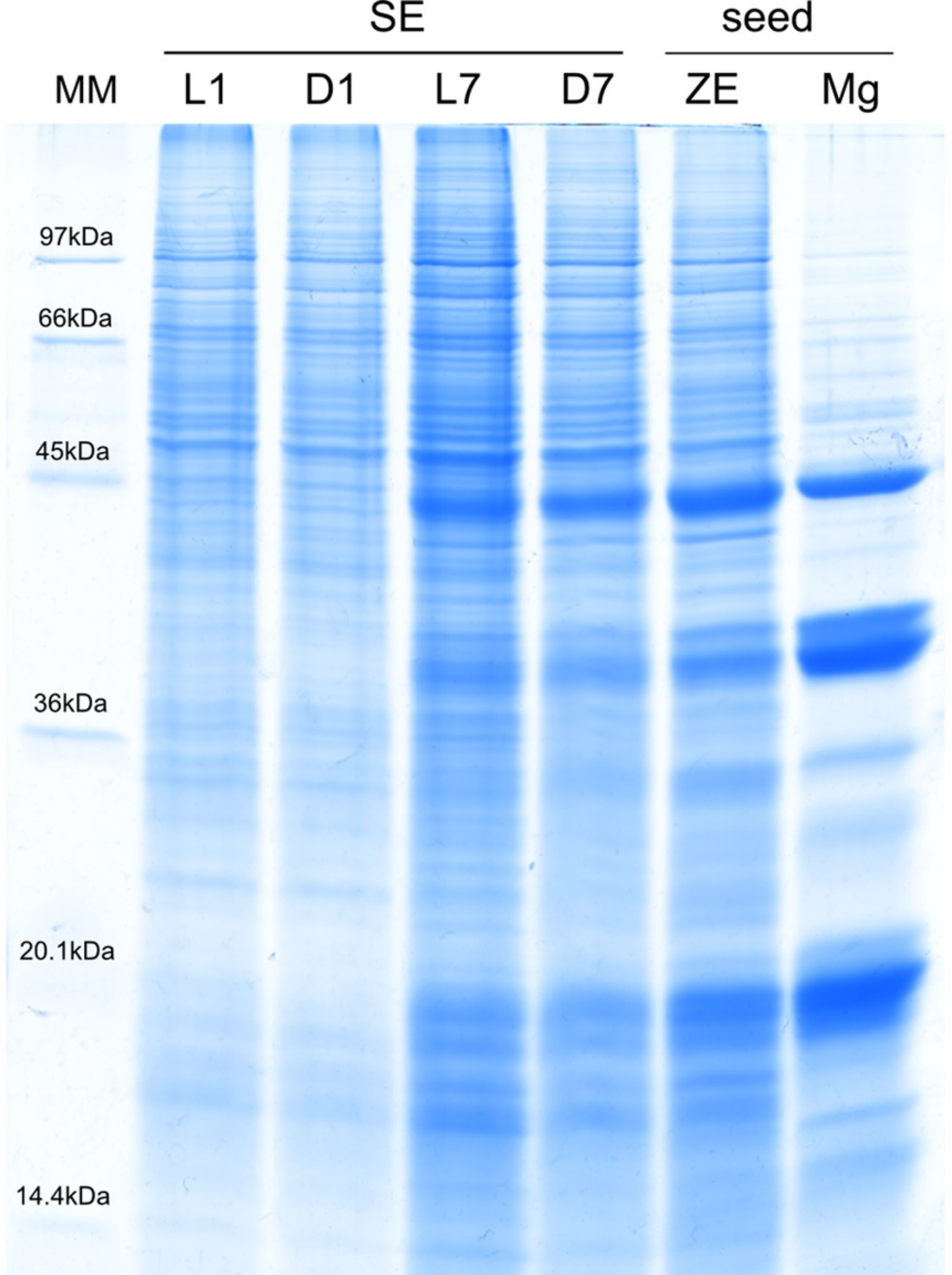


Figure 2

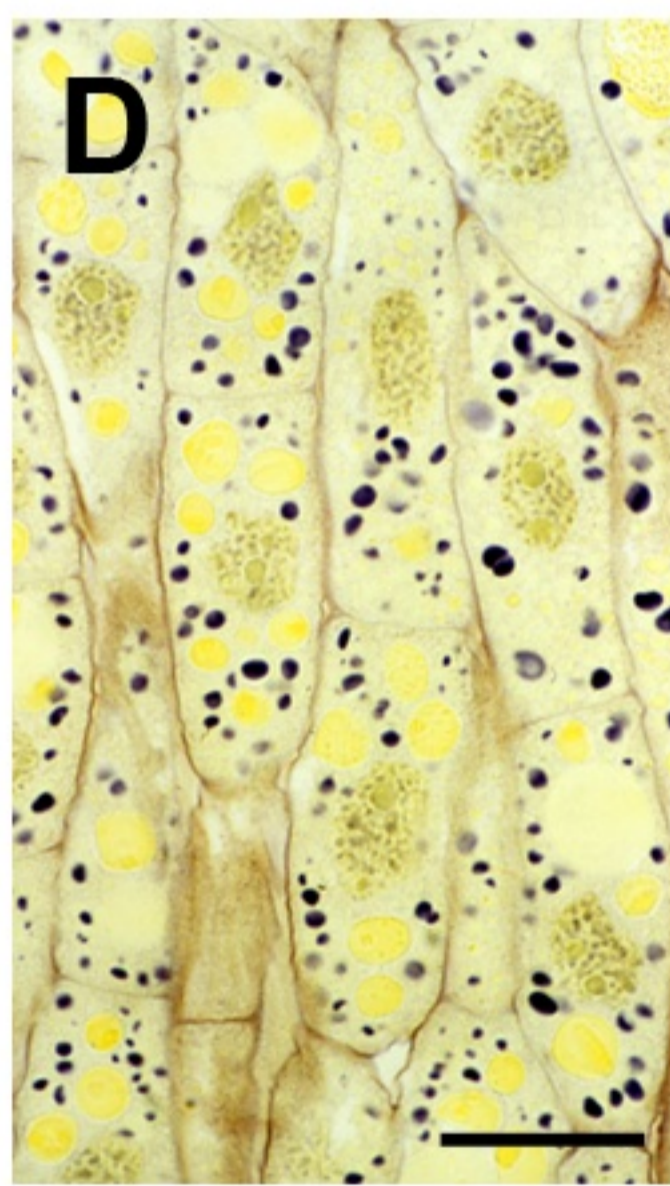
