

STORAGE PROTEIN GENE EXPRESSION IN ZYGOTIC AND SOMATIC
EMBRYOS OF INTERIOR SPRUCE

by

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ABSTRACT

Storage proteins from interior spruce (*Picea glauca/engelmannii* complex) were identified, partially characterized and used as markers to compare the developmental fidelity between zygotic and somatic embryos. The major storage proteins expressed in both embryo types had molecular weights of approximately 41, 35, 33, 24 and 22 kD. The 41 kD protein was buffer and low salt-soluble, whereas the 35-33 kD and 24-22 kD proteins were high salt-soluble and disulfide linked. All of the proteins possessed several isoelectric variants. Based on solubility and disulfide linkage characteristics, as well as cDNA sequences, these storage proteins were homologous to angiosperm vicilin-type (41 kD) and legumin-type (35-33 kD, 24-22 kD) storage proteins.

Somatic embryos of different genotypes matured on 40 μ M ABA accumulated significant levels of storage protein, similar to or higher than levels found in zygotic embryos. Somatic embryos on 10 μ M ABA displayed initial storage protein accumulation, but the levels did not reach those found in zygotic embryos or somatic embryos matured on 40 μ M ABA.

Zygotic embryos and somatic embryos differentiated on 40 μ M or 10 μ M ABA displayed differential storage protein accumulation, with the legumin-type proteins apparent before the vicilin-type, although all showed major accumulations during cotyledon development. Zygotic embryos displayed a rapid, transient period of storage protein accumulation, with maximum storage protein levels attained at least 1 month prior to mature seed shed. In contrast, somatic embryos differentiated on 40 μ M ABA displayed a

more prolonged, gradual accumulation of storage proteins, which were still on the increase after 9 weeks of maturation on ABA. Somatic embryos on 10 μ M ABA initially accumulated storage proteins, but these were rapidly degraded as the embryos germinated precociously.

Analysis of storage protein mRNAs indicated they were present by torpedo stage in zygotic embryos and somatic embryos matured on 40 μ M and 10 μ M ABA. In all cases, the transcripts increased during development, with those of legumin reaching high levels prior to those of vicilin. Transcript levels in zygotic embryos increased during cotyledon development and then declined rapidly to very low levels at least 1 month prior to mature seed shed. Somatic embryos on 40 μ M ABA displayed high transcript levels for a prolonged period, and these were still present after 9 weeks, although they had declined to 50% of maximum levels. Low levels of storage protein transcripts also appeared in somatic embryos on 10 μ M ABA, but declined during precocious germination, although they were still detectable after several weeks of precocious germination.

Osmotic stress, caused by the culture of somatic embryos on medium containing 15% mannitol, induced storage protein and storage protein transcript accumulation. This could be inhibited by inclusion of the ABA-biosynthetic inhibitor, fluridone, suggesting that the increase was due to osmotic stress-induced ABA biosynthesis.

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1. INTRODUCTION

Forestry is the primary natural resource industry in British Columbia and throughout Canada. Spruce species (*Picea* spp.) form the second largest standing timber volume, after pines, and provide the second largest log source in B.C. (Council of Forest Industries of B.C. 1987). The propagation of these economically important conifers is thus an essential part of any national effort to ensure the long term replacement of harvested trees. Tissue culture methods are widely believed to hold the best prospect of achieving the required mass propagation of superior genotypes (Cheliak and Rogers 1990, Karnosky 1981) which will provide the opportunity to maintain and even enhance the quality of the forest stock. Tissue culture propagation by somatic embryogenesis (embryo differentiation from somatic cells) allows rapid production of large numbers of plants and the fast exploitation of genetic gains that have been achieved by conventional forest tree breeding programs (Cheliak and Rogers 1990). Use of somatic embryogenesis improves the economic feasibility of propagation via tissue culture, because it provides large scale production and "bulk up" of the selected stock. It also offers the potential for the genetic engineering of single cells, for such traits as herbicide resistance and production of the anti-spruce budworm *Bacillus thuringiensis* toxin, and their regeneration to plants.

Now that the technologies for somatic embryogenesis have been developed for spruce (Attree *et al.* 1990ab, Becwar *et al.* 1989, Hakman and von Arnold 1988, Krogstrup 1990, Lelu and Bornman

1990, Roberts *et al.* 1990ab, Tremblay 1990, von Arnold and Hakman 1988, Webster *et al.* 1990) and other conifer species (Becwar *et al.* 1990, Bourgakd and Favre 1988, Durzan and Gupta 1987, Finer *et al.* 1989, Gupta and Durzan 1987, Nørgaard and Krogstrup 1991, von Aderkas and Bonga 1988), emphasis has moved to ensure that the derived plants are both healthy and vigorous. Evaluation of alfalfa artificial seed (Redenbaugh *et al.* 1986) showed that somatic embryos that produced vigorous germinants more closely resembled their zygotic counterparts than did the embryos that produced less vigorous plants. This has led to the current wisdom that the potential of somatic embryos to produce vigorous plants can be predicted by determination of their biochemical, physiological and morphological similarity to normal zygotic embryos.

The periods of synthesis and deposition of storage material are critical for zygotic embryogenesis, seed development, germination and development of robust seedlings. In mature conifer embryos, proteins and lipids (triglycerides) are the most prominent reserves (Bewley and Black 1985, Cyr *et al.* 1991, Flinn *et al.* 1989), and are excellent biochemical markers of zygotic and somatic embryogenesis. However, there are few reports concerning biochemical development in conifer zygotic embryos (see for example Janick *et al.* 1991, Johnson *et al.* 1987) and only recently, during the course of this present study have other labs reported identification and characterization of conifer storage proteins (Gifford 1988, Gifford and Tolley 1989, Green *et al.* 1991, Misra and Green 1990, Misra and Green 1991). Clearly,

we need to understand the biochemical processes that occur during conifer zygotic embryogenesis before we can evaluate conifer somatic embryos.

The involvement of plant growth regulators in the control of storage reserve deposition during angiosperm seed development has been well documented (Bewley and Black 1985). Endogenous abscisic acid (ABA) levels increase during the period of reserve synthesis and deposition in developing seeds (Bewley and Black 1985, Finkelstein *et al.* 1985) and exogenous ABA can induce storage protein accumulation (Barratt 1986, Crouch and Sussex 1981, Goffner *et al.* 1990). Furthermore, the addition of ABA to cultures during somatic embryo differentiation prevents precocious germination, increases the number of embryos that mature, and reduces the occurrence of morphologically-abnormal embryos (Ammirato 1974, Roberts *et al.* 1990a, von Arnold and Hakman 1988, Webster *et al.* 1990).

While most studies have been carried out with angiosperms, little is known about the role of ABA in conifer embryogenesis. Most of the angiosperm studies have shown that patterns of reserve substance deposition in somatic embryos differs temporally and/or quantitatively from those in zygotic embryos (Avjioglu and Knox 1989, Crouch 1982, Shoemaker *et al.* 1987, Krochko *et al.* 1989, Stuart *et al.* 1988). No comparable information is available from conifers, although Feirer *et al.* (1989) and Cyr *et al.* (1991) found that spruce somatic embryos contained less triglyceride reserves than zygotic embryos and Hakman and von Arnold (1988) and von Arnold and Hakman (1988)

showed that spruce somatic embryos contained storage protein bodies and protein profiles similar to those of zygotic embryos (Hakman *et al.* 1990). These studies did not provide developmental comparisons of storage protein gene expression between zygotic and somatic embryos, nor did they report the role of ABA on these reserves.

The hypothesis of this present thesis is that somatic embryos matured in the presence of high, adequate levels of ABA will resemble their zygotic counterparts with respect to patterns of storage protein gene expression and overall embryo development than somatic embryos matured using low levels of ABA. To test this hypothesis, the goals of this study will be to: 1) Identify the major storage proteins of spruce zygotic and somatic embryos; 2) Compare the developmental expression of these proteins at the protein and gene transcript level during zygotic and somatic embryogenesis and; 3) Examine the effect of high and low levels of ABA on the formation of these reserves during spruce somatic embryo development.

2. LITERATURE REVIEW

2.1. SOMATIC EMBRYOGENESIS IN SPRUCE

2.1.1. General introduction

The advances in conifer tissue culture during the past 6-7 years have led to the production of somatic embryos in species of *Larix* (Klimaszewska 1989, Nagmani and Bonga 1985, von Aderkas and Bonga 1988, von Aderkas et al. 1990), *Abies* (Nørgaard and Krogstrup 1991, Schuller et al. 1989), *Picea* (Attree et al. 1990a, Becwar et al. 1989, Gupta and Durzan 1986a, Hakman and von Arnold 1985, Krogstrup et al. 1988, Lelu and Bornman 1990, Roberts et al. 1990ab, Roberts et al. 1991, Tautorius et al. 1990, Tremblay 1990, von Arnold and Hakman 1988, von Arnold and Woodward 1988, Webb et al. 1989, Webster et al. 1990), *Pinus* (Becwar et al. 1990, Becwar et al. 1991, Finer et al. 1989, Gupta and Durzan 1986b, Gupta and Durzan 1987, Jain et al. 1989, Lainé and David 1990), *Pseudotsuga* (Durzan and Gupta 1987) and *Sequoia* (Bourgkard and Favre 1988). Since most of the work has been carried out with *Picea* species and interior spruce (*Picea glauca/engelmannii* complex) was the object of this study, the following literature review deals only with spruce.

2.1.2. Somatic embryo induction

Somatic embryo induction depends on a variety of factors. Explant age plays a major role in the ability to respond to morphogenic stimuli in culture and somatic embryogenesis has been obtained primarily from immature embryos (for review see Attree and Fowke 1991). There is commonly a window of competence, in which specific developmental stages are more amenable to

induction, and a declining capacity for embryogenic induction with increasing embryo age (Hakman *et al.* 1985, Lu and Thorpe 1987, Webb *et al.* 1989). Generally, in *Picea*, early cotyledonary embryos are the most responsive. The loss of competence is attributed to morphological, physiological and biochemical changes that occur during embryo maturation (Roberts *et al.* 1989).

Culture media commonly used for induction are modifications of the LP medium (von Arnold and Eriksson 1981) used at either full- or half-strength (Hakman and von Arnold 1988, von Arnold and Hakman 1988, Webb *et al.* 1989), although optimal media strength appears to be species dependent (Tautorus *et al.* 1990). Somatic embryogenesis has also been obtained using modified forms of Murashige and Skoog's (1962) medium (Gupta and Durzan 1986a, Krogstrup *et al.* 1988) and Litvay's (1985) medium (Tremblay 1990, Tremblay and Tremblay 1991ab). The latter medium is a formulation based on the chemical composition of conifer seed megagametophyte. Since total N, reduced N, and $\text{NO}_3:\text{NH}_4$ are critical for conifer organogenesis (David *et al.* 1982, Flinn *et al.* 1986) and angiosperm embryogenesis (Gleddie *et al.* 1983, Walker and Sato 1981, Wetherell and Dougall 1976), the different amounts and ratios of nitrogen in the different media formulations are probably an important factor.

