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Effect of Abscisic Acid, Osmoticum, and Desiccation on Synthesis of Storage Proteins during the Development of White Spruce Somatic Embryos

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The effect of abscisic acid (ABA), non-permeating osmoticum and desiccation treatment on storage protein synthesis during maturation of somatic embryos of *Picea glauca* (Moench) Voss. was examined. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis demonstrated that some of the major crystalloid and matrix polypeptides were absent from somatic embryos maturing on medium containing ABA and low osmoticum. However, treatment with polyethylene glycol-4000 (PEG) in combination with ABA resulted in the synthesis of a spectrum of storage polypeptides resembling that of mature zygotic embryos. These storage proteins accumulated throughout an 8-week culture period, resulting in a threefold higher protein content than somatic embryos maturing for the same time in the absence of PEG. The structure and distribution of protein bodies in cells of these osmotically treated somatic embryos was similar to that in cells of mature zygotic embryos. Treatment with 5·0–7·5% PEG prevented catabolism of the accumulated storage polypeptides during desiccation. The optimal culture conditions for somatic embryo maturation and storage protein deposition was 16 μM ABA and 7·5% PEG for 8 weeks followed by desiccation. Analysis of mRNAs by *in vitro* translation and immunoprecipitation of translated products showed that the crystalloid protein mRNA profiles of zygotic and those of somatic embryos maturing on 16 μM ABA in the absence of PEG were similar. The differences observed in the pattern of accumulated polypeptides in these somatic embryos and those of mature zygotic embryos, therefore, indicates that storage-protein synthesis in response to osmoticum is in part regulated at the translational level. During regeneration of somatic embryos to plantlets the storage polypeptides were rapidly utilized in a manner similar to that in zygotic seedlings.

Key words: Desiccation, osmotic stress, storage proteins, *Picea*, embryogenesis—somatic, mRNA (crystalloid protein).

INTRODUCTION

The development of techniques for somatic embryogenesis in conifers has led to rapid advances in the ability to culture conifer tissues *in vitro* (see Attree and Fowke, 1991). Somatic embryogenesis now provides a means to clonally propagate commercially valuable conifers of several species, including white spruce (see Attree, Dunstan and Fowke, 1991*a*), and offers an *in vitro* experimental system for studying embryo development. A number of laboratories have reported differentiation and development of conifer somatic embryos on abscisic-acid (ABA) containing media (Attree *et al.*, 1990, 1991*b*; Hakman *et al.*, 1990; Joy *et al.*, 1991; Flinn, Roberts and Taylor, 1991). In the absence of ABA, the development of somatic embryos is very limited.

Abscisic acid and water stress are important for maintaining embryos of angiosperms in a maturation state, and embryo drying occurs naturally during seed development (for a recent review see Kermode, 1990). Recently, Attree *et al.* (1991*b*) found that a non-plasmolysing moisture stress effected by 5·0–7·5% polyethylene glycol (PEG), when included with ABA in the maturation protocol, yielded white-spruce somatic embryos that closely resembled zygotic embryos in terms of low moisture content, high degree of quiescence and ability to survive desiccation to 30% moisture content. In addition, PEG caused a three-fold increase in the number of somatic embryos that underwent maturation, and increased the dry weights of the somatic embryos, indicating increased storage reserve accumulation. Plasmolysing osmotica such as sucrose were detrimental to maturation. Thus, the combination of ABA and non-plasmolysing osmoticum was an effective treatment for maturation of conifer somatic embryos. Desiccation was essential for promoting plantlet regeneration following maturation with high osmoticum.

Both ABA and osmoticum are known to promote synthesis of a number of proteins in developing embryos (reviewed by Skriver and Mundy, 1990), including storage proteins. Barratt and Clark (1989) observed a cumulative effect of ABA and osmoticum in promoting maturation of pea embryos. Recent reports indicate that the effect of osmoticum is not mediated through a rise in endogenous levels of ABA and that each treatment imparts a distinct metabolic response on the developing embryo (Goffner, This and Delseny, 1990; Xu, Coulter and Bewley, 1990; Rivin and Grudt, 1991). For example, Xu *et al.* (1990) showed that both ABA and high osmotic potential prevented precocious germination of developing alfalfa embryos in culture; however, only the osmoticum promoted the maintenance of a protein pattern which was typical of

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developing embryos *in situ*. In maize, a high level of ABA and the product of the viviparous-1 gene were both required early in the maturation phase for the accumulation of storage globulin and for germination suppression (Rivin and Grudt, 1991). However, suppressing precocious germination with a high osmoticum was not sufficient to initiate globulin-1 protein synthesis, although continued accumulation was contingent upon this inhibition.

The storage proteins of seeds are localized within protein bodies (Bewley and Black, 1985). In white spruce, these protein bodies are composed of a buffer-soluble matrix in which water-insoluble crystalloids and globoids are frequently found (Misra and Green, 1990). The latter study showed that crystalloids are the major storage protein of white spruce zygotic seed. Synthesis of these proteins is developmentally regulated with maximal synthesis occurring between early-to-late cotyledonary stages (Misra and Green, 1991).

Several workers have followed the accumulation of storage products in maturing somatic embryos of conifers (Feirer, Conkey and Verhagen, 1989; Hakman *et al.*, 1990; Flinn *et al.*, 1991; Joy *et al.*, 1991; Attree, Pomeroy and Fowke, 1992). In *Picea abies*, SDS–PAGE analysis showed that protein composition of somatic embryos maturing on media containing just 7·5 μM ABA was very similar to that of zygotic embryos (Hakman *et al.*, 1990). In interior spruce (*P. glauca*, *P. engelmannii* an intermediate hybrid), a high level of ABA (40 μM) was required to promote storage-protein synthesis typical of zygotic embryos and to suppress precocious germination under low osmotic conditions (Flinn *et al.*, 1991).

The effects of a non-plasmolysing osmoticum and desiccation on protein synthesis in maturing conifer somatic embryos have not been studied. The aim of the present study, therefore, was to observe the effect of ABA, PEG, and desiccation treatment on synthesis and deposition of storage protein (a marker of embryo maturation) during maturation of white spruce somatic embryos.

MATERIALS AND METHODS

Culture media

The medium used for maturation of white-spruce suspension cultured somatic embryos (line WSI; Attree *et al.*, 1991*b*) was the basal medium (BM) of von Arnold and Eriksson (1981). The BM was used at half-strength and the maturation medium contained 90 mM sucrose and 16 μM ABA (product No. A 2784; Sigma Chemical Co.) and 0·8% agar (Difco Bitek, Detroit, MI, USA). During maturation all cultures were maintained in the dark. During plantlet regeneration cultures were maintained at low light intensity (2 Wm^{-2}, 12 h photoperiod, 20 W cool-white fluorescent lamps). Plantlet regeneration medium consisted of half-strength BM but with 60 mM sucrose, 0·6% agar and no plant growth regulators. The above media were adjusted to pH 5·7. Petri-dishes (10 cm diameter) containing 15–20 ml medium were sealed with Parafilm (American Can Co., Greenwich, CT, USA) and cultures were incubated at 25°C.

Maturation of somatic embryos

Somatic embryos were matured following methods developed by Attree *et al.* (1991*b*, 1992). To observe the effects of PEG-4000 Fluka (AG, Ronkonkoma, NY, USA) on storage protein synthesis by the somatic embryos the following concentrations were included with the ABA in the maturation medium: 0, 2·5, 5·0, 7·5, and 10% (w/v). Somatic embryos were maintained on these media for 4 weeks prior to protein analysis.

To test the effect of culture time on storage protein synthesis, somatic embryos were maintained on maturation medium which contained ABA and 7·5% PEG for 2, 4 and 8 weeks prior to protein analysis. The protein contents were also compared with immature embryos from suspension culture.