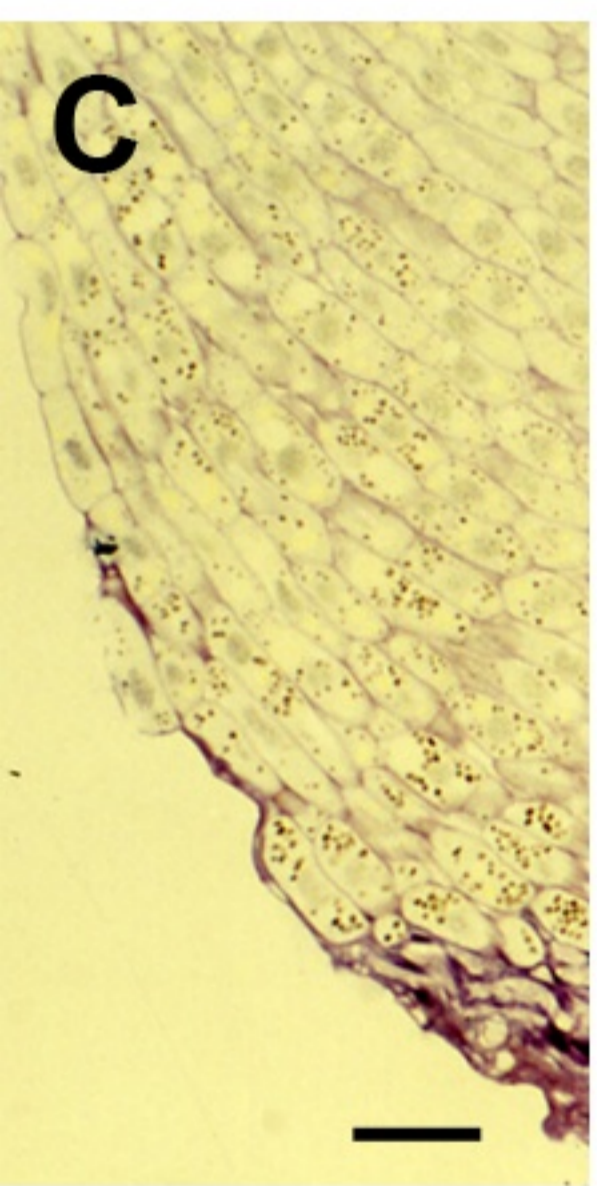
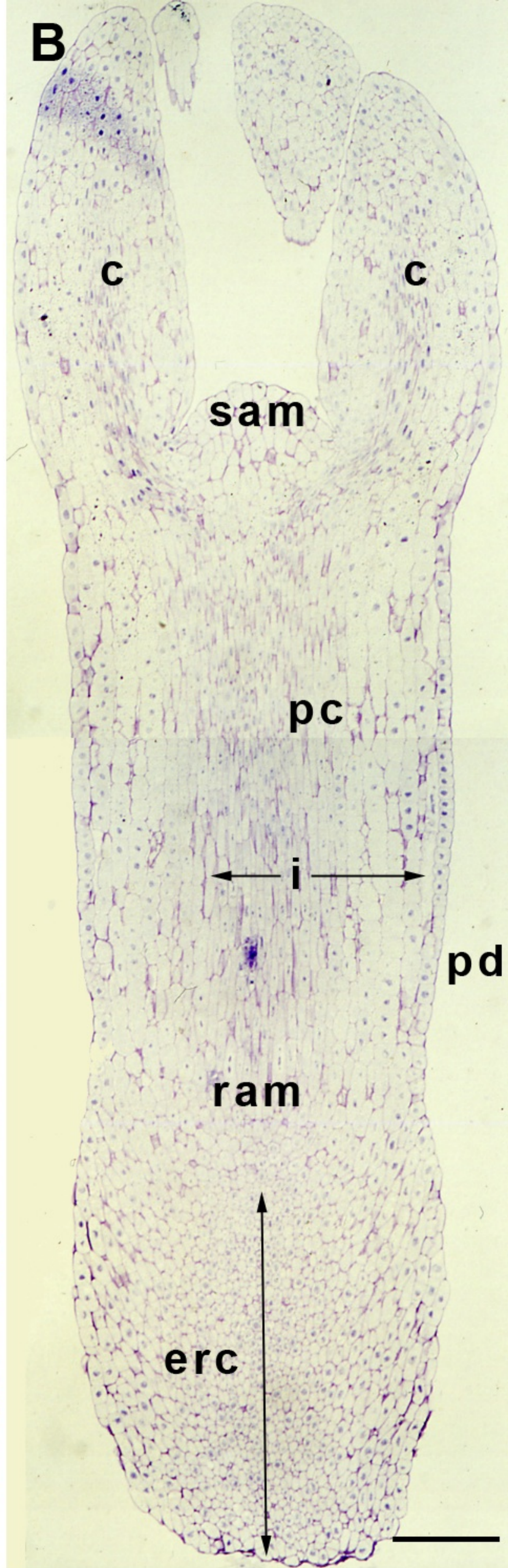