Early studies with mature embryos revealed that induction frequencies were lower than from immature embryo explants. However, more recently, induction frequencies similar to those from immature embryos have been obtained (Tremblay 1990, Verhagen

and Wann 1989, von Arnold 1987). The expansion of the window of competence to include mature embryos has been obtained by various media manipulations. Half-strength media formulations are often used (Tautorus *et al.* 1990, Tremblay 1990, Verhagen and Wann 1989, von Arnold 1987, von Arnold and Woodward 1989). The manipulation of NH_4NO_3 levels, pH and amino acid composition has substantially enhanced induction from mature embryos (Tautorus *et al.* 1990, Verhagen and Wann 1989, von Arnold 1987).

Somatic embryogenesis has also been achieved from germinated seedlings ranging from several days (Lelu and Bornman 1990, Lelu *et al.* 1990) to 3-5 weeks old (Attree *et al.* 1990a, Mo and von Arnold 1991). Induction was achieved by manipulating media and cultural environment conditions. Some studies have suggested that explant pretreatment with cytokinin enhanced the embryogenic response (Lelu and Bornman 1990, Lelu *et al.* 1990), although others found this not to be beneficial (Attree *et al.* 1990, Mo and von Arnold 1991).

While explant developmental stage and media composition may affect the capacity for embryogenic induction, other factors are also involved. Cold storage of immature cones enhanced subsequent induction from the excised embryos (Hakman and von Arnold 1985, Hakman and Fowke 1987), although Tremblay (1990) reported that low temperature exposure of mature excised embryos strongly inhibited embryogenesis. The duration of mature seed storage significantly decreased embryogenic potential (Tremblay 1990). Furthermore, the vigour of the explant (Tremblay 1990),

as well as its genetic background (Hakman and von Arnold 1988, Tremblay 1990, Webb *et al.* 1989) are important.

Auxin-cytokinin combinations are used to initiate embryogenesis. While 2,4-dichlorophenoxyacetic acid (2,4-D) and N⁶-benzyladenine (BA) (see Attree and Fowke 1990) are most commonly used, other workers have found combinations of other cytokinins with 2,4-D to be effective (Gupta and Durzan 1986a, Krogstrup *et al.* 1988). As well, somatic embryos have been induced using other auxins in combination with cytokinins (Lu and Thorpe 1987, Verhagen and Wann 1989, von Arnold and Hakman 1988).

Another important component included in the tissue culture medium is the carbohydrate source, with sucrose the most commonly used. The optimum sucrose concentration varies between 1% and 3%, depending on the basal medium (von Arnold and Hakman 1986, von Arnold 1987) and may also vary between different seed sources used for explants (Webb *et al.* 1989). The sucrose level used can also influence the morphogenic response. Webb *et al.* (1989) found that low sucrose (1%) favoured embryogenic induction from immature embryos, while higher levels (3-4%) favoured the induction of adventitious shoots.

The culture environment during induction is also important. Kvaalen and von Arnold (1991) reported that the gaseous environment of the culture vessel affected induction. Low O₂ in combination with full strength medium stimulated induction, while on half-strength medium, high O₂ was better. High CO₂ levels also promoted embryo induction. This effect was attributed to a stimulation of embryogenic tissue growth or to an inhibitory

effect by CO₂ on ethylene synthesis, since ethylene is known to inhibit the growth of embryogenic tissue (Kumar *et al.* 1989).

It has been suggested that light inhibits embryogenic tissue induction. Von Arnold (1987) reported that culture in the dark was better for induction and most workers follow this practice (Hakman and Fowke 1987, Gupta and Durzan 1986a, Webb *et al.* 1989). However, Verhagen and Wann (1987) reported no significant differences between light and dark treatments on induction.

2.1.3. Somatic embryo maturation

The embryogenic cultures described in the above studies consist of small, single cells and cell aggregates, as well as numerous proembryo-like structures with small, opaque, densely cytoplasmic embryos (Hakman *et al.* 1987, von Arnold and Woodward 1988, Webb *et al.* 1989). Culture of embryogenic tissues under conditions used for induction results in the continued growth and proliferation of these proembryonal structures and the prevention of subsequent development. Further embryo development requires the transfer to conditions allowing maturation. Embryo maturation involves cell division and the expansion of the embryonal cells to form globular embryos, which then elongate and develop cotyledon primordia, followed by cotyledon and hypocotyl elongation (Dunstan *et al.* 1988, Hakman and von Arnold 1988). Developmental progress to maturation or to precocious germination depends on the manipulation of media and other culture conditions.

In all cases, the continued maturation of spruce somatic embryos past the proembryo stage requires, at the least, the

reduction or removal of embryo-inducing growth regulator levels. Some studies have used reduced 2,4-D levels for further development (Gupta and Durzan 1986a, Lu and Thorpe 1987, Tremblay 1990), although embryo quality and yield were low. Better yield and maturation have been obtained in the absence of 2,4-D and with the use of ABA alone or in combination with an auxin. However, Lu and Thorpe (1987) reported no improvement of somatic embryo development by ABA. Low levels of ABA and an auxin (1 μM each) increased the number of embryos produced and the number reaching maturity (Becwar et al. 1989, Jain et al. 1988). Furthermore, buthionine sulfoximine (an inhibitor of reducing agents) tripled somatic embryo maturation in combination with the above growth regulators (Jain et al. 1988). Higher ABA concentrations (5-16 μM) have been used by others to promote embryo maturation (Attree et al. 1990b, Krogstrup et al. 1988, von Arnold and Hakman 1988), but levels in excess of this range reduce regeneration (von Arnold and Hakman 1988) and lower maturation and germination values (Attree et al. 1990b). Comprehensive studies on the effects of ABA on quality and maturation of interior spruce somatic embryos revealed that the optimum ABA level for most genotypes was between 40 and 60 μM , although there were genotype-related differences in maturation (Webster et al. 1990). The incorporation of low levels (0.1-10 μM) of indole-3-butyric acid (IBA) with ABA enhanced embryo production, cotyledon development and morphology (Roberts et al. 1990a). ABA levels in the range of 10-20 μM resulted in fewer embryos, most of which germinated precociously (Roberts et al.

1990a). It is evident that a wide range of ABA levels have been used for maturation. Boulay *et al.* (1988) reported that the number of subcultures on ABA and the concentration required to maximize somatic embryo recovery was dependent on the number of previous subcultures under proliferation conditions.

In addition to ABA, ABA analogues have been tested for their effects on spruce somatic embryo maturation (Dunstan *et al.* 1988, 1991). Of those tested, abscisyl alcohol produced results similar to ABA, while the others were inhibitory.

Most studies have utilized ABA exposures of 4-5 weeks. Prolonged exposures have been reported to cause swelling (von Arnold and Hakman 1988), inhibit hypocotyl and cotyledon elongation (Boulay *et al.* 1988) and result in poor root development (Dunstan *et al.* 1988). However, Dunstan *et al.* (1991) reported that 9 weeks of ABA exposure stimulated epicotyl development in the resulting plantlets.

While ABA influences embryo maturation, other factors, such as carbohydrates, are also important. Von Arnold and Hakman (1988) found that 90 mM (3%) sucrose was optimal for development. High sucrose levels (120-150 mM; 4-5%) stimulated early embryo development but repressed further maturation. Other workers have reported that 6% sucrose was optimal for somatic embryo development (Lu and Thorpe 1987, Tremblay 1990, Tremblay and Tremblay 1991a). Individual carbohydrates may act differently on embryo maturation in different species. Tremblay and Tremblay (1991a) reported that 6% fructose was more effective than glucose

or sucrose for maturation in red spruce, whereas in black spruce all three carbohydrates were equally effective at the 6% level.

Some of the carbohydrate supplied during maturation may be replaced by an osmoticum (Lu and Thorpe 1987, Tremblay and Tremblay 1991a), indicating that the carbohydrate effect on maturation is partly osmotic. Roberts (1991) reported that low levels of osmoticum (2-6% mannitol) promoted globular embryo formation, and that ABA was required for development to the cotyledonary stage. After cotyledon development, high levels of osmoticum (13-20% mannitol) could replace ABA as an inhibitor of precocious germination. Furthermore, a 1 week mannitol pulse in combination with ABA, followed by maturation on ABA alone, doubled the production of late cotyledonary stage embryos (Roberts 1991).

The culture environment during maturation also affects the extent of embryo development. Embryo maturation was stimulated by combinations of low O₂ and high CO₂ on medium containing 7.6 μ M ABA (Kvaalen and von Arnold 1991). This effect was not as evident at the higher (60 μ M) ABA levels tested. Light can also influence embryo development, with maturation in the light favouring the production of a greater number of mature embryos compared to darkness (Tremblay and Tremblay 1991b).

Thus, while several factors must be considered during somatic embryo induction and maturation, it is possible to obtain well developed spruce somatic embryos from a number of species. This capability provides the potential for biotechnological applications to spruce species, as well as a system to study

factors that affect conifer embryo development without complications that arise from the effects of embryo excision from the seed, and the presence of the surrounding maternal tissues.

2.2. PROTEIN RESERVES DURING EMBRYOGENESIS

2.2.1. Regulation of angiosperm storage protein accumulation

Most mature seeds contain protein, lipid and carbohydrate reserves which are used by the embryo during germination and post germination processes. Most studies have concentrated on cereal and legume reserves, due to their economic importance. However, little emphasis has been placed on the study of the major conifer reserve materials. Since proteins are prominent conifer seed reserves, they represent useful biochemical markers to study and compare development in zygotic and somatic embryogenesis.

Storage proteins are synthesized during embryo development and are degraded during germination to supply amino acids, nitrogen and carbon skeletons to the developing seedling. These deposits occur in distinct protein bodies and are confined largely to the embryo and surrounding storage tissue (megagametophyte in gymnosperms). Many storage proteins undergo post-translational modifications during deposition to convert them to their correct size for deposition (Müntz 1989, Shotwell and Larkins 1989).

Storage protein synthesis tends to be limited to the cell expansion phase, following cell division and prior to maturation and desiccation (Bewley and Black 1985). Some pea legumin storage protein accumulation has been noted during the cell division phase (Domoney et al. 1980), but it was not known if this occurred in dividing cells or a relatively few non-dividing

cells. Recent work with pea, using *in situ* hybridization, showed that storage protein mRNAs only accumulated in cells lacking mitotic activity (Hauxwell *et al.* 1990).

Most seeds contain more than one class of storage protein, each of which has a distinct temporal accumulation pattern. In rapeseed, the 2S protein, napin, and the 11S protein, cruciferin, start to accumulate during early embryo development, with napin detectable slightly earlier than cruciferin (Crouch and Sussex 1981, Murphy *et al.* 1989). These proteins accumulate rapidly between 5 and 7 weeks post-anthesis, after which accumulation slows down and levels off (Crouch and Sussex 1981, Murphy *et al.* 1989). Napin accumulation ends when the embryo water content begins to decline, while cruciferin accumulation continues until seed maturity (Crouch and Sussex 1981).