To observe the effects of embryo desiccation on storage proteins, somatic embryos were subjected to a mild drying treatment. Somatic embryos were first matured for 4 weeks on maturation medium containing 0 or 7·5% PEG and ABA. Drying was then accomplished by transferring these somatic embryos on filter-paper supports (Whatman no. 2, Maidstone, UK) to unsealed Petri-dishes in an environment of 81% relative humidity (RH) maintained by a saturated solution of $(NH_4)_2SO_4$ as described previously (Attree *et al.*, 1991*b*). Total desiccation treatment time was 2 weeks. Embryos were then analysed for crystalloid proteins. Embryos were compared to non-desiccated controls matured under the same conditions.

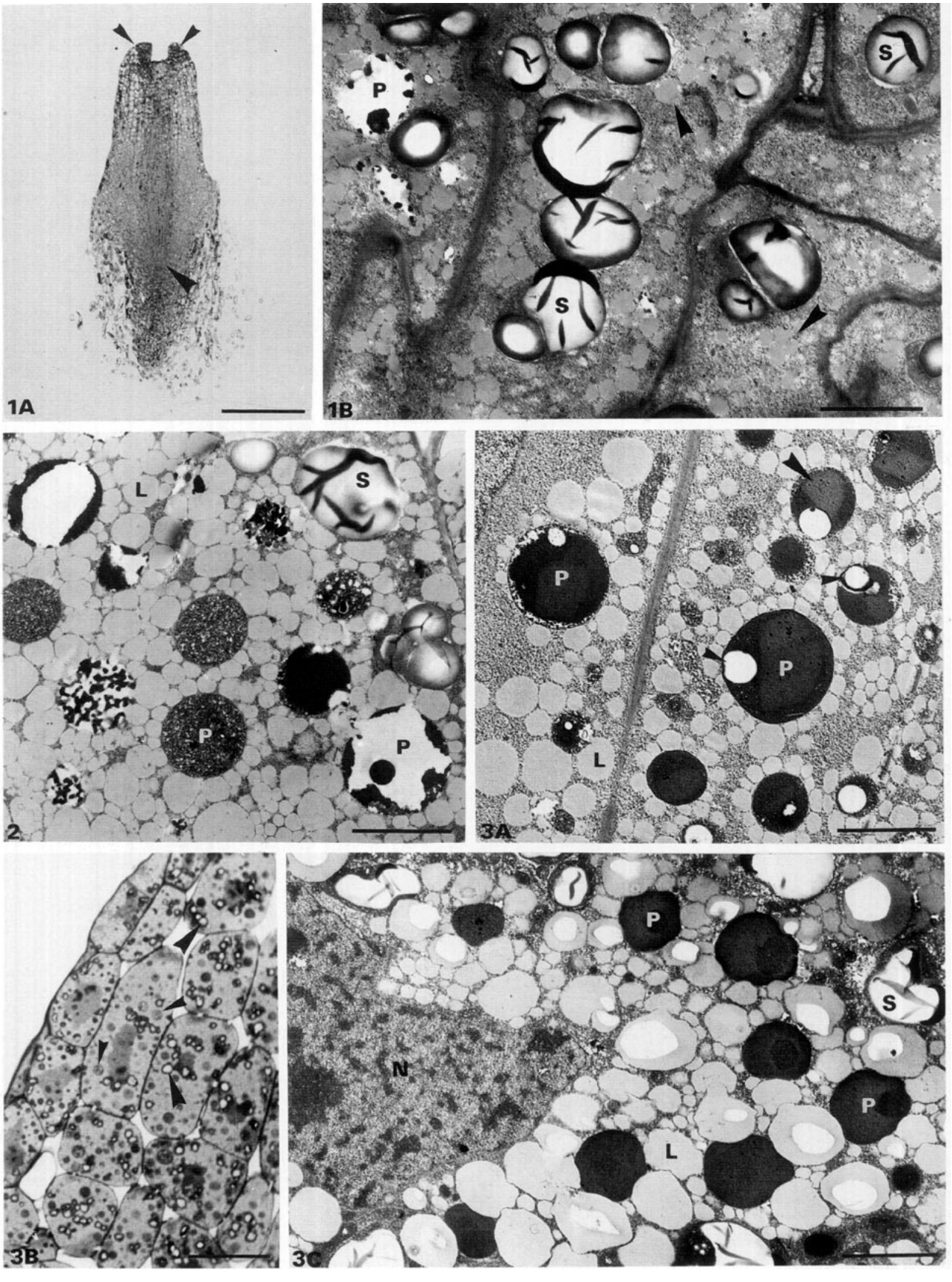
Plantlet regeneration

Non-desiccated somatic embryos from 0% PEG treatments were separated individually from the cultures directly following the maturation treatments. They were placed

FIG. 1. A, Light micrograph of an early cotyledonary stage somatic embryo after 4 weeks on 16 μM ABA (no PEG), showing young cotyledons (small arrows) and root meristem region (large arrow). Bar = 200 μm. B, Electron micrograph showing numerous starch granules (S) and lipid bodies (arrows) and few immature protein bodies (P) in root meristem region cells of a cotyledonary stage somatic embryo after 4 weeks on 16 μM ABA (no PEG). Bar = 2 μm.

FIG. 2. Electron micrograph showing an increased level (compare Fig. 3B) of protein bodies (P) and lipid bodies (L) in a cell of root meristem region of a cotyledonary stage embryo after 4 weeks on 16 μM ABA plus 7·5% PEG. S, Starch. Bar = 3 μm.

FIG. 3. A, Electron micrograph showing mature protein bodies (P) and lipid bodies (L) in the root meristem region of a cotyledonary stage somatic embryo after 8 weeks on 16 μM ABA and 7·5% PEG. Note that the protein bodies contain distinct protein crystalloids (large arrow), globoids (small arrows) and are more densely packed with protein than observed in the root meristem region of 4 week embryos (compare Figs 3 and 4). Bar = 3 μm. B, Light micrograph showing storage product accumulation in cotyledonary cells of a somatic embryo after 8 weeks on 16 μM ABA and 7·5% PEG. Both protein bodies (small arrows) and starch granules (large arrows) are recognizable. Bar = 40 μm. C, Electron micrograph of cell in same cotyledon shown in Fig. 3B. The cell is packed with lipid bodies (L) and contains numerous mature dense protein bodies (P) as well as some starch (S). N, Nucleus. Bar = 3 μm.



FIGS 1–3. For legend see facing page.

horizontally on fresh plantlet regeneration medium. PEG-treated desiccated somatic embryos were gently imbibed on the filter paper supports moistened with liquid plantlet regeneration medium as described by Attree *et al.* (1992). Imbibed desiccated somatic embryos were transferred individually to fresh plantlet regeneration medium after 1 week.

Protein analysis

The following developmental stages of somatic embryos were identified and their proteins analysed and compared to zygotic embryos dissected from mature seeds: (*a*) immature somatic embryos from suspension culture; (*b*) early globular (1–2 weeks on maturation medium); (*c*) late globular (1–3 weeks on maturation medium); (*d*) early cotyledonary (4 weeks on maturation medium); (*e*) late cotyledonary (6–8 weeks on maturation medium); and (*f*) regenerated plantlets (somatic embryos after 1, 2, or 4 weeks on plantlet regeneration medium).

Protein extractions and sodium dodecyl sulphate–polyacrylamide gel electrophoresis

Protein extractions were carried out under non-reducing conditions at 4 °C. All extraction buffers contained the protease inhibitors 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma) and 10 μM leupeptin (Sigma). Fractionation of proteins into soluble matrix and insoluble crystalloids was carried out according to Misra and Green (1990). Briefly, soluble matrix proteins were extracted using 0·05 M sodium phosphate buffer (pH 7·5). Insoluble crystalloid proteins were solubilized by boiling the pellet for 5 min in 65 mM Tris (hydroxymethyl) aminomethane (Tris) buffer (pH 6·8), containing 2% SDS and 10% glycerol. Extractions were performed using a tissue weight to buffer volume ratio of 100 mg: 1 ml.