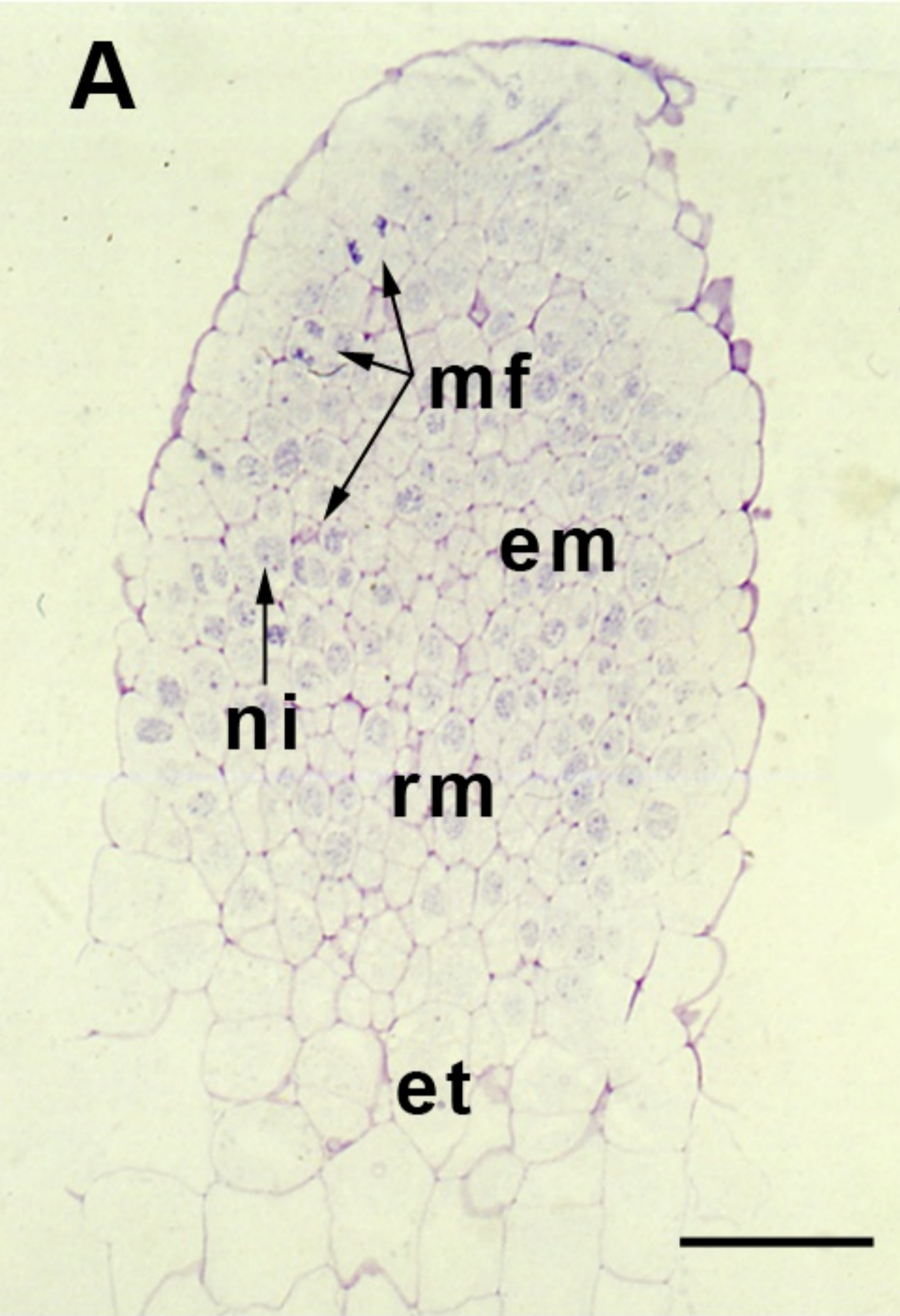


Figure 3