In pea, accumulation of the 7S protein, vicilin, begins one day earlier than that of the 11S protein, legumin. During the first few days, vicilin synthesis predominates (Boulter *et al.* 1987), reaches a maximum by 14/15 days after flowering (DAF) and then declines. During the period from 18/19 to 20 DAF, legumin synthesis and accumulation peaks and remains constant.

In soybean, the 7S β -conglycinin α - and α' -subunit proteins are detectable 18 to 20 days after anthesis (DAA), while the 11S glycinin subunit proteins start to accumulate between 19 and 21 DAA. However, the β -subunit of β -conglycinin does not accumulate until after the onset of glycinin accumulation (Meinke *et al.* 1981).

The temporally distinct accumulation patterns described above for storage proteins also occur for their mRNA levels. In rapeseed, napin mRNA is detectable by 18 DAA, achieves its maximum at 27 DAA and remains high until 40 DAA. In contrast, cruciferin mRNA is detected 3 days later at 21 DAA and peaks at 40 DAA (Finkelstein *et al.* 1985). In pea, vicilin mRNA is detectable before legumin mRNA, peaks at 14 DAF, then declines. Legumin mRNA is detectable about 1 day after vicilin, peaks at 18 DAF, then declines (Boulter *et al.* 1987, Yang *et al.* 1990). Similarly, in soybean, β -conglycinin mRNA is detectable several days prior to glycinin mRNA (Walling *et al.* 1986).

Members of the same protein family may or may not exhibit the same temporal mRNA accumulation patterns. In soybean, each glycinin gene is expressed in the same temporal framework during embryo development (Nielsen *et al.* 1989). In contrast, the soybean β -conglycinin gene family shows differences. The mRNAs for the α - and α' -subunits accumulate prior to β -subunit mRNA (Harada *et al.* 1989). In rapeseed, one subfamily of napin (gNa) has mRNA levels that peak and decline earlier than those of the other members of the napin family (Blundy *et al.* 1991).

The level of storage protein mRNAs appears to be controlled primarily at the transcriptional level. Storage protein genes are transcriptionally activated early in embryogenesis, attain relatively high transcription rates by mid-maturation and are repressed prior to desiccation and dormancy (Delisle and Crouch 1989, Evans *et al.* 1984, Harada *et al.* 1989, Nielsen *et al.* 1989,

Walling et al. 1986). These changes roughly parallel those in endogenous mRNA levels.

Apart from transcriptional regulation, post-transcriptional regulation also plays a role in controlling storage protein mRNA levels (Delisle and Crouch 1989, Harada et al. 1989, Nielsen et al. 1989, Walling et al. 1986). In soybean, Gy2, Gy5 and G* members of the glycinin gene family are transcriptionally activated and repressed at the same developmental stages. By 35 DAF, Gy2 and Gy5 show similar transcription rates, which are lower than that of G*. However, steady state Gy2 and Gy5 mRNA levels are higher than those of G*, suggesting that the G* mRNA is more unstable (Nielsen et al. 1989). With soybean β -conglycinin, the α/α' - and β -subunit genes, which are also transcriptionally activated at the same developmental stage, show similar transcription rates by 25 DAF, although the α/α' -subunit mRNA levels are higher than β -subunit mRNA (Harada et al. 1989). These results suggest that at this developmental stage, the β -subunit mRNA is less stable. In rapeseed both napin and cruciferin transcription rates are on the decline by 38 DAA. However, cruciferin mRNA levels remain elevated while napin mRNA levels drop, suggesting that cruciferin mRNA is more stable during the later stages of embryo maturation (Delisle and Crouch 1989). All of these above studies suggest that developmentally-specific changes in mRNA stability may affect steady state mRNA levels.

While storage protein accumulation can be regulated by mRNA availability, it can also be regulated at the level of protein

synthesis. In developing oat seeds (Chesnut *et al.* 1989), the amounts of avenin storage protein mRNA are equal to or greater than globulin storage protein mRNA during most of development, although globulin is the predominant storage protein in the seed. The high proportion of globulin suggests some type of post-transcriptional regulation, which is believed to be translational, because there is a greater proportion of globulin message incorporated into polysomes (Fabijanski and Altosaar 1985). Post-translational regulation also regulates storage protein accumulation (Shuttuck-Eidens and Beachy 1985). These workers used pulse:chase-labelling to detect the synthesis of β -conglycinin β -subunit protein well before its accumulation during embryo development. However, this protein was rapidly turned over by proteolysis during the early stages of cotyledon maturation. As maturation proceeded, the stability of the protein increased, allowing its accumulation.

2.2.2. Spatial patterns of storage protein accumulation

Seed storage proteins are differentially expressed, both quantitatively and qualitatively in various tissues, and in different cells within the same tissue during development. In soybean, the relative proportions of 7S and 11S proteins differ between embryonic axes and cotyledons. Axes contain very little 11S storage protein, as well as reduced levels of β -conglycinin α -subunit and no β -subunit. In addition, axes contain a β -conglycinin subunit that is not found in cotyledons (Meinke *et al.* 1981). In maize endosperm, storage protein composition varies with increasing distance from the aleurone layer. Cells

adjacent to the aleurone accumulate β - and γ -zeins, but little or no α -zein, whereas cells further away from the aleurone contain more α -zein (Lending and Larkins 1989). The 2S storage proteins of *Arabidopsis thaliana* also show differential expression patterns (Guerche *et al.* 1990). Of the 4 members of the at2S gene family, at2S2, at2S3 and at2S4 are expressed throughout the embryo, while at2S1 is strongly expressed only in the axis. A recent study using *in situ* hybridization pointed out the changing cellular patterns of storage protein expression during rapeseed embryo development (Fernandez *et al.* 1991). Napin and cruciferin mRNAs accumulated initially in the cortex of the axis during late heart stage, then in the outer face of the cotyledons during torpedo stage, followed by a "wave-like" spread to the inner face of the cotyledons. No expression occurred in the root or shoot meristem during the early stages of embryogenesis, but during maturation drying, both mRNAs were detected in the shoot meristem.

The results of the above studies indicate that the accumulation of storage proteins during embryogenesis and seed development is a highly regulated event, in which gene expression is controlled at a variety of levels both temporally and spatially.

2.2.3. Storage protein accumulation during conifer embryogenesis

It is well known that conifer seed embryos contain prominent protein bodies (Flinn *et al.* 1989, Green *et al.* 1991, Mia and Durzan 1974), but qualitative descriptions of conifer storage proteins have only been reported over the past few years,

(Gifford 1988, Gifford and Tolley 1989, Green *et al.* 1991, Misra and Green 1990, Misra and Green 1991, Stabel *et al.* 1990) during the course of this present work. Few biochemical studies have been carried out during conifer embryogenesis. Johnson *et al.* (1987) followed changes in buffer-soluble protein in developing red and white pine embryos. However, this study did not deal specifically with storage proteins, and since most conifer seed proteins are buffer-insoluble and require SDS or urea for solubilization (Gifford 1988, Gifford and Tolley 1989, Green *et al.* 1991), this study did not examine the major protein accumulation patterns. Recently, Janick *et al.* (1991) followed changes in embryo length, dry weight and fatty acid content during loblolly pine seed development, but storage protein accumulation was not addressed specifically. Therefore, the biochemistry, molecular biology and developmental regulation of conifer storage proteins requires further study.

2.2.4. Comparison of storage protein accumulation in zygotic and non-zygotic embryos

The studies described in the previous sections dealt with zygotic embryogenesis. Only a few studies have concentrated on storage protein gene expression in non-zygotic (somatic and microspore-derived) embryos and have shown that non-zygotic embryo storage protein expression differs temporally and/or quantitatively from the patterns observed in zygotic embryos. Shoemaker *et al.* (1987) noted that the pattern of storage protein synthesis, processing and accumulation in cotton somatic embryos paralleled that reported in zygotic embryos, although somatic

embryos accumulated their proteins at earlier stages and in smaller amounts. Similar observations were reported for microspore-derived rapeseed embryos (Crouch 1982). In alfalfa somatic embryos, Stuart *et al.* (1988) found that both 7S and 11S storage proteins accumulated to only 10% of the level found in zygotic embryos, while Krochko *et al.* (1989) found that alfalfa somatic embryos contained altered proportions of the 7S and 11S storage proteins.

However, none of the above studies included ABA during their differentiation protocols. Since ABA is known to influence embryo maturation and storage protein gene expression (see section 2.3 of Literature Review), the absence of exogenous ABA could explain the altered storage protein expression found in these embryos. Recent evidence may support this view. Rapeseed microspore-derived embryos treated with ABA displayed similar storage protein mRNA induction to that observed in equivalent stage zygotic embryos, however, storage protein accumulation was not examined. The developmental timing of storage protein gene expression was also similar to that of zygotic embryos (Wilén *et al.* 1990).

The few investigations carried out with conifer somatic embryos showed that those of Norway spruce and white spruce contained distinct protein bodies (Von Arnold and Hakman 1988, Hakman and von Arnold 1988). In addition, Hakman *et al.* (1990) reported that Norway spruce somatic embryos contained similar proteins to those found in zygotic embryos, and Joy IV *et al.* (1991) found that white spruce somatic embryos that

differentiated on low ABA levels contained less lipid and total protein, but more starch than their zygotic counterparts.

The work to date with conifer somatic embryos has not included developmental comparisons of storage protein accumulation and gene expression. These studies are required if we are to make comparisons between zygotic and somatic embryos and increase our understanding of conifer embryonic gene expression.

2.3. ABSCISIC ACID, OSMOTIC STRESS AND EMBRYO DEVELOPMENT

The plant growth regulator ABA is believed to modulate numerous aspects of plant growth and development (for reviews see Creelman 1989, Zeevaart and Creelman 1988). It has been implicated in such diverse processes as stomatal closure, seed and bud dormancy, stress adaptation, gravitropism and growth inhibition. For the purpose of this review, discussion will be limited to detailed studies of ABA effects on seed/embryo development.

Absciscic acid has been found to increase during angiosperm seed development, showing one or two peaks of accumulation, followed by a decline during late embryo development and desiccation (Ackerson 1984a, Finkelstein et al. 1985, Galau et al. 1987, Pence 1991). It is commonly considered to function as a growth inhibitor. Immature zygotic embryos will germinate precociously when cultured *in vitro* on growth regulator-free medium, conditions which allow depletion of the endogenous ABA pool (Ackerson 1984b, Finkelstein et al. 1985, Rivin and Grudt 1991). Furthermore, the continued culture of these embryos on ABA-containing medium prevents this germination (Eisenberg and

Mascarenhas 1985, Finkelstein *et al.* 1985, Rivin and Grudt 1991). Application of exogenous ABA also prevents the precocious germination of somatic embryos (Ammirato 1974, Boulay *et al.* 1988, Roberts *et al.* 1990a). Further support for the role of endogenous ABA in the control of embryo germination comes from the study of ABA-deficient and ABA-insensitive mutants, such as the maize viviparous (*vp*) mutants, which are characterized by an uninterrupted progression from embryogenesis to germination (Kriz *et al.* 1990, Rivin and Grudt 1991).