Electrophoresis of proteins was performed according to the method of Laemmli (1970) with minor modifications. Prior to electrophoresis, extracts of both soluble (matrix) and insoluble (crystalloid) proteins were boiled for 5 min in sample buffer [65 mM Tris (pH 6·8), 2% (w/v) SDS, 10% (w/v) glycerol] and when reducing conditions were required, 5% (v/v) β-mercaptoethanol. Electrophoresis was carried out using 'mini gels' (Bio-Rad mini Protean II gel apparatus, Bio-Rad, Ontario, Canada). Approximately 2·5 μg total protein was loaded per lane. After SDS–PAGE the gels were stained with Brilliant Blue G250 (Serva, Feinbiochemica, Heidelberg, Germany) according to the method of Weber and Osborne (1969). For quantitative determinations the Bradford (1976) protein assay was employed. The following molecular-weight (Mr) protein standards (Bio-Rad, Ont., Canada) were used: phosphorylase B, 97·4 kDa; bovine serum albumin, 66·2 kDa; ovalbumin, 42·7 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21·5 kDa; and lysozyme, 14·4 kDa.

Western blot (immunoblot) analysis of storage protein profiles

The electrophoretically purified SDS-dissociated non-reduced crystalloid protein complexes with Mr's of 43 and 57 kDa were collectively used as antigen. Polyclonal antibodies were prepared as described in Misra and Green (1991). For Western blot analysis polypeptides separated by SDS–PAGE were electrophoretically transferred to nitrocellulose membranes at 100 V for 1·1 h using a mini-Trans-Blot Cell (Bio-Rad, Ont., Canada). The membranes were blocked in 1% (w/v) bovine calf serum (BSA), 0·5% (v/v) Tween 20 in PBS, pH 0·4, for 1 h at room temperature (RT) or overnight at 4 °C with gentle shaking. Membranes were incubated with antiserum against the white spruce dissociated crystalloid protein complex for 2 h at RT. Antiserum was diluted 1:5000 in first antibody buffer (10% [v/v] BSA, 10% [v/v] glycerol, 1 M D-glucose, 0·5% [v/v] Tween 20 in PBS, pH 7·4). After washing 3 × 15 min with PBS/0·5% [v/v] Tween 20 the membranes were incubated for 1 h at RT with gentle shaking with goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (AP) (Tago Inc., Burlingame, CA, USA) using a 1:4000 dilution in blocking buffer. After washing (as above) the membranes were stained in the following manner: membranes were washed in AP buffer (100 mM Tris, pH 9·5, 100 mM NaCl, 50 mM $MgCl_2$) then stained with nitro-blue tetrazolium (0·33 mg ml^{-1}; Sigma, St. Louis, MO, USA) and 5-bromo-4-chloro-3-indolyl-phosphate (0·165 mg ml^{-1}; Boehringer Mannheim, Mannheim, Germany) in AP buffer without shaking. Colour development was halted by washing the membranes in stop solution (20 mM Tris, pH 7·5, 5 mM EDTA) followed by running tap water.

RNA isolation

Total RNA was extracted from white spruce somatic zygotic embryos by a modification of the procedure of Verwoerd, Dekker and Hoekema (1989). After grinding 100 mg of frozen tissue, 0·5 ml of hot extraction buffer [Phenol-100 mM LiCl, 100 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS (1:1) at 80 °C] and 0·25 ml of chloroform-isoamyl alcohol (24:1) were added and vortexed. The aqueous phase was removed by centrifugation and mixed with one volume of 4 M LiCl. After precipitation overnight at 4 °C, the RNA pellet was collected by centrifugation then suspended in 0·25 ml of water, 0·1 volume of 3 M NaOAc pH 5·2 and the RNAs were precipitated with 2 volumes of cold 95% ethanol. Yields of between 30–50 μg of total RNA were routinely obtained from 100 mg of tissue.

In vitro *translation and immunoprecipitation*

Cell-free translation of 10 μg of total RNA as a source of mRNA was carried out in a rabbit reticulocyte lysate translation system (BRL, Gaithersberg, MD., USA). The reaction mixture contained [^{35}S]-methionine using the conditions given by the supplier. To measure incorporation of methionine or leucine into polypeptides 2 μl of the mixture was spotted onto filter-papers (Whatman, Maidstone, U.K.). Proteins were precipitated in cold TCA and the filters were dried. Protein samples with 20000 cpm were separated in one dimension on 12% SDS poly-

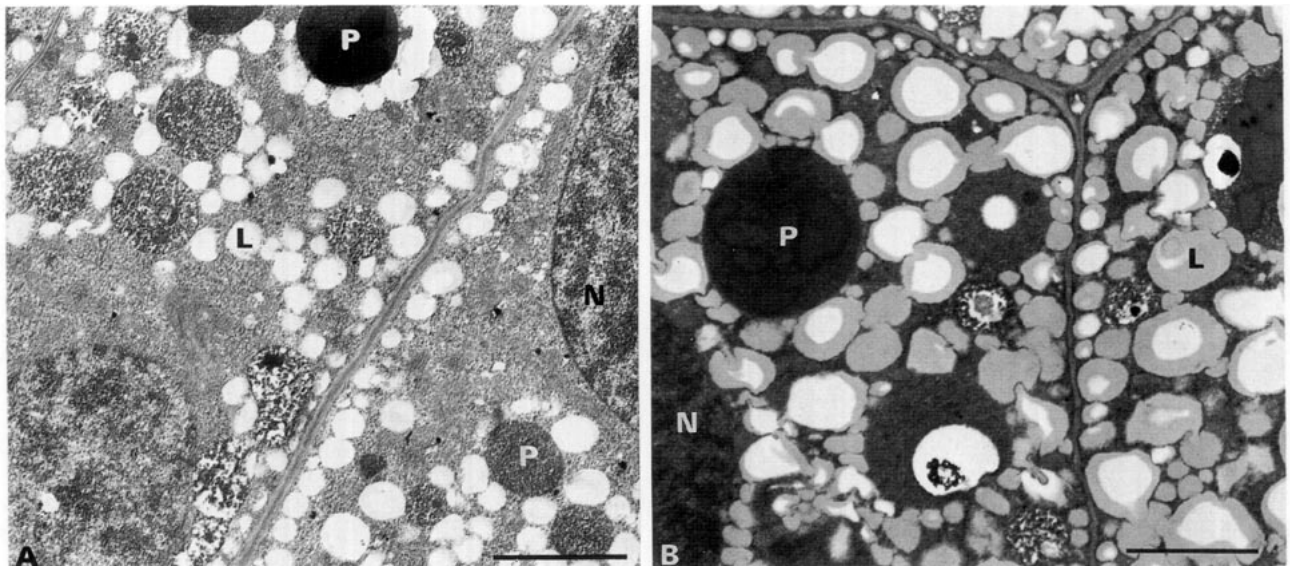


FIG. 4. A, Electron micrograph showing cell of root meristem region of zygotic embryo. Both loosely and tightly packed protein bodies (P) as well as lipid bodies (L) are present (compare Fig. 3). N, Nucleus. Bar = 3 μm. B, Electron micrograph showing a cotyledon cell of a mature zygotic embryo. The structure and distribution of protein bodies (P) and lipid bodies (L) are similar to those observed in cotyledons of somatic embryos after 8 weeks on ABA and PEG (compare Fig. 3C). N, Nucleus. Bar = 3 μm.

acrylamide gels and then visualised by fluorography and autoradiography.