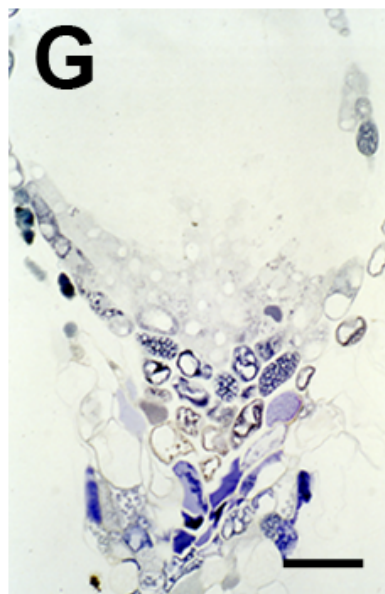
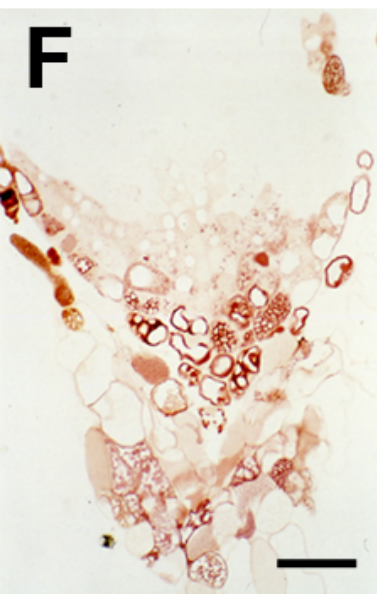