While these studies suggest that ABA is inhibitory during the later stages of development, it also has a promotory role in embryo maturation. Crouch and Sussex (1981) found that ABA promoted embryo growth in rapeseed. Similarly, soybean embryos cultured in the presence of ABA showed a close correlation between ABA levels and growth, with a stimulation of growth and dry weight accumulation during early phases of embryogenesis, and growth suppression by mid-stage embryo development (Ackerson 1984b). A further promotory effect has been noted in somatic embryo differentiation systems, where ABA enhances both the number and morphological normality of the embryos (Ammirato 1974, Kamada and Harada 1981, Roberts *et al.* 1990a).

Abscissic acid has also been implicated in the development of desiccation tolerance during embryogenesis. Late Embryogenesis Abundant (Lea) proteins accumulate during the mid to later stages of embryo development, just prior to maturation drying (Galau *et al.* 1987). These proteins, because of their structure and timing of accumulation, are believed to serve a protective role during

desiccation (Dure et al. 1989). The expression of some of the Lea mRNAs mirror changes in endogenous ABA levels, while others do not (Galau et al. 1987), and the precocious appearance of these proteins can be induced by exogenous ABA (Galau et al. 1986). Furthermore, excised zygotic or somatic embryos treated with ABA show enhanced tolerance to partial or complete desiccation (Bochicchio et al. 1991, Kim and Janick 1991, Roberts et al. 1990b, Senaratna et al. 1990). Koornneef et al. (1989) found that ABA was required for the development of desiccation tolerance in *Arabidopsis thaliana* mutants. Using recombinants from crosses containing mutations for ABA deficiency (*aba*) and reduced ABA sensitivity (*abi3*), these workers found that desiccation intolerance in seeds only occurred when both maternal and embryonic genotypes were double recessive; ie. seeds homozygous recessive for both ABA deficiency and reduced ABA sensitivity (*aba/abi3*) that arose from the self-fertilization of an individual heterozygous for ABA deficiency and homozygous recessive for reduced ABA sensitivity (*aba/ABA, abi3/abi3*) were still capable of desiccation and were viable.

In addition to the above effects on seed/embryo development, ABA has also been implicated in dormancy induction. The most compelling evidence for this again comes from the study of *Arabidopsis* mutants. Mutants deficient in ABA or with reduced ABA sensitivity display reduced dormancy (Karssen et al. 1983, Koornneef et al. 1984). Furthermore, the use of double mutants has shown that dormancy induction in the developing seed requires embryonic ABA. Maternal ABA or exogenously applied ABA did not

induce dormancy in seeds that were homozygous for the *aba* mutation (Koornneef *et al.* 1989).

Different stages of seed growth and development are characterized by patterns of differential gene expression. It has been suggested that approximately 20,000 diverse genes are expressed at the mRNA level at any particular stage of seed development (Goldberg *et al.* 1989). Several proteins that are expressed during embryo development have been found to be affected by ABA (Bartels *et al.* 1991, Bochicchio *et al.* 1991, Galau *et al.* 1986, Hatzopoulis *et al.* 1990, Williamson and Quatrano 1988). The most highly expressed genes during seed development are those of the storage proteins, whose mRNAs can represent at least 50% of total mRNA at mid-maturation (Goldberg *et al.* 1989). The potential role of ABA in the regulation of expression of these genes has been studied intensively.

Evidence for the *in situ* regulation of storage protein accumulation by ABA in zygotic embryos is based primarily on the fact that endogenous ABA levels increase during embryo development and are correlated with the synthesis of storage reserves (Bewley and Black 1985, Finkelstein *et al.* 1985). Most work with zygotic embryos has utilized excised embryos cultured on ABA-containing media. This type of experiment has shown that ABA induces storage protein accumulation in immature embryos of rapeseed (Crouch and Sussex 1981), mustard (Croissant-Sych and Bopp 1988), soybean (Ackerson 1984a, Eisenberg and Mascarenhas 1985) and wheat (Williamson *et al.* 1985), as well as in cultured broad bean cotyledons (Barratt 1986) and endosperm of *Solanum*

species (Smith and Desborough 1987). However, this characteristic is not true for all plants, as ABA does not appear to be associated with storage protein expression in cotton, either *in vivo* or *in vitro* (Dure and Galau 1981, Galau et al. 1987).

In embryos where ABA does affect storage protein accumulation, the response to ABA varies with developmental stage. Younger embryos are more responsive to ABA, while older embryos are less so (Bray and Beachy 1985, Croissant-Sych and Bopp 1988, Eisenberg and Mascarenhas 1985, Finkelstein et al. 1985), suggesting a decline in ABA sensitivity during maturation. In addition, while ABA is required for 11S storage protein and mRNA accumulation in early and mid-maturation cultured soybean embryos, it causes a decline in 11S protein mRNA (Eisenberg and Mascarenhas 1985) at younger stages.

In embryos that do show storage protein response to ABA, not all storage proteins are equally affected. In cultured soybean cotyledons, the accumulation of the β -subunit protein and mRNA of β -conglycinin is stimulated by ABA, while the α/α' -subunits are not (Bray and Beachy 1985). Also, legumin storage protein accumulation by cultured pea embryos is not stimulated by ABA (Davies and Bedford 1982), whereas pea vicilin synthesis is enhanced by exogenous ABA application to seeds (Schroeder 1984).

The enhancement of storage protein accumulation by ABA appears to be due to increased storage protein mRNAs (Finkelstein et al. 1985, Goffner et al. 1990, Kriz et al. 1990, Taylor et al. 1990). These increases are dose-responsive, with higher ABA causing

increased mRNA levels (Finkelstein et al. 1985). Different storage protein mRNAs are increased to different degrees by ABA, as seen in cultured 27 DAA rapeseed embryos, where cruciferin mRNA levels were increased 2-fold and napin mRNA levels were increased 4-fold by 1 μ M ABA (Delisle and Crouch 1989). In addition, Eisenberg and Mascarenhas (1985) noted that the mRNAs of different members of the soybean glycinin gene family accumulated to different degrees in response to ABA.

The mechanism by which ABA maintains or stimulates storage protein synthesis and accumulation is complex. The response to ABA appears primarily transcriptional (Mansfield and Raikhel 1990, Williamson and Quatrano 1988), with the higher mRNA levels paralleled by higher transcription rates (Delisle and Crouch 1989). However, post-transcriptional, translational and post-translational regulation have also been suggested. Williamson and Quatrano (1988) noted that wheat E_m protein (an albumin storage protein) mRNA showed ABA-mediated stabilization, suggesting post-transcriptional regulation. However, E_m protein synthesis rates closely paralleled E_m mRNA levels, suggesting no control by ABA at the translational level. Some post-translational regulation is implicated, because, in the absence of ABA, E_m synthesis continued but no appreciable accumulation occurred. This suggests that ABA may help to stabilize the protein and allow its accumulation (Williamson et al. 1985). Finkelstein et al. (1985) found that excised rapeseed embryos cultured on 1 μ M ABA accumulated cruciferin at the same rate as in seed embryos, although the mRNA levels were lower in the

cultured embryos. These workers suggested that ABA may have enhanced cruciferin translation, since there was less cruciferin mRNA but more cruciferin protein accumulated per embryo at increased ABA concentrations.

The previously described studies used exogenous ABA to study effects on storage protein accumulation. Further insight into ABA effects on storage proteins have been gained from ABA mutants. Groot *et al.* (1991) noted that tomato mutant *sitiens*, which has strongly reduced ABA levels, did not differ from wild type in seed fresh weight, dry weight or storage protein content. While this suggested no role for ABA in seed development, there were indications that this mutant was "leaky", with enough endogenous ABA to allow normal development. Koornneef *et al.* (1989) had examined this proposal earlier by constructing *Arabidopsis* double mutants using recombinants of ABA-deficient *aba* with ABA-insensitive *abi1* or *abi3*. They found that *aba*, *abi3* double mutants did not display storage protein accumulation and that aberrant seed development occurred when the reduced ABA level of the *aba* mutant was combined with the reduced sensitivity of the *abi3* mutant. Finkelstein and Somerville (1990) found that *Arabidopsis* wild type and *abi1* and *abi2* mutants had similar levels of storage protein, but *abi3* mutants contained significantly less storage protein and storage protein mRNA. These results indicated that the *abi3* locus is important for seed responses to ABA and that ABA levels and sensitivity are important for storage protein gene expression during embryo development.

The normal process of seed development includes a loss of water content. It has been suggested that changes in the internal osmotic environment of the seed play a role in embryo maturation. In support of this, numerous studies have shown that osmotic stress influences embryo development. In somatic embryo differentiation systems, osmotic stress during early development can enhance the number of globular embryos produced (Litz 1986, Roberts 1991). However, development past this point may require subculture to medium of lower osmolarity (Litz 1986). In addition, later stages of embryo development can be enhanced and accelerated (Nadel *et al.* 1989) and precocious germination inhibited by osmotic stress (Finkelstein and Crouch 1986, Roberts 1991, Xu *et al.* 1990).

Apart from morphological development, osmotic stress has been shown to stimulate storage protein accumulation in excised zygotic (Finkelstein and Crouch 1986, Goffner *et al.* 1990, Xu *et al.* 1990) and microspore-derived embryos (Wilén *et al.* 1990). Since osmotic stress can cause elevated ABA levels in vegetative tissues (Skriver and Mundy 1990), it has been suggested that osmotic effects on embryo development are mediated via increased ABA levels. Some studies support this. Wilén *et al.* (1990) noted that rapeseed microspore-derived embryos cultured on 12.5% sorbitol showed induction of storage protein mRNAs, albeit at slower rates than after exogenous ABA application. Measurements of endogenous ABA changes during sorbitol treatment revealed an increase in ABA that preceded storage protein mRNA induction, suggesting that osmotically-induced ABA stimulated storage

protein gene expression. Similarly, broad bean cotyledons cultured on high osmoticum (18% sucrose) accumulated vicilin and legumin storage proteins (Barratt 1986). The combined effect of fluridone (an inhibitor of endogenous ABA biosynthesis) with the high osmoticum treatment inhibited the storage protein increase. Exogenous ABA counteracted this inhibition. Additionally, maize ABA-deficient *vp5* mutants accumulated globulin storage proteins after exposure to ABA, whereas those cultured on high osmoticum alone did not (Rivin and Grudt 1991). This was attributed to the fact that these embryos could not synthesize ABA in response to osmotic stress.