The *in vitro* translation mixture was first precleared as described in Sambrook, Fritsch and Maniatis (1989) by pretreating the lysate with preimmune serum drawn from the same rabbit that was immunized against the 43 and 57 kDa white spruce crystalloid protein complexes (Misra and Green, 1990). After the preclearing treatment, the lysate samples were immunoprecipitated with anti-white spruce crystalloid protein complex antibodies essentially as described by the procedure of Howard and Buckley (1985). After boiling 50000 cpm of each lysate sample for 2 min in 0·1 ml of immunoprecipitation buffer (1% SDS, 50 mM Tris–HCl pH 8, 1 mM EDTA), each sample was diluted with 4 volumes of 1·25% Triton X-100, 190 mM NaCl, 60 mM Tris–HCl containing 0·1 mM PMSF, 1 mg BSA and 20 μl of a 10% (w/v) *Staphylococcus aureus* cell solution. After 1 h incubation at 4 °C, the samples were centrifuged for 15 min and the supernatants were transferred to new microfuge tubes. To each sample 2 μl of anti-white spruce crystalloid protein complex antibodies was added and immune complexes were allowed to form overnight. The immune complexes were then collected by centrifugation with a *S. aureus* cell solution [10% (w/v) previously incubated for 15 min in 30 mg ml^{-1} BSA in phosphate-buffered saline], washed, suspended in sample buffer (62·5 mM Tris, pH 6·8, 2% SDS, 10% glycerol, and 0·1 M DTT) and subjected to electrophoresis on a 12% SDS polyacrylamide gel. Gels were then subjected to fluorography and autoradiography.

Electron microscopy

Somatic and zygotic embryos were prepared for transmission electron microscopy (TEM) according to previously published methods (Fowke, 1984). Embryos were first cut longitudinally to ensure subsequent penetration of fixatives and resin. In addition to thin sections, thick sections (i.e. 1 μm) were cut from the plastic embedded material and stained with toluidine blue (1%, w/v, in 1% sodium tetraborate solution) for observations by light microscopy.

RESULTS

Microscopy

During maturation, immature white spruce somatic embryos increased in size and developed through globular to cotyledonary stages. Cotyledonary stage somatic embryos matured for 4 weeks on 16 μM ABA were characterized by distinct cotyledon primordia surrounding the shoot apical meristem (Fig. 1A). Cells in the hypocotyl and root meristem regions (Fig. 1B) contained numerous lipid bodies and considerable starch reserves, but very few protein bodies. The level of starch accumulation was distinctly higher than that observed in zygotic embryos (compare Fig. 4A).

Somatic embryos matured for 4 weeks in the presence of 7·5% PEG along with 16 μM ABA, in contrast contained considerably more lipid and protein bodies in their root meristem regions (Fig. 2) than somatic embryos matured without PEG, but again very few lipid and protein bodies were observed in the shoot apical meristem and cotyledonary regions.

Somatic embryos matured for 8 weeks with both PEG and ABA contained similar numbers of lipid and protein bodies in the root meristem region (Fig. 3A) compared to the 4 week treatment; however, considerably more of the protein bodies were densely packed with protein and many contained distinct protein crystalloids and globoids. The

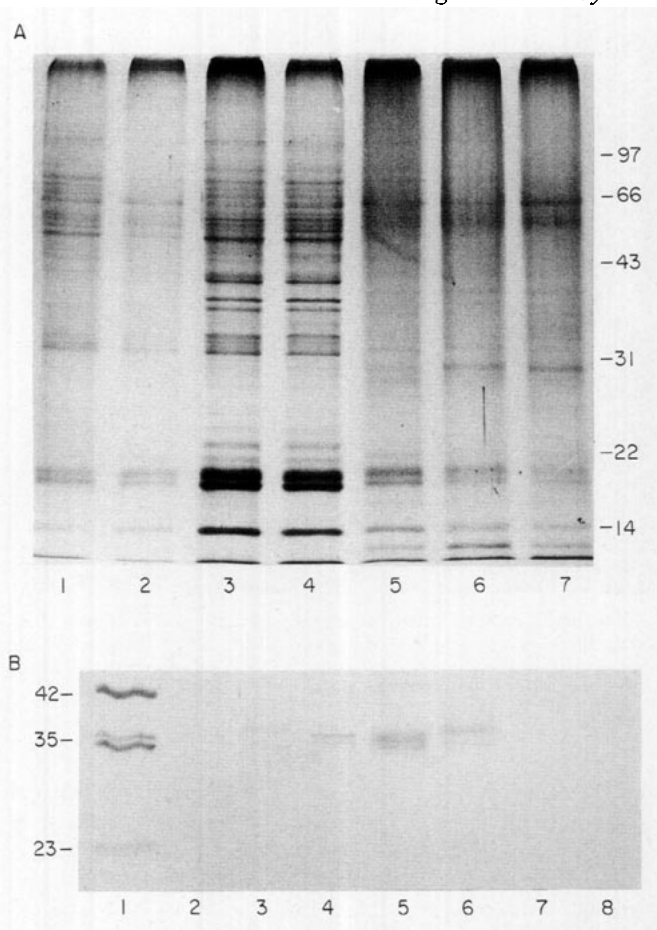


FIG. 5. SDS-PAGE and immunoblot analysis of proteins isolated from the somatic embryos developing for up to 4 weeks with 16 μM ABA and regenerating plantlets. A, Coomassie Blue stained SDS-PAGE profiles of proteins. Relative molecular weights in kDa are shown. Lane 1, immature somatic embryos from suspension culture; 2, early globular; 3, late globular; 4, early cotyledonary. Somatic embryos on a plantlet regeneration media (no ABA) for 1 week, lane 5; 2 weeks, lane 6 and 4 weeks, lane 7. B, Western blot of crystalloid proteins separated by SDS-PAGE, transferred to nitrocellulose membrane and processed with antibodies against white spruce crystalloid proteins from mature zygotic embryos. Lane 1, mature zygotic embryo; 2, immature somatic embryos; 3, early globular; 4, late globular; 5, early cotyledonary. Plantlets regenerated from somatic embryos after 1 week, lane 6; 2 weeks, lane 7 and 4 weeks, lane 8 culture on plantlet regeneration medium.

clearest difference noticed in the somatic embryos matured for 8 weeks with 7·5% PEG compared to other treatments was the presence of substantial lipid and protein reserves in the apical shoot region and throughout the cotyledons (Fig. 3B, C). The protein bodies in root meristem region and cotyledons of these somatic embryos were similar in structure and distribution to those in fully mature zygotic embryos (Fig. 3, compare Fig. 4).

SDS–PAGE and Western blot analysis of storage proteins in developing somatic embryos

ABA treatment. Figure 5A shows Coomassie Blue stained SDS-PAGE profiles of proteins in developing somatic embryos (lanes 1–4) and in regenerated plantlets (lanes 5–7). A number of new polypeptides were synthesized by somatic embryos after 3 (Fig. 5A, lane 3) and 4 weeks (lane 4) culture on 16 μM ABA (no PEG). Following transfer of 4-week-old somatic embryos to plantlet regeneration medium (Fig. 5A, lane 5), a number of the polypeptides ranging in molecular weights from 35–43 to 55–57 kDa disappeared within 1 week. The 18–20 and approx. 14 kDa polypeptides were reduced to lower levels in the plantlets (lanes 6–7). In the regenerated plantlets synthesis of some of the polypeptides (60–66 and 27 kDa) was increased.