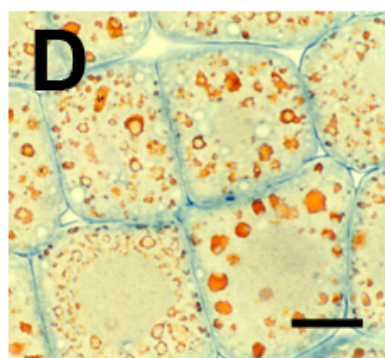
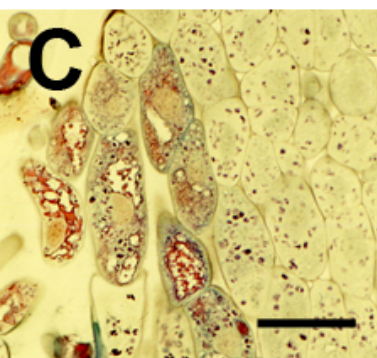
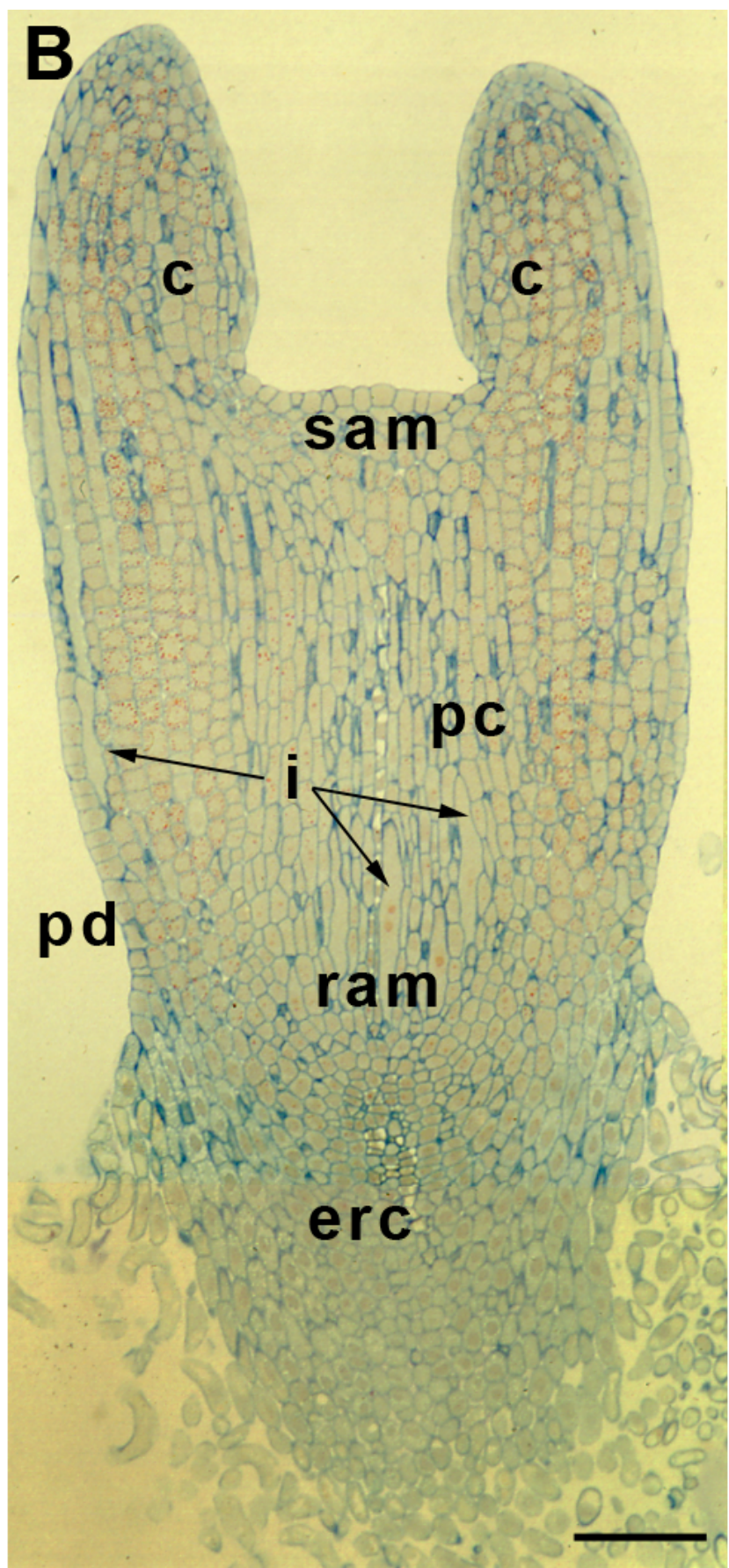
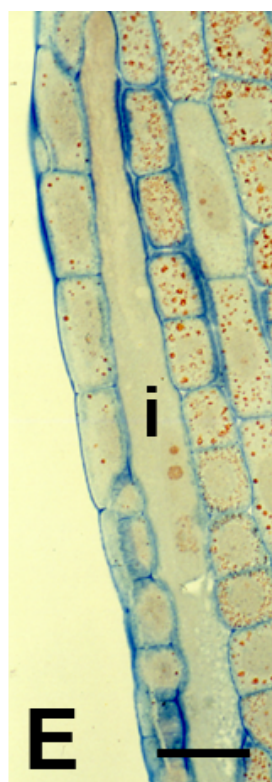