All of these studies suggest that osmotic stress-induced alterations in ABA levels are responsible for the changes in gene expression, and that ABA is directly involved in this response. In contrast, other workers have found that ABA changes do not occur in response to osmotic stress. Finkelstein and Crouch (1986) noted that rapeseed embryos cultured on sorbitol accumulated storage protein and storage protein mRNA, but no transient increase in ABA content was observed. In sunflower, 11S storage protein and mRNA increased during culture on sorbitol (Goffner *et al.* 1990). The increase in mRNA level was more pronounced for culture on sorbitol than on ABA, and no stimulation of ABA levels or ABA biosynthesis was detected in response to the osmotic stress. Barratt *et al.* (1989) found that pea pods cultured for 14 days on medium containing ABA or ABA plus fluridone contained no differences in legumin or vicilin translatable mRNA, suggesting that ABA did not play a role in pea

storage protein gene expression. Finally, Xu *et al.* (1990) noted that alfalfa embryos cultured on ABA or osmoticum remained embryonic and did not germinate, but only embryos cultured on osmoticum synthesized embryo developmental proteins (including storage proteins). This suggested that the osmotic prevention of germination and maintenance of developmental gene expression was not mediated through ABA.

Since ABA and osmoticum do inhibit water uptake (Finkelstein and Crouch 1986, Schopfer and Plachy 1984), it may be that the involvement of ABA in the above responses is indirect, and that the major effector causing changes in developmental gene expression is the water status of the embryo, which is altered by osmotic stress or by ABA.

It is evident that the role of ABA in the regulation of gene expression is still unclear. Further work is required to identify putative ABA receptors and elucidate the signal transduction pathways. The identification of upstream regulatory regions of ABA-responsive and osmotically-induced genes should help to clarify the role of ABA and osmotic stress in gene regulation.

2.4. SUMMARY

Major advances in tissue culture have made the attainment of conifer somatic embryos and plants a reality. In order to gauge the quality of these embryos and plants, biochemical and physiological comparisons between zygotic embryo- and somatic embryo-derived material is required. Comparisons of storage proteins in angiosperm zygotic and somatic embryos revealed

several differences between the two systems. These differences may be attributed to the absence of ABA during differentiation *in vitro*. Work to define storage protein accumulation patterns and the effects of ABA on storage proteins at both the protein and molecular level during conifer embryogenesis has still to be addressed.

3. MATERIALS AND METHODS

3.1. ZYGOTIC EMBRYO MATERIAL

Interior spruce from the interior of British Columbia is a mixture of two closely related species *Picea glauca* (Moench) Voss and *Picea engelmannii* Parry (Owens and Molder 1984). Seed cones were collected during 1988 and 1989 at the British Columbia Ministry of Forests Kalamalka Research Station, Vernon, B.C. from source tree EK10, which was open pollinated and possessed a *Picea engelmannii* maternal background. Due to poor EK10 cone yield during 1991, seed cones were collected from the adjacent tree EK11. Cones were received the day after collection and stored at 4°C until embryo excision, which took place within a few days of receipt. Embryos were classified according to their developmental stage based on morphological criteria established by Buchholz and Stiemert (1945) and stored at -80°C until required. Embryos with suspensors and small embryonal heads (proembryos) were obtained in very small numbers. Stage 2 embryos were torpedo-shaped, possessed a visible apical dome but lacked cotyledon primordia. Stage 3 embryos possessed cotyledons that had not developed above the apical dome. Embryos collected during 1989 were abundant enough to allow further sub-classification into Stage 3A (cotyledons visible as small stubs, but not very large) and Stage 3B (cotyledons larger with distinct clefts between them, but not overgrowing the shoot apex). Collections during 1988 and 1991 did not contain sufficient embryos to allow this separation, so all Stage 3 embryos were grouped together. Stage 3-4 embryos had cotyledons that had

overgrown the shoot apex, but had not yet completely covered the apical dome. Stage 4 embryos had cotyledons that completely enclosed the apical dome. Collections were made weekly during 1989 and 1991, with the last dated collection being mature seed embryos. Collections made during 1988 were not as frequent, and Stage 4 embryos (excluding mature desiccated embryos) were only obtained at 3 collection dates, which represented increasing stages of embryo age (Stage 4-1: collected July 13; Stage 4-2: collected July 27; Stage 4-3: collected August 24).

To study the effects of germination on embryo proteins, seeds were surface sterilized in 10% (v/v) commercial bleach containing 0.1% (v/v) Tween 80 for 15 min, washed three times with sterile distilled water and imbibed overnight at 4°C in the dark. Imbibed seeds were placed on a water-saturated Kimpak (Seedburo Equipment Co., Chicago, IL) inside a Magenta GA7 vessel (Magenta Corp., Chicago, IL) and germinated at 27°C in a 16-h photoperiod with a light intensity of 30 $\mu\text{E}/\text{m}^2/\text{sec}$. Seeds were examined at three-day intervals and classified according to extent of radical emergence. Germinants of similar development were removed and stored at -80°C.

3.2. SOMATIC EMBRYO MATERIAL

All culture media used were autoclaved, all growth regulators were added prior to sterilization and all media were solidified with 0.6% Noble agar (Difco Laboratories, Detroit, MI) unless otherwise stated. The ABA used in experiments was (+,-)-cis, trans-Abscisic acid (Sigma Chemical Co., St. Louis, MO). All cultural manipulations were carried out under sterile conditions

and all cultures were grown at 27°C in either the dark or light (25-40 $\mu\text{E}/\text{m}^2/\text{sec}$).

Somatic embryo cultures were available which had been induced from individual zygotic embryos of trees EK10 and PG118 (Webb *et al.* 1989), and identified as distinct lines (W29, W74, W76, W77 from EK10; W70 and W46 from PG118). Embryogenic cultures were maintained in the dark on Litvay's medium (Litvay *et al.* 1985) using half-strength macronutrients containing 1% sucrose (1% $\frac{1}{2}$ LM medium) and 10 μM 2,4-D and 5 μM BA. Cultures were subcultured to fresh media every 2 weeks.

Somatic embryo differentiation was carried out in the light, and initially used the protocol of Roberts *et al.* (1990a). Embryogenic tissues containing proembryos were subcultured to VE medium (von Arnold and Eriksson 1981), containing 3.4% sucrose (3.4% VE medium), no growth regulators and 1% charcoal, for one week. The ABA-dependent developmental profile was obtained from embryogenic tissues (approximately 150 mg pieces) that were placed onto 3.4% VE medium containing 1 μM indole-3-butyric acid (IBA) and various levels (0, 20, 40 or 60 μM) of ABA for 5 weeks, with subculture to fresh medium every 2 weeks. Following this maturation, embryos were classified according to morphological appearance (Roberts *et al.* 1990a) as mature (remained opaque with well-developed cotyledons) or precociously germinating (exhibited extensive greening and elongation of the cotyledon-hypocotyl axis). Aberrant structures that appeared as an abnormal elongation of cotyledons from a basal callus were classified as shooty structures. The number of visible structures

were counted after 5 weeks. All subsequent somatic embryo differentiation was performed using 40 μM ABA, except for the developmental study to compare effects of high and low ABA levels, in which case 10 μM ABA was also used.

Somatic embryos on 40 μM ABA were classified according to developmental stage (Table 1), using a modification of the scheme described by von Arnold and Hakman (1988). Early cotyledonary somatic embryos were designated Stage 3 embryos. Once the cotyledons had overgrown the shoot apex (Stage 3-4), the somatic embryos resembled Stage 4 zygotic embryos, and were classified as Stage 4 somatic embryos. Somatic embryos reached maximum length after approximately 6 weeks on ABA and did not lengthen much more during the time of these experiments, and were thereafter classified as 7-week (Stage 4-7), 8-week (Stage 4-8) or 9-week (Stage 4-9) embryos. In order to compare the pattern of storage protein accumulation during the later stages of maturation, zygotic and somatic embryos were identified on the basis of time since onset of cotyledon development.

Somatic embryos on 10 μM ABA developed in a similar manner to those on 40 μM ABA, but once the cotyledons had overgrown the shoot apex (Stage 4-2), the embryos showed the extensive chlorophyll accumulation and hypocotyl/cotyledon elongation that was characteristic of precocious germination. These embryos could not be classified using the length/morphology criteria applied to non-germinating embryos on 40 μM ABA and hence were referred to as germinants (Germ 1-6). For the developmental comparisons, embryos cultured on both 10 and 40 μM ABA were

TABLE 1. Somatic embryo developmental stages on 40 μ M ABA

| | Stage | Weeks after onset of cotyledon development | Description |
|--------------------|-------|--|---|
| Early Cotyledon | Pe | | Proembryos on maintenance calli |
| | Glob | | Embryos with globular heads |
| | Round | | Torpedo embryos with round heads |
| | Flat | | Torpedo embryos with flat heads |
| | 3-1 | 0 | Small cotyledon primordia barely visible |
| | 3-2 | 0.2 | Primordia enlarged with clefts visible between them, but not overgrown the shoot apex |
| | 3-3 | 1.0 | Cotyledons enlarged and slightly overgrown the shoot apex, but embryo less than 1 mm long |
| | 3-4 | 1.4 | Embryos 1.0-1.25 mm long |
| | 4-1 | 1.7 | Embryos 1.3-1.5 mm long |
| | 4-2 | 2.1 | Embryos 1.55-1.75 mm long |
| | 4-3 | 2.4 | Embryos 1.8-2.0 mm long |
| | 4-4 | 2.9 | Embryos 2.05-2.25 mm long |
| | 4-5 | 3.3 | Embryos 2.3-2.5 mm long |
| | 4-6 | 4.1 | Embryos 2.55-2.75 mm long |
| | 4-7 | 4.6 | Embryos 2.55-2.75 mm long |
| Late Cotyledon | | | and on ABA for 7 weeks |
| | 4-8 | 5.6 | Embryos 2.55-2.75 mm long and on ABA for 8 weeks |
| | 4-9 | 6.6 | Embryos 2.55-2.75 mm long and on ABA for 9 weeks |

collected for analysis after the same exposure time to ABA. Germ 1 (2-2.25 mm in length) precociously germinating embryos were collected at the same time as Stage 4-3 embryos, and Germ 6 (8+ mm in length) advanced precocious germinants were collected at the same time as Stage 4-8 embryos. The intermediate stages formed a developmental continuum of increasing size.

Embryos differentiated during the 40 μ M/10 μ M ABA developmental comparison used a modified differentiation protocol. Embryogenic tissues were grown for 1 week in suspension culture (10% w/v) in liquid 1% $\frac{1}{2}$ LM medium containing 2,4-D and BA rotating at 120 rpm. The suspensions were then filtered through 2 layers of Miracloth (Calbiochem Corp., La Jolla, CA) and washed 3 times with 1% $\frac{1}{2}$ LM medium containing no growth regulators. The resulting tissue mass was resuspended (20% w/v) in growth regulator-free 1% $\frac{1}{2}$ LM medium. The resuspended tissue (750 μ l) was plated onto black 0.8 μ m filter discs (Millipore Corp., Bedford, MA), with a filter paper absorbant backing to remove excess medium. The filter disc was separated from the absorbant pad and placed onto solid 3.4% VE medium containing 1 μ M IBA and 40 μ M ABA as described previously.