The identity of the crystalloid polypeptides in the somatic embryos was confirmed by immunoblotting proteins from the mature zygotic embryos as well as the somatic embryo stages (Fig. 5B, lanes 1–8). The antibodies recognized a 42, 35, and 22·5 kDa range of polypeptides in extracts from mature zygotic embryos (lane 1). These polypeptides were not detected in immature somatic embryos from suspension culture (lane 2) and in 2 week matured (lane 3) somatic embryos. However, in 3 (lane 4) and in 4 week matured (lane 5) somatic embryos the antibodies recognized a 35 kDa range of polypeptides. In somatic embryos transferred to plantlet regeneration media (no ABA), the amount of these proteins declined after 1 week (lane 6) followed by complete disappearance in 2 week regenerated plantlets (lane 7). A comparison of crystalloid polypeptides from 4 week old somatic embryos (lane 5) with mature zygotic embryos (lane 1) revealed that polypeptides of 42, and 20–23 kDa were not detected in the somatic embryos cultured for up to 4 weeks on ABA containing media. This crystalloid protein profile of somatic embryos was similar to that of immature zygotic embryos corresponding to the globular-torpedo shaped stages (Misra and Green, 1991).

Effect of ABA and osmoticum on storage proteins. Figure 6 shows the effect of 4 weeks maturation with ABA and PEG osmoticum followed by subsequent desiccation (lanes 1–3; see below) and without subsequent desiccation (lanes 4–7) on crystalloid proteins. The Coomassie Blue stained SDS–PAGE gel shows that non-desiccated somatic embryos maturing on 2·5% PEG (Fig. 6A, lane 4); 5·0% PEG (lane 5); 7·5 PEG (lane 6) (all with 16 μM ABA) possessed similar protein profiles with prominent polypeptides of 41–42, 34–35, 24–25, and 18–20 kDa range. In 10% PEG (lane 7) accumulation of these polypeptides were decreased. Analysis of the protein levels accumulated in the somatic embryos revealed that embryos cultured with 7·5% PEG continued to accumulate proteins for at least 8 weeks, reaching a level of 4·3 μg mg fresh weight^{-1} (Table 1). This amount was threefold higher than the protein content of 8 week-old somatic embryos maturing on 16 μM ABA in the absence of PEG.

Western blot analysis of proteins from somatic embryos maturing on PEG containing media, which included 16 μM ABA (Fig. 6B), shows distinct differences from those of somatic embryos maturing in the absence of PEG (Fig. 5). Figure 6B shows that 2·5 to 7·5% PEG (lanes 4–6) induced synthesis and accumulation of 42, 35, 23, and 8 kDa range crystalloid polypeptides. In somatic embryos maturing without PEG (e.g. Fig. 5B) only 34–35 kDa range polypeptides were detected by Western blotting.

Figure 7 shows the effect of ABA and osmoticum followed

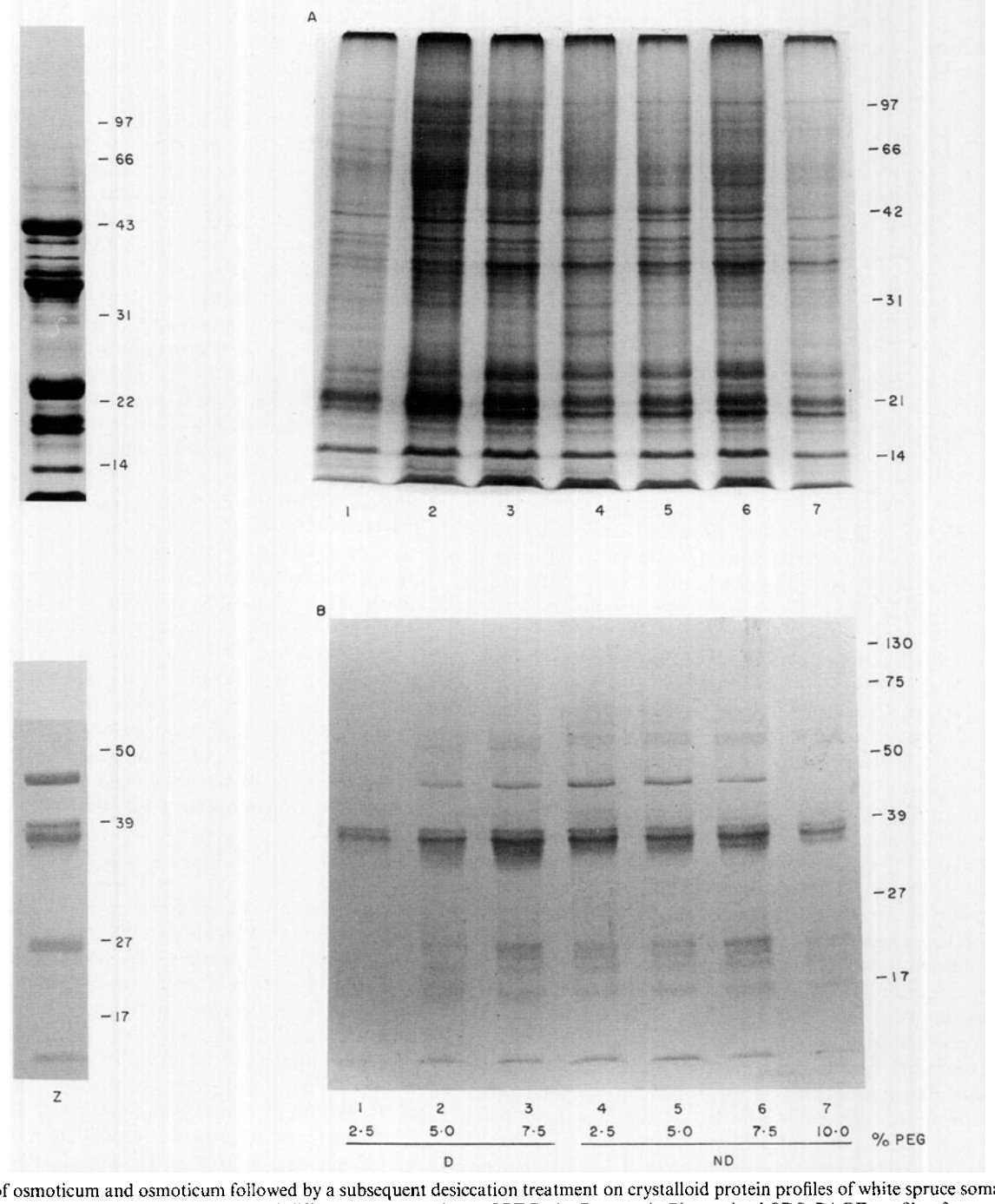


FIG. 6. Effect of osmoticum and osmoticum followed by a subsequent desiccation treatment on crystalloid protein profiles of white spruce somatic embryos matured for 4 weeks on 16 μM ABA and different concentrations of PEG. A, Coomassie Blue stained SDS–PAGE profile of proteins. Relative molecular weights in kilodaltons are shown. Lane 1, 2·5% PEG + 80% RH; 2, 5% PEG + 80% RH; 3, 7·5% PEG + 80% RH; 4, 2·5% PEG; 5, 5% PEG, 6, 7·5% PEG; 7, 10% PEG. Z, Proteins isolated form mature zygotic embryos. D, Desiccated; ND, non-desiccated. B, Corresponding Western blot of crystalloid proteins from somatic embryos. Labels as in Fig. 6A.

by subsequent desiccation (lanes 1–3; see below) and without subsequent desiccation (lanes 4–7) on matrix polypeptides. In the matrix protein fraction of mature zygotic embryos, three major polypeptides of 42, 27–30, and 18 kDa are evident (Fig. 7A, lane 8). Each of these groups of polypeptides can be identified in non-desiccated somatic embryos maturing for 4 weeks in the presence of 5·0% (lane 5), 7·5% (lane 6); and 10% PEG (lane 7). On the other hand, in the presence of 2·5% PEG, the 42 kDa polypeptide was not detected (Fig. 7A and B, lane 4). It would thus appear that accumulation of the 42 kDa polypeptide was enhanced by the PEG treatment. In addition to the 42 kDa polypeptide, synthesis of 32–35, 27–30, and 20 kDa polypeptides was also induced or levels increased in response to the PEG treatment (e.g. Fig. 7A, lanes 2, 3, 5, 6,7, compare with Fig. 5).