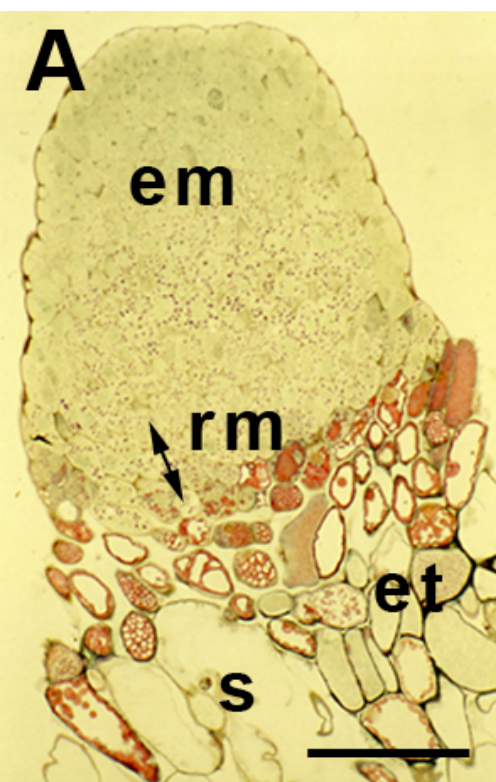
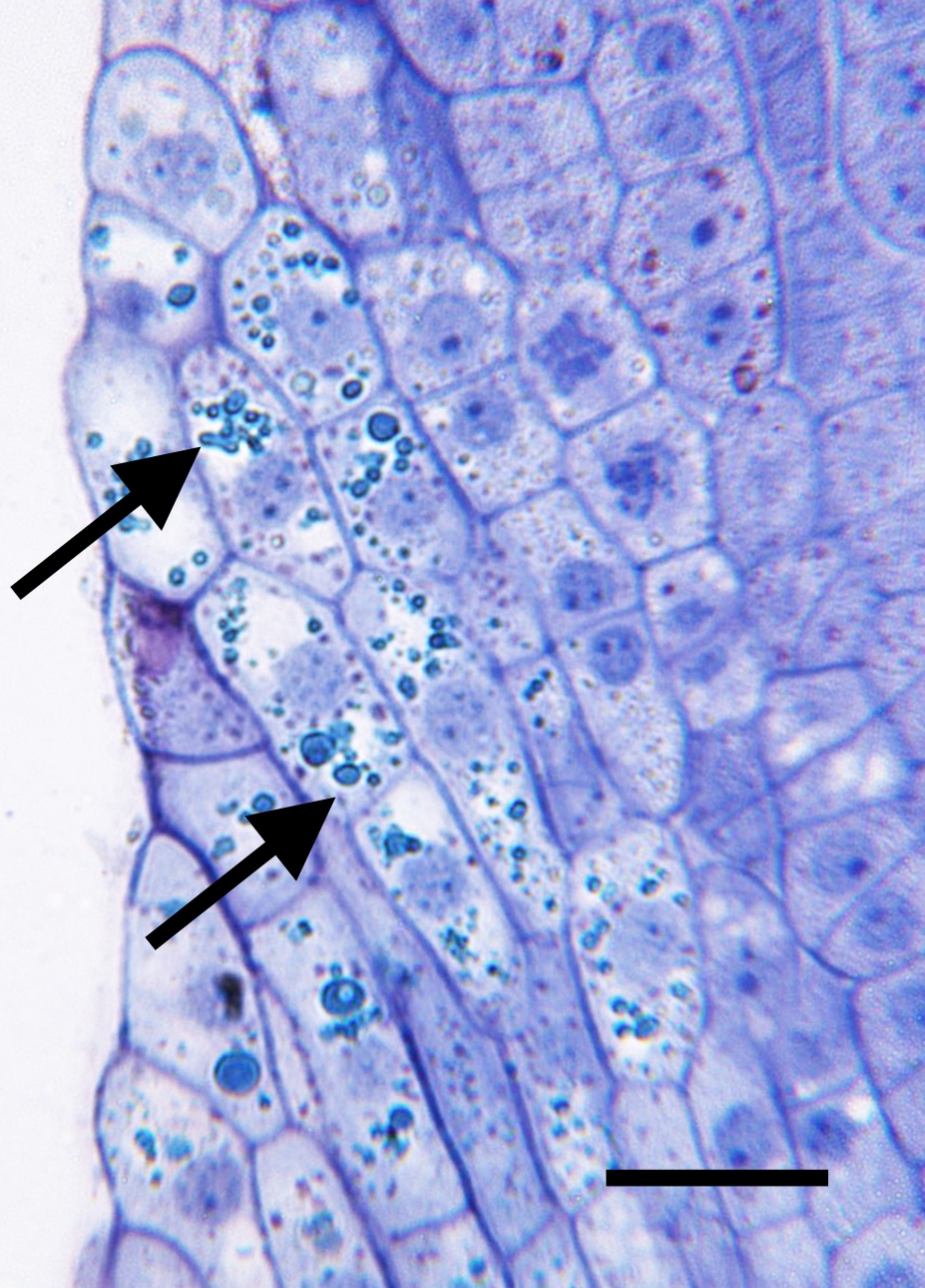