For the osmotic stress experiments, embryogenic cultures obtained via the suspension culture method were differentiated on solid 3.4% VE medium containing 1 μ M IBA and 40 μ M ABA. Embryogenic tissues containing Stage 3-4 embryos were removed and placed onto solid 3.4% VE medium containing no growth regulators, 40 μ M ABA, fluridone, 15% mannitol or combinations of the above. Fluridone (kindly supplied by Dr. Franklin Fong, Texas A & M

University) was made up as a 2.5 mg/ml stock solution in acetone and added as required. All of the above compounds were added to the media prior to autoclaving. Cultures were grown for a further 2 weeks and then embryos were removed and stored at -80°C until required. Four cultures were used per treatment.

3.3. PROTEIN EXTRACTION AND ELECTROPHORESIS

All protein and immunoblot analyses were performed using line W29 except where indicated. For analysis of proteins by sodium dodecylsulfate-polyacrylamide electrophoresis (SDS-PAGE), embryonic tissues were homogenized in Eppendorf tubes with 30-40 μl 4x SDS sample buffer [562.5 μl 0.5 M Tris-HCl pH 6.8, 225 μl 2-mercaptoethanol, 225 μl glycerol, 9% (w/v) SDS, 0.5 mg bromophenol blue] per mg tissue. Samples were boiled for 7 min, centrifuged (10 min; 14,000 rpm) in a bench top microfuge and stored at -80°C until needed. Protein was determined by the modified method of Ghosh et al. (1988), in which 2 μl of sample was dot-blotted, incubated in 1 ml 1% (w/v) SDS overnight and measured spectrophotometrically at 595 nm. Bovine serum albumin (BSA) was used as a standard.

For two-dimensional electrophoresis, extracts in 4x SDS sample buffer were dialyzed overnight at 4°C against 4 L deionized water, frozen at -80°C and lyophilized. The samples were resuspended in 13 μl modified 2D-MH (Mayer et al. 1987) buffer [9.6 ml of buffer contained 100 μl Pharmalyte 3-10 (Pharmacia Inc., Montreal, QU), 175 mg NaCl, 3.7 mg Na_2EDTA , 3.8 mg EGTA, 200 μl Triton X-100, 8.8 mg ascorbic acid, 154 mg dithiothreitol (DTT), 100 μg leupeptin, 100 μg α -macroglobulin, 2% (w/v) SDS and

deionized water] containing 9.5 M urea. Just before sample application, 0.5 μ l Pharmalyte (5-8 or 3-10) was added.

For one-dimensional SDS-PAGE analysis, gels consisted of a 12% separating gel and a 5% stacking gel, with the Laemmli buffer system (Laemmli 1970). For two-dimensional analysis, samples were run into a 10 cm x 2 mm tube gel. The gel solution consisted 4.86 g urea, 2.88 ml deionized water, 1.18 ml acrylamide-bis-acrylamide (31% T, 4.3% C), 2.03 ml 10% (v/v) Triton X-100 and 500 μ l Pharmalytes (Pharmalyte 3-10 or 450 μ l Pharmalyte 5-8 + 50 μ l Pharmalyte 3-10), and was polymerized with 2 μ l 20% ammonium persulfate and 3 μ l TEMED per ml of gel solution. The sample was applied at the cathode end and covered with overlay buffer [2 ml 10% (v/v) Triton X-100, Pharmalytes (500 μ l Pharmalyte 3-10 or 450 μ l Pharmalyte 5-8 + 50 μ l Pharmalyte 3-10), 7.5 ml deionized water]. The cathode solution was 0.1 N NaOH and the anode solution was 0.06% phosphoric acid. Gels were run at 400 V constant voltage to a total of 7100 Vh. After electrophoresis, tubes were placed on ice for 20 min, the gels extruded, incubated in 4x SDS sample buffer for 20 min and applied to a stacking gel. The tube gel was overlaid with 1% (w/v) agarose in 12.5 mM Tris-HCl pH 6.8. After electrophoresis, gels were fixed with aqueous 40% methanol + 10% acetic acid and stained with Coomassie R-250 or silver reagent (Wray et al. 1981).

The gels were photographed using Ektachrome 160 (Kodak, Toronto, ON) colour slide film and quantification of protein bands was carried out by scanning densitometry of each colour

slide at 560 nm using a DU-64 Spectrophotometer (Beckman Instruments Inc., Mississauga, ON). Results from scans of 3 individual total protein profiles were used to calculate the percentage of the major storage proteins relative to total protein.

3.4. PROTEIN BODY ISOLATION AND ANALYSIS

Protein bodies were isolated from whole embryos based on the technique of Chrispeels *et al.* (1982). Approximately 80-100 mature zygotic embryos or 50 Stage 4-9 somatic embryos were placed on ice in a petri dish containing 2 ml Medium A (100 mM Tris-HCL pH 7.8, 1 mM EDTA), 12 % (w/w) sucrose and 2 mM $MgCl_2$. Embryos were macerated and the resulting slurry filtered through one layer of 53 μm nylon mesh. The petri dish was rinsed with 1 ml of the same medium. This 3 ml filtrate was layered over 8 ml Medium A containing 16% (w/w) sucrose and 2 mM $MgCl_2$ in a centrifuge tube and centrifuged (40 min: 2,800 rpm) at 4°C, using a SW41 swinging bucket rotor in an L8-M Ultracentrifuge (Beckman Instruments Inc., Mississauga, ON). The protein body pellet was resuspended in 80-100 μl 4x SDS sample buffer, and the sample treated as described above for SDS-PAGE. Extractions under non-reducing conditions were performed in 4x SDS sample buffer minus 2-mercaptoethanol. The non-reduced samples were analyzed under reducing conditions by cutting slices from non-reducing gels following SDS-PAGE and incubating them in 4x SDS sample buffer. They were then placed on top of the stacking gel and electrophoresed under standard conditions.

Buffer-soluble and buffer-insoluble proteins were separated from the protein body pellet by extraction with 35 μ l of 0.05 M sodium phosphate buffer (pH 7.5) and centrifugation (10 min; 14,000 rpm). The supernatant was removed, the pellet re-extracted and centrifuged, and the supernatant fractions were pooled to give the buffer-soluble fraction. This was mixed with an equal volume of 4X SDS sample buffer and treated as described previously for SDS-PAGE. The insoluble pellet was extracted with 100 μ l of a 1:1 mixture of sodium phosphate buffer:4x SDS sample buffer and treated for SDS-PAGE as described previously. The solubility characteristics of the storage proteins were examined using Osborne's (1924) criteria. The protein body pellet was extracted twice with 25 μ l of either deionized water, sodium phosphate buffer, buffer containing 0.2 M NaCl or buffer containing 1 M NaCl. The extracts were mixed with 50 μ l of 4x SDS sample buffer and treated for SDS-PAGE as described above.

3.5. MICROSCOPY

Embryos from late August seed collections were excised, fixed overnight in 25 mM potassium phosphate buffer (pH 6.8) containing 2.5% (v/v) glutaraldehyde and post-fixed for 2 hr in buffered 1% (w/v) osmium tetroxide. Specimens were dehydrated through a graduated acetone series, transferred to propylene oxide, then propylene oxide:Spurr's resin (1:1) (Spurr 1969) and embedded in Spurr's resin. After polymerization of the resin for 24 hr at 60°C, sections (1 μ m thick) were cut, stained using the periodic acid-Schiff's (PAS) reagent (Jensen 1962) for polysaccharides,

and counter-stained with aniline blue-black (Jensen and Fisher 1968) for proteins.

3.6. ANTIBODY PRODUCTION

The 41 kD or 24 + 22 kD bands were excised from SDS-PAGE gels of protein body extracts, dried, ground to a fine powder in liquid nitrogen and added to a 1:1 mixture of phosphate-buffered saline:Freund's complete adjuvant. A total of 100-200 μ g protein was injected into New Zealand white rabbits. Booster injections of 100-200 μ g protein in a mixture of phosphate-buffered saline:Freund's incomplete adjuvant (1:1) were given 4 weeks later. Rabbits were bled 1 week later, the blood allowed to clot overnight at 4°C then the supernatant was removed and centrifuged (15 min; 4,000 rpm). The straw-coloured supernatant anti-serum was removed and used for subsequent immunoblots. The 41 kD anti-serum, which displayed slight cross-reactivity with a high molecular weight embryo protein, was affinity-purified by the method of Smith and Fisher (1984).

3.7. IMMUNOBLOTTING

Proteins separated by SDS-PAGE were blotted onto nitrocellulose overnight at 30 V using the Transblot apparatus (Bio-Rad, Mississauga, ON) and transfer buffer consisting of stock buffer [glycine 100 g/L, Tris base 80 g/L pH 8.7, 2 g/L SDS]:methanol:deionized water (1:2:7). The filters were treated with Tris-buffered saline pH 7.4 (TBS) containing 3% (w/v) Carnation powdered skim milk (CM) for 1 hr, washed 3 times for 5 min each with TBS + 0.5% (v/v) Tween 80, then incubated overnight in TBS containing 1% (w/v) CM and a 1:400 dilution of primary

antibody solution. Filters were rinsed 3 times for 5 min each with TBS + Tween, incubated for 1 hr in TBS + 1% (w/v) CM containing a 1:3000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad, Mississauga, ON). Filters were then rinsed 3 times for 5 min each with TBS + Tween and colour developed following the Bio-Rad protocol. The blots were photographed and scanned by densitometry of photographic slides as described previously.

3.8. CHLOROPHYLL ANALYSIS

Chlorophyll analysis of somatic embryos was based on the protocol of Hiscox and Israelstam (1979). Somatic embryos of lines W29 and W70 were collected at Stages 4-6 and 4-9. Embryos were placed into screw-capped vials containing 20 μ l dimethyl sulfoxide (DMSO)/mg fresh weight, that had been heated at 65°C for 10 min. Samples were shaken to submerge the embryos in solvent, and incubated at 65°C for 45 min. The vials were centrifuged (14,000 rpm) for a few seconds to recover solvent droplets condensed on the side, and the absorbence of each sample was measured at 645 and 663 nm against a DMSO blank. Total chlorophyll was calculated using the equation:

$$\text{Total [Chlorophyll] in mg/g} = \frac{(20.2 D_{645} + 8.02 D_{663}) \times V \text{ (ml)}}{1000 \times W \text{ (g)}}$$

Where D = absorbence at the wavelength stated

V = total volume of the chlorophyll solution

W = weight of the fresh tissue extracted

Analyses were carried out twice with samples from separate experiments.