TABLE 1. *Quantitative analysis of storage proteins in non-desiccated white spruce somatic and zygotic embryos from maturing dry seed. Somatic embryos were matured for 4 or 8 weeks with 16 μM ABA and a range of PEG concentrations*

Treatment (% PEG)	μg protein mg tissue^{-1}*
4 weeks maturation	
0	1·07
2·5	1·05
5·0	2·61
7·5	2·20
10·0	1·34
8 weeks maturation	
7·5	4·30
0	1·46
zygotic embryo (mature)	7·2

* Values are means of triplicate assays on duplicate extractions. Total protein values are the sum of soluble and insoluble protein fractions.

Effect of desiccation. The crystalloid and matrix protein profiles of PEG-treated somatic embryos following desiccation are shown in Fig. 6A and B, (lanes 1–3); and Fig. 7A and B, (lanes 1–3), respectively. Results showed that desiccation of 2·5% PEG treated somatic embryos affected the accumulation of crystalloid polypeptides of 42, 23, and 8 kDa, Fig. 6B (lane 1) and the 42 kDa, Fig. 7B (lane 1) buffer soluble polypeptides, as these polypeptides were not detected in these treatments. Five percent PEG was partially effective in stabilizing the storage proteins as judged by the intensity of the immunoreactive bands (Fig. 6B, lane 2; Fig. 7B, lane 2); however, accumulated crystalloid polypeptides (Fig. 6B, lane 3) as well as the 42 kDa matrix protein (Fig. 7B, lane 3) were fully stable during a 2 week desiccation of 7·5% PEG treated somatic embryos.

Mobilization of storage proteins in desiccated somatic embryos. Crystalloids and the matrix storage proteins are mobilized following the germination of mature dry seed of white spruce (Misra and Green, 1990). To determine the function of soluble and insoluble reserves, protein profiles were examined during rehydration and plantlet formation in four week matured somatic embryos. Figure 8A, B shows that following rehydration of desiccated somatic embryos major soluble proteins in the matrix fractions (lanes M 1–3) as well as a number of insoluble proteins in the crystalloid fraction (lanes C 5–7) were mobilized. These proteins were absent from 2-week old plantlets (lanes 3 and 7). Immunoblot analysis of storage polypeptides confirmed these results. The 42 kDa matrix polypeptide (Fig. 8B, lane M 3) and 42, 35, 23, and 8 kDa range polypeptides in the crystalloid fraction (lane 7) were completely mobilized in 2-week-old plantlets. This is consistent with the storage function of these polypeptides in the somatic embryos.

Analysis of crystalloid protein mRNAs in somatic and zygotic embryos

Using *in vitro* translation of RNA and immunoprecipitation of translated products with anti-crystalloid serum, we have characterized the profiles of translatable crystalloid storage protein mRNAs in developing zygotic and somatic embryos of white spruce. Analysis of the various stages of somatic embryos maturing on 16 μM ABA without PEG show that the mRNAs isolated from immature somatic embryos from suspension culture directed synthesis of a number of polypeptides *in vitro* (Fig. 9A, lane 2). Upon transfer to ABA containing media, mRNAs from 4 week cultured somatic embryos directed synthesis of a new set of polypeptides of 60, 55, 21 and 19 kDa (Fig. 9A, lane 5). These polypeptides were immunoprecipitated using anti-crystalloid serum (Fig. 9B, lane 5). None of these polypeptides were immunoprecipitated in samples of immature somatic embryos from suspension culture (lane 2), 1–2 week (lane 3), 2–3 week (lane 4), cultured somatic embryos or in plantlets derived from 4-week old somatic embryos (lane 6). In zygotic embryos, crystalloid protein mRNAs could be detected at the early cotyledonary stage (Fig. 9C, lanes 2–3). The major polypeptides immunoprecipitated were of 55–60 kDa and 18–21 kDA range. In later stages of embryogenesis the crystalloid protein mRNAs were not detected (lanes 4–7).

A comparison of the pattern of immunoprecipitated polypeptides of 4 week old somatic embryos maturing on 16 μM ABA in the absence (Fig. 9B, lane 5), or in the presence (Fig. 9D, lane 2), of 7·5% PEG, with those of zygotic embryos (Fig. 9C, lane 2, 3) reveals that the crystalloid protein mRNAs of the two treatments were very similar. This is in contrast to the accumulated crystalloid protein profiles of somatic embryos maturing on 16 μM ABA without PEG (Fig. 5A, B), which differs markedly from that of mature zygotic embryos and from somatic embryos matured on media containing 16 μM ABA and 7·5% PEG.

DISCUSSION

A non-plasmolysing osmotic treatment effected by 7·5% PEG-4000 in combination with 16 μM ABA was optimal for maturation of white spruce somatic embryos. At this concentration the osmoticum produced a normal spectrum of storage polypeptides and suppressed precocious germination. Maturation continued for up to 8 weeks, and protein synthesis was increased threefold. The high osmotic treatment prevented catabolism of the accumulated polypeptides during desiccation treatments. These conditions were also found to promote accumulation of lipids by white spruce somatic embryos (Attree *et al.*, 1992), and plantlet regeneration frequencies of up to 81% were obtained from the desiccated somatic embryos. Analysis of the mRNAs by *in vitro* translation in our study revealed that despite the differences observed in the patterns of accumulated proteins between zygotic embryos, and somatic embryos maturing on medium containing ABA with low osmoticum, the crystalloid protein mRNA products were very similar. Storage protein synthesis therefore, was initiated in response to the ABA treatment in differentiated somatic embryos; however, high osmoticum was required for continued accumulation of the storage polypeptides. Inhibition of precocious germination in response to 7·5% PEG (Attree *et al.*, 1991*b*) appears to have a positive effect on crystalloid protein synthesis as well as preventing the catabolism of