Figure 4

A



B

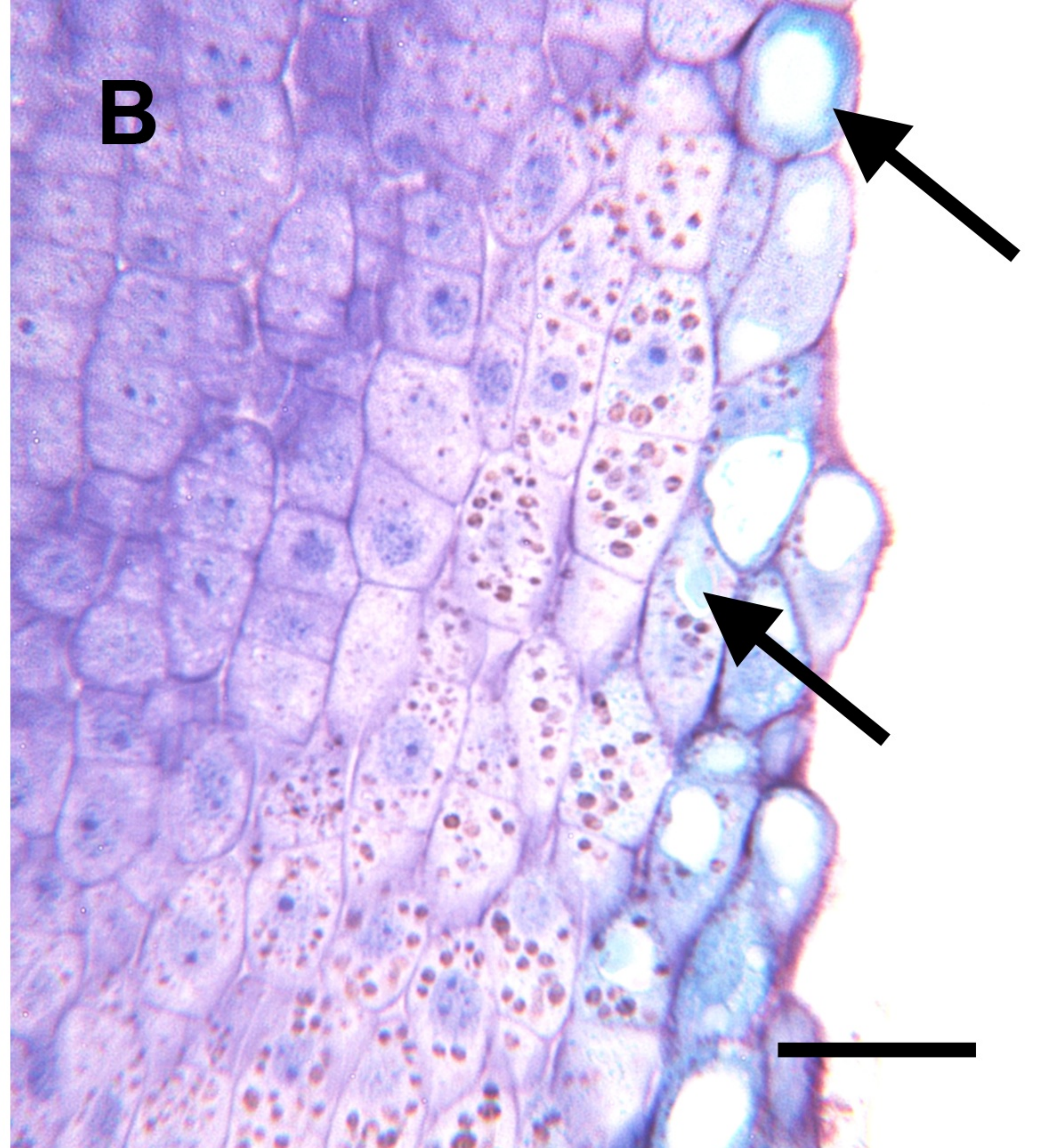


Figure 5