3.9. IN VIVO PROTEIN LABELLING AND IMMUNOPRECIPITATION

Stage 4-7 somatic embryos of line W29 were placed into a sterile Eppendorf tube containing 30 μ Ci of Tran ^{35}S -Label (a mixture of 70% L-methionine, 15% L-cysteine; ICN Biomedicals, Mississauga, ON; 1011 Ci/mmol) in 300 μ l of liquid 3.4% VE medium containing 1 μ M IBA and 40 μ M ABA. Embryos were allowed to label for 4 hr at room temperature, the solution was removed, the embryos were rinsed with medium containing 1 mM unlabelled methionine and cultured in 500 μ l of medium containing 10 μ M unlabelled methionine. Embryo samples were collected after 0 (immediately after the rinse), 2, 4, 8, 16 and 24 hr of chase. Proteins were extracted from embryos using 4x SDS sample buffer as described previously and the trichloroacetic acid (TCA)-precipitable radioactivity determined. Briefly, five μ l of each sample was spotted onto a filter paper disc, allowed to dry, and incubated in cold 10% (w/v) TCA containing 1 mg/ml methionine for 10 min. The disc was boiled in hot 10% (w/v) TCA for 10 min, washed by vacuum suction with cold 10% (w/v) TCA followed by 95% ethanol, and allowed to dry. The sample spots were cut out, placed into a scintillation vial containing 1 ml of 0.1% (w/v) SDS and allowed to stand for 2 hr. Following this, 10 ml of Aquasol (Du Pont Corp., Mississauga, ON) was added and the radioactivity was determined using an LS 1801 Liquid Scintillation Counter (Beckman Instruments Inc., Mississauga, ON).

Total labelled protein profiles were obtained by application of samples containing equal TCA-precipitable radioactivity to

each gel, followed by SDS-PAGE as described previously. Following fixation, gels were rinsed twice (30 min each) in deionized water to remove excess fixative, placed in fluor (Enlightning; Du Pont Corp., Mississauga, ON) containing 10% (v/v) glycerol for 1 hr, dried at 60°C for 2 hr, and allowed to expose at -80°C using Kodak X-Omat AR film.

For immunoprecipitation, equal amounts of TCA-precipitable radioactivity were used for each reaction. For each individual sample, 11-15 μ l of labelled protein extract was added to 1200 μ l of BBS [10 mM sodium borate, 160 mM NaCl pH 7.9]. To this were added 36 μ l of 16.5% (w/v) BSA, 60 μ l of 10% (v/v) Triton X-100, 60 μ l of 250 mM methionine and 5 μ l (to give a final 1:275 dilution) of 41 kD protein antiserum. The reaction mixture was incubated for 1 hr with mixing at 37°C, and kept overnight at 4°C. The whole solution was added to a 20 μ l bed volume (approximately 11.5 mg) of Protein A-Sepharose CL4B (Pharmacia Inc., Montreal, QU), which had been washed 3 times with 100 μ l each of BBS. This mixture was shaken gently for 1 hr at room temperature and centrifuged (2 min; 14,000 rpm). The supernatant was discarded and the Protein A-Sepharose washed 3 times with BBS containing 10% (v/v) Triton X-100 and 250 mM methionine, followed by a single wash with 0.3x BBS. Fifteen μ l of 4x SDS sample buffer was added to the Protein A-Sepharose, the sample boiled for 7 min, centrifuged (5 min; 14,000 rpm) and the supernatant, containing the immunoprecipitated protein, removed. Ten μ l of each immunoprecipitated sample was analyzed by SDS-PAGE and the gel processed for fluorography as described above.

3.10. 41 kD STORAGE PROTEIN cDNA ISOLATION

A cDNA library constructed using poly (A) RNA from late cotyledonary somatic embryos cultured on 40 μ M ABA was obtained from Dr. Craig Newton at B.C. Research. Inserts had been cloned into the *Sma* I site of pUC 18 and plasmids transformed into competent SURE cells (Stratagene Cloning Systems, La Jolla, CA). The cDNA library was partially screened using 2 plates, each containing approximately 7500 colony forming units. Colonies were grown overnight at 37°C on YT medium [8 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 5 g/L NaCl] containing 100 μ g/ml ampicillin (YT + amp medium) and solidified with 15 g/L agar.

Replica filters of the plates were made using nitrocellulose (Bio-Rad, Mississauga, ON) and placed colony side up on YT + amp plates covered with 50 μ l of 100 mM Isopropyl β -D-Thiogalactopyranoside (IPTG). After overnight growth at 37°C, colonies on the filters were lysed for 30 min in chloroform vapour by suspension in a TLC tank equilibrated 30 min with chloroform. Filters were placed in a solution of 50 mM Tris-HCl pH 7.5, 150 mM NaCl and 5 mM MgCl₂, containing 1 μ g/ml DNase, 40 μ g/ml lysozyme and 3% (w/v) CM, and incubated overnight with agitation. They were rinsed 3 times with TBS-Tween, then incubated in TBS-Tween containing a 1:400 dilution of affinity purified 41 kD antibody. Washing, secondary antibody incubation and colour development of filter immunoblots was as described for SDS-PAGE immunoblots.

An antibody-positive colony was identified on the replica filter, the corresponding colony sampled and inoculated into 2 ml

of YT + amp broth for overnight growth. Following this, a dilution series was made and plated onto YT + amp plates. One hundred μ l of a dilution containing 10^2 colonies/ml was plated, grown overnight, a replica filter made and the immunoscreening procedure repeated as described previously. Several antibody-positive colonies were isolated, grown overnight in YT + amp broth and their plasmid DNA isolated by the alkaline lysis method described by Sambrook et al. (1989). Briefly, for each sample, 1.5 ml of bacterial culture was centrifuged (30 sec; 14,000 rpm), the medium removed and the pellet resuspended with 100 μ l of Solution I [50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA pH 8]. Following this, 200 μ l of freshly prepared Solution II [0.2 N NaOH, 1% (w/v) SDS] were added and the contents mixed by inverting the tube rapidly 5 times, and then 150 μ l of ice cold Solution III [60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml deionized water] were added, mixed, the tube kept on ice for 5 min and centrifuged (5 min; 14,000 rpm) at 4°C. The supernatant was transferred to a fresh tube, extracted with an equal volume of phenol:chloroform (1:1), mixed, centrifuged (5 min; 14,000 rpm), and the aqueous phase removed and mixed with 2 vol of 95% ethanol. The DNA was allowed to precipitate for 2 min at room temperature, the sample centrifuged (15 min; 14,000 rpm) at 4°C, the pellet washed with 95% ethanol, then 70% ethanol, vacuum dried and resuspended with 50 μ l TE buffer [10 mM Tris-HCl, 1 mM EDTA pH 8].

The plasmid cDNA inserts were excised using restriction enzyme digests. All restriction enzymes were used with the appropriate

buffers and conditions described by the suppliers, and all plasmid DNA samples were treated with 5% (v/v) of 10 mg/ml RNase during digestion to destroy contaminating RNAs. Two μ l aliquots of the plasmid DNA samples isolated above were digested with *Eco* RI-*Pst* I and analyzed by agarose gel electrophoresis. Ten μ l of each digest mix was added to 1 μ l Ficoll dye mix [0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 15% (w/v) Ficoll (Pharmacia) in water], and separated on a 1.2% (w/v) agarose gel in 1x TAE [50x TAE = 2 M Tris-acetate, 0.05 M EDTA pH 8], containing 0.5 μ g/ml ethidium bromide, with the electrophoresis buffer consisting of 1x TAE. Examination of samples under UV light revealed that they all contained the same sized insert fragment (1.7 kb). One colony was chosen for further use and designated as II5A.0. This plasmid DNA was digested with *Eco* RI-*Pst* I and the insert fragment separated on an agarose gel. The insert band was visualized under UV light, cut out and gel purified using the Prep-A-Gene method (Bio-Rad, Mississauga, ON). Briefly, the gel fragment was placed into an Eppendorf tube, the volume estimated and 3 vol binding buffer [60 mM sodium perchlorate, 1 mM EDTA, 50 mM Tris-HCl pH 7.8] was added. The sample was heated at 37-55°C for several minutes to dissolve the agarose, after which 10 μ l of Prep-A-Gene binding matrix was added. The sample was mixed by vortexing, then shaken for 10 min at room temperature. The sample was centrifuged (30 sec; 14,000 rpm), the supernatant removed and the pellet rinsed 3 times with 1 ml of a 1:1 mixture of 95% ethanol:wash buffer [400 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl pH 7.4], followed by suspension with TE

buffer. This was mixed by vortexing, centrifuged as described above, and the supernatant TE transferred to a fresh tube. One μ l of the TE fraction was diluted to 100 μ l with fresh TE buffer and the DNA quantified spectrophotometrically at 260 nm. The gel-purified DNA was used for II5A.0 probe. Other gel purified probes (XI5H and 18S rRNA) were obtained from Dr. Craig Newton.

3.11. GENERATION OF DELETION CONSTRUCTS FOR SEQUENCING

Two hundred ml of YT + amp broth was inoculated with bacteria containing the II5A.0 insert. Following overnight incubation at 37°C, nucleic acids were isolated using the alkaline lysis procedure. The resulting pellet was resuspended in 2.4 ml of TE buffer and the plasmid DNA isolated using cesium chloride-ethidium bromide (CsCl-EtBr) gradient centrifugation. Briefly, CsCl (4.2 g) and 400 μ l of 10 mg/ml EtBr were added to the 2.4 ml plasmid solution, and centrifuged (5 min; 6,000 rpm) at room temperature in a J2-21 Centrifuge (Beckman Instruments Inc., Mississauga, ON). A Ti70.1 centrifuge tube was partially filled with 8 ml of light CsCl solution (63 g/100 ml), and the plasmid solution placed at the bottom of this tube. The tube was balanced, filled with light CsCl solution, sealed and centrifuged overnight (18 hr; 40,000 rpm) in an L8-M Ultracentrifuge. Following this, the lower band was removed from the tube using a 21-gauge needle and extracted 4 times with an equal volume of water-saturated isobutanol. The lower, aqueous phase was transferred to a clean tube, 3 vol of deionized water and 2 vol of 95% ethanol were added, and the DNA allowed to precipitate for 30 min at -80°C. The sample was centrifuged (15 min; 14,000

rpm), the pellet washed with ethanol, dried under vacuum and resuspended with TE buffer.

The resulting DNA preparation was used for deletion construct formation. In order to sequence both strands, the cDNA insert was required in both orientations. The II5A.0 insert was excised by *Eco* RI-*Sal* I double restriction digestion and the resulting fragment gel purified and blunt-ended. To blunt end the fragment, 500 ng of II5A.0 DNA in 3 μ l of TE buffer was added to a solution containing 6 μ l of reaction buffer [1.5 mM DTT, 30 mM magnesium acetate, 200 mM potassium acetate, 100 mM Tris-acetate pH 7.9], 2 μ l dNTP mix [125 μ M each of dATP, dCTP, dGTP, dTTP] and 1 unit of Klenow DNA polymerase, and incubated for 15 min at 37°C. This was extracted with phenol:chloroform, the aqueous phase was mixed with 0.5 vol 7.5 M ammonium acetate and precipitated with 2 vol of 95% ethanol at -80°C. The pellet obtained following centrifugation (15 min; 14,000 rpm) was washed with ethanol, dried, resuspended with TE buffer and the DNA content was determined.