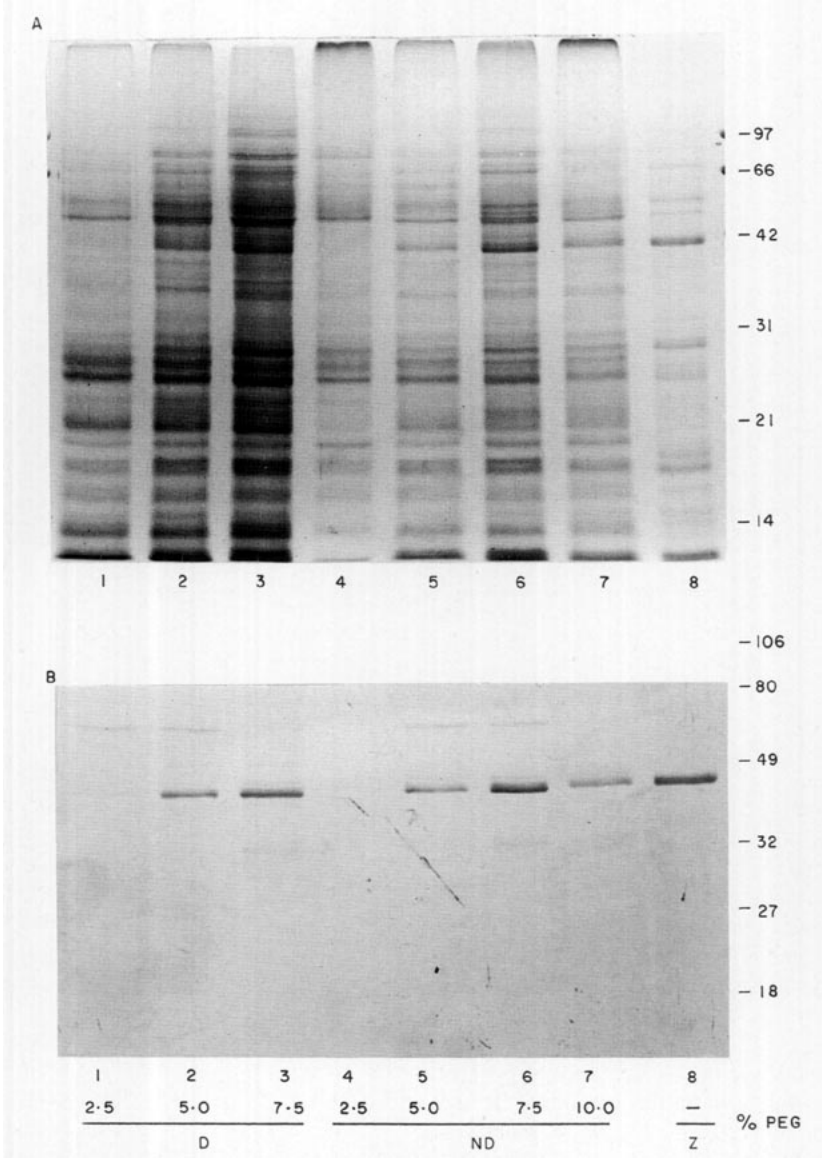


FIG. 7. Effect of osmoticum followed by a subsequent desiccation treatment (81% RH) or osmoticum alone on matrix protein profiles of white spruce zygotic and somatic embryos matured for 4 weeks on 16 μM ABA and different concentrations of PEG. A, Coomassie Blue stained SDS-PAGE profiles of proteins. Lane 1, 2·5% PEG + desiccation; 2, 5% PEG + desiccation; 3, 7·5% PEG + desiccation; 4, 2·5% PEG; 5, 5·0% PEG; 6, 7·5% PEG; 7, 10% PEG; 8, mature zygotic embryos. D, Desiccated; ND, non-desiccated. B, Corresponding Western Blot of matrix proteins from somatic and zygotic embryos. Labels as in Fig. 7A.

storage polypeptides during maturation and desiccation, as also reported for alfalfa (Xu *et al.*, 1990) and maize (Rivin and Grudt, 1991) zygotic embryos.

In white spruce zygotic embryos, the synthesis of crystalloid proteins was initiated during the early stages of development and these proteins accumulated to high levels during mid and late stages (Misra and Green, 1991). They showed that the crystalloids are made up of four groups of polypeptides: 42, 34·5–35, 22–23, and 8 kDa, linked by disulphide bonds. In our study the pattern of proteins synthesized by white spruce somatic embryos cultured in the presence of ABA and increased osmoticum was similar to the storage protein composition observed by Misra and Green (1991) in the mid-to-late stages of white spruce zygotic embryogenesis, and was distinctly different from those of embryos maturing on ABA and no osmoticum. Thus, even in the presence of just 2·5% PEG, all four groups of crystalloid proteins could be detected by Western blot analysis after 4 weeks maturation. Also, the synthesis of the 42 kDa polypeptide was observed in the matrix protein fraction. The storage protein profiles of 4 week matured somatic embryos were similar to those at 8 weeks, but protein levels were greater after the longer maturation period. This was also reflected in the ultrastructure of the osmotically treated somatic embryos which closely resembled that of mature zygotic embryos.

Only the 35 kDa range of crystalloid polypeptides was detected in white spruce somatic embryos matured for 4

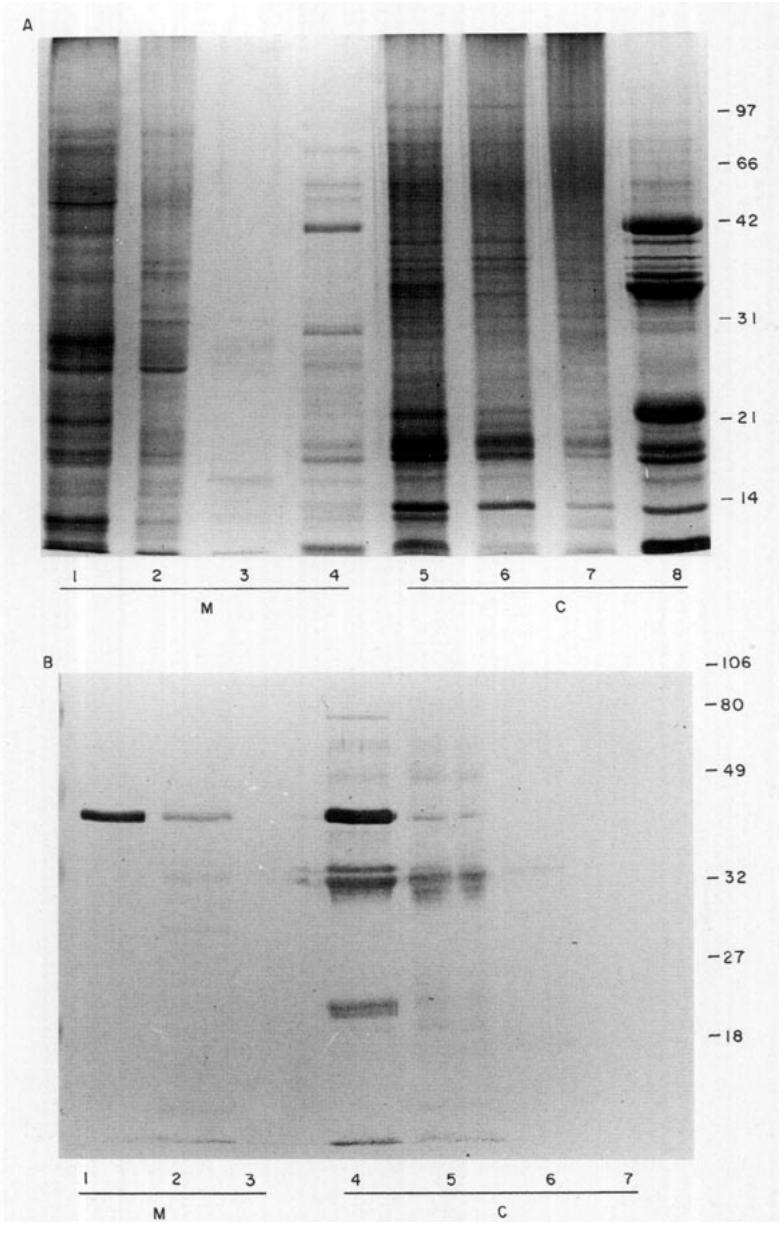


FIG. 8. A, Coomassie Blue stained SDS–PAGE profiles of matrix (M) and crystalloid (C) proteins extracted from: Lanes 1 and 5, desiccated somatic embryos; 2 and 6, desiccated then rehydrated somatic embryos; 3 and 7, 2-week regenerated plantlets; 4 and 8, mature zygotic embryos. Somatic embryos for all the above were matured for 4 weeks on 16 μM ABA with 7·5% PEG then desiccated for 2 weeks at 81% RH prior to transfer to phytohormone-free plantlet regeneration medium for plantlet development. B, Western blot of matrix (M) and crystalloid (C) proteins extracted from: lanes 1 and 5, desiccated somatic embryos; 2 and 6, rehydrated somatic embryo; 3 and 7, 2-week-old plantlets; 4, mature dry zygotic embryos.

weeks on 16 μM ABA with low osmoticum, suggesting the other polypeptides were not accumulated. Also as seen by the intensity of immunoreactivity, the amount of storage protein synthesis in the somatic embryos was much lower than in their zygotic counterparts. This crystalloid protein profile was similar to the protein profile of immature zygotic embryos (Misra and Green, 1991), where the 35 kDa range of polypeptides made up a higher proportion of the total crystalloids. The lack of maturity and somatic embryos matured with low osmoticum was also reflected in the structure of the protein bodies, which contained only diffuse deposits of proteins similar to early stages of protein body development in white spruce zygotic embryos (Joy *et al.*, 1991). The identity of these early deposited proteins has been confirmed by immunoelectron microscopy (unpubl. res.).

Treatment with high osmoticum in the absence of ABA did not lead to maturation of white spruce somatic embryos (not shown). The effect of osmoticum on storage protein synthesis during conifer embryogenesis is probably at a translational and post-translational level, since the crystalloid protein mRNAs were transcribed in white spruce

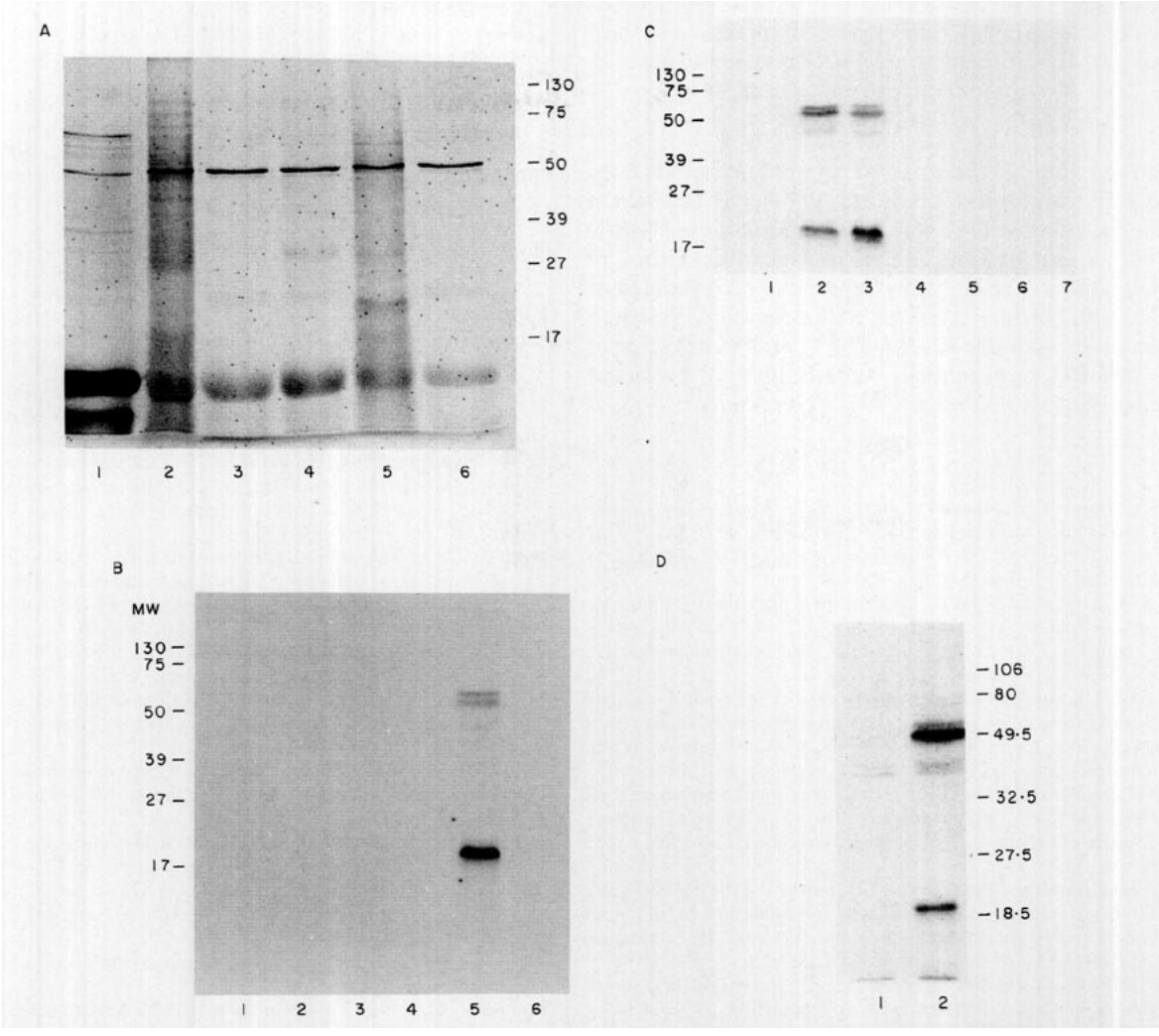


FIG. 9. Fluorographs of proteins synthesized *in vitro* using bulk RNA extracted from zygotic and somatic embryos cultured on media containing 16 μM ABA. Translation was in a rabbit reticulocyte cell-free system charged with [^{35}S]-methionine. Relative molecular weights in kDa are shown. A, Cell free translation products after *in vitro* translation of bulk RNA extracted from somatic embryos. Lane 1, control globin mRNA; 2, immature embryo from suspension culture; 3, early globular; 4, late globular; 5, early cotyledonary; 6, germinated plantlets. B, Immunoprecipitation of *in vitro* translated proteins of somatic embryos with subsequent SDS–PAGE and fluorography. Lane 1, globin mRNA; 2, immature somatic embryos; 3, early globular; 4, late globular; 5, cotyledonary; 6, germinated plantlets. C, Immunoprecipitation of *in vitro* translated proteins of zygotic embryos. Lane 1, globin mRNA; 2, Jul. 5, immature club-shaped embryos; 3, early cotyledonary—Jul. 17; 4, cotyledonary—Aug. 2; 5, late cotyledonary—Aug. 15; 6, mature embryo—Sep. 5; 7, germinated embryo. D, Immunoprecipitation of *in vitro* translated proteins of somatic embryos matured on 16 μM ABA with 7·5% PEG. Lane 1, early globular; 2, cotyledonary embryos.

somatic embryos matured on low osmoticum, in a pattern identical to mature zygotic embryos. Previous reports support an independent action of ABA and osmoticum on storage protein synthesis (Goffner *et al.*, 1990; Wilen *et al.*, 1990; Xu *et al.*, 1990; Rivin and Grudt, 1991). Both ABA and osmoticum independently maintained steady state levels of storage protein mRNAs in zygotic embryos of sunflower (Goffner *et al.*, 1990) and in microspore derived haploid embryos of *B. napus* (Wilen *et al.*, 1990).

The desiccated white spruce somatic embryos in our study displayed metabolic events similar to those reported in desiccated embryos of angiosperms (Misra, Kermode and Bewley, 1985; Kermode, 1990). The protein profile of desiccated white spruce somatic embryos was similar to that of undesiccated somatic embryos and desiccated zygotic embryos. During plantlet regeneration the storage proteins of the somatic embryos were rapidly mobilized in a manner similar to germinated zygotic embryos and germination-related gene activity was observed.

In summary our results show that osmotic treatment enhanced storage protein synthesis in white spruce somatic embryos both quantitatively and qualitatively. The resulting protein profiles and protein body structure following desiccation were the same as those of zygotic embryos from mature dry seed. The response of conifer somatic embryos *in vitro* to ABA and osmoticum at the level of protein synthesis appears to be comparable to that of angiosperm zygotic embryos, indicating common regulatory mechanisms in these two distant groups of plants. The fact that in the absence of high osmoticum, mRNAs of conifer somatic embryos matured with ABA were similar to those of maturing zygotic embryos, is intriguing and warrants further

analysis of translational/transcriptional control of storage protein synthesis in response to ABA and osmoticum.

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