Ten μ g of pEMBL 8 vector (without insert) was digested with *Sma* I, and the resulting linearized plasmid treated with calf intestinal alkaline phosphatase (CIP) to prevent plasmid recircularization. Briefly, following restriction digestion, the sample was extracted with phenol:chloroform, the aqueous phase mixed with ammonium acetate and precipitated with 95% ethanol as described previously. The resulting pellet was resuspended in 90 μ l of 10 mM Tris-HCl (pH 8.3). To this was added 10 μ l of 10x CIP buffer [10 mM ZnCl_2 , 10 mM MgCl_2 , 100 mM Tris-HCl pH 8.3] and

1 μ l (22 units) CIP. The sample was incubated at 37°C for 15 min, followed by the addition of 1 μ l of CIP and incubation at 55°C for 45 min. CIP was inactivated by heating at 65°C for 1 hr in the presence of 5 mM EDTA (pH 8), after which the sample was extracted with phenol:chloroform, the aqueous phase mixed with ammonium acetate and precipitated with 95% ethanol as described above. The pellet was resuspended with TE buffer and the DNA content determined.

The vector and insert were ligated using a 3:1 (300 ng:100 ng) ratio of insert:vector. The insert + vector were contained in a total of 3 μ l of TE buffer, to which was added 1 μ l of 10x ligase buffer [500 mM Tris-HCl pH 7.6, 100 mM MgCl₂, 10 mM ATP], 5 μ l of 10 mM DTT and 1 μ l of T4 DNA ligase (5 units). Ligation was allowed to proceed overnight at room temperature, then the 10 μ l ligation mixture was added to 100 μ l of competent SURE cells (Stratagene Cloning Systems, La Jolla, CA), mixed gently, incubated for 30 min on ice, heated at 42°C for 2 minutes and then placed on ice for 2 min. Two hundred μ l of SOC broth [20 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 0.5 g/L NaCl, 3.6 g/L glucose] was added, the sample warmed to 37°C for 5 min and shaken at 37°C for 1 hr. Cells were plated onto YT + amp medium and grown overnight at 37°C. Ten individual colonies were removed, the plasmid DNA isolated by alkaline lysis, the DNA digested with *Bam* HI, and separated on an agarose gel. Samples were compared to *Bam* HI-digested plasmid containing II5A.0 in the forward orientation. A colony containing altered fragment sizes

expected from the reverse orientation was identified and designated as RII5A.0.

Plasmids containing II5A.0 and RII5A.0 were used for unidirectional deletion construct formation following the Promega Erase-A-Base system (Promega Corp., Madison, WI). Plasmid DNA (5-10 μ g) was digested with *Sal* I overnight, and a small amount was analyzed by agarose gel electrophoresis to assay for complete digestion. After completion, the plasmid DNA was digested with *Pst* I for 6 hr, and extracted with an equal volume of phenol:chloroform (1:1). The aqueous phase was mixed with ammonium acetate and precipitated with 95% ethanol as described previously. The pellet was resuspended in 30 μ l of 1x Exo III buffer [10x Exo III buffer = 660 mM Tris-HCl pH 8, 6.6 mM MgCl₂], heated at 35°C for 10 min, and then 250 units of Exo III added. Digestion was carried out at 35°C. After a 25 sec lag period, 2.5 μ l aliquots were collected every 30 sec for up to 5 min. These were placed into tubes containing 7.5 μ l of S1 mix [172 μ l deionized water, 27 μ l 7.4x S1 buffer (0.3 M potassium acetate pH 4.6, 2.5 M NaCl, 10 mM ZnSO₄, 50% glycerol), 60 units S1 nuclease] and incubated at room temperature for 30 min, then 1 μ l S1 stop buffer [0.3 M Tris base, 0.05 M EDTA] was added and the samples heated at 70°C for 10 min. The extent of digestion was determined by analysis of 2 μ l of each sample by agarose gel electrophoresis. Samples were heated to 37°C and 1 μ l of Klenow mix [30 μ l 1x Klenow buffer (20 mM Tris-HCl pH 8, 100 mM MgCl₂), 5 units Klenow DNA polymerase] was added, the samples incubated for 3 min, followed by the addition of 1 μ l dNTP mix [125 μ M each

of dATP, dCTP, dGTP, dTTP] and incubation for 5 min. Samples were moved to room temperature, 40 μ l of ligase mix [890 μ l deionized water, 100 μ l 10x ligase buffer (500 mM Tris-HCl pH 7.6, 100 mM MgCl₂, 10 mM ATP), 10 μ l DTT, 5 units T4 DNA ligase] added, and ligation allowed to proceed overnight. Ligation mix (10 μ l) was then used to transform 100 μ l of competent cells. These were plated onto YT + amp medium and grown overnight. Ten individual colonies from each deletion time point were used for plasmid DNA isolation by alkaline lysis. The resulting DNA was double digested with *Eco* RI-*Hind* III to excise the insert, and analysed by agarose gel electrophoresis. Colonies containing deletions were identified in this manner. The deletions obtained spanned the entire length of the II5A.0 and RII5A.0 inserts.

3.12. DNA SEQUENCING

DNA sequences were obtained using the dideoxy method. For each colony representing a deletion time point to be sequenced, plasmid DNA was isolated from a 2-4 ml YT + amp broth overnight culture using alkaline lysis. The DNA was resuspended in 100 μ l of TE buffer and then treated with 1 μ l RNase (10 mg/ml) for 1 hr at 37°C to digest contaminating RNA. Following this, 60 μ l of 2.5 M NaCl/20% PEG 8000 was added, the contents mixed, left at room temperature for 15 min, centrifuged (10 min; 14,000 rpm) at 4°C and the supernatant (containing the RNA) removed. The pellet was resuspended in 100 μ l TE buffer, extracted with an equal volume of phenol:chloroform, the aqueous phase removed and mixed with ammonium acetate and precipitated with 95% ethanol as

described previously. The pellet was resuspended in TE buffer and the DNA quantified.

A portion of the template DNA isolated above (2-4 μg) in 16 μl TE buffer was added to 4 μl of 1 M NaOH, incubated at room temperature for 10 min, and precipitated with 0.5 vol of 7.5 M ammonium acetate and 2 vol of 95% ethanol at -80°C . The pellet obtained following centrifugation was washed with ethanol, dried and resuspended in a solution of 5 μl deionized water, 1 μl 10x sequencing sample buffer [500 mM Tris-HCl pH 7.5, 100 mM NaCl, 100 mM MgCl_2] and 1 μl of 2 ng/ μl forward primer ($5'\text{GTAAAACGACGGCCAGT}3'$). This was mixed by vortexing, centrifuged briefly to collect the sample and incubated at $37-45^{\circ}\text{C}$ for 15 min. The sample was placed on ice and 1 μl of 15 μM dATP, 1 μl 100 mM DTT, 1.5 μl α - ^{32}P -dATP (NEN-Du Pont Corp., Mississauga, ON; 3000 Ci/mmol) and 4.5 μl of a 0.5 unit solution of Klenow DNA polymerase added. This solution was the template/primer/label mix.

In a petri dish on ice, 1.5 μl of each dideoxy nucleotide/deoxynucleotide (ddN/dN) mix was spotted in the dish [(G mix: 18 μl 1 mM ddG, 16 μl 0.1 mM dGTP, 32 μl each of 1 mM dTTP and 1 mM dCTP, 102 μl deionized water), (A mix: 23 μl 1 mM ddA, 22 μl each of 1 mM dGTP, 1 mM dTTP and 1 mM dCTP, 111 μl deionized water), (T mix: 12.5 μl 10 mM ddT, 32 μl 1 mM dGTP, 26 μl 0.1 mM dTTP, 32 μl 1 mM dCTP, 97 μl deionized water), (C mix: 75 μl 1 mM ddC, 32 μl each of 1 mM dGTP and 1 mM dTTP, 41 μl 0.1 mM dCTP, 20 μl deionized water). The template/primer/label mix (3.5 μl) was added to each of the ddN/dN spots, and the petri

dish placed onto a water bath at 37-42°C for 5 min. Following this, the petri dish was placed back on ice, 1 μ l of chase solution [1 mM each of dGTP, dATP, dTTP, dCTP] added to each spot and, the dish placed onto the water bath for a further 5 min. Reactions were stopped by placing the petri dish on ice and adding 5 μ l formamide dye mix [98% (v/v) deionized formamide, 10 mM EDTA pH 8, 0.025% (w/v) xylene cyanol FF, 0.025% (w/v) bromophenol blue] to each spot. The petri dish was placed on a boiling water bath and the samples heated for 2 min. Samples (1-2 μ l) were then loaded onto sequencing gels.

Sequencing gels were made using 37.5 g urea, 7.5 ml 10x modified TBE [162 g/L Tris base, 27 g/L boric acid, 9.4 g/L Na₂EDTA.2H₂O], 11.25 ml 40% (w/v) acrylamide and 32 ml deionized water, to give a final urea concentration of 8 M and acrylamide concentration of 6%. The gel mix was filtered, degassed for 30 min and polymerized with 300 μ l 10% ammonium persulfate and 15 μ l TEMED. Gels were run at 2000 V using 1x TBE as the electrophoresis buffer. Following electrophoresis, gels were dried at 80°C for 1 hr and allowed to expose at -80°C using Kodak X-Omat RP film. Sequences for both strands were obtained and the coding strand sequence compared to sequences in the EMBL data bank using PC/GENE (IntelliGenetics Inc., Mountain View, CA).

3.13. GENOMIC DNA EXTRACTION, ELECTROPHORESIS AND BLOTTING

Spruce needles (5 g) were ground in liquid nitrogen, suspended in 90 ml of extraction buffer [50 ml/L 1 M Tris-HCl pH 8, 10 ml/L 0.5 M EDTA, 63.78 g/L sorbitol, 100 g/L PEG 8000, 5 g/L spermidine, 5 g/L spermine, 1 ml/L 2-mercaptoethanol] and

filtered through 12 layers of cheesecloth. The solution was filtered through 1 layer of Miracloth and 1 layer of 53 μ m nylon mesh, and centrifuged (20 min; 10,000 rpm) at 4°C in a J2-21 Centrifuge. The supernatant was removed, the pellet resuspended in 3.6 ml CsCl-lauryl sarcosine solution [17.5 g CsCl in 10 ml sarcosine solution (50 mM Tris-HCl pH 7.8, 10 mM EDTA, 0.5% (w/v) sodium lauryl sarcosine)], and heated for 10 min at 60°C. Ethidium bromide (400 μ l of a 10 mg/ml solution) was added and the sample centrifuged (10 min; 10,000 rpm) at room temperature. The clear solution was removed and placed at the bottom of a Ti70.1 centrifuge tube containing 8 ml of light CsCl solution, and the sample centrifuged as described previously for CsCl-EtBr gradient centrifugation. The DNA band was removed, extracted with isobutanol, precipitated as described previously and resuspended in TE buffer.

Five μ g of DNA were used for each restriction digest, and, following digestion, the DNA was precipitated with ammonium acetate and ethanol as described previously. The resulting pellet was resuspended in 20 μ l TE buffer containing 5 μ l Ficoll dye mix, and the DNA separated on a 1% agarose gel. The gel was then rinsed 3 times (10 min each) with 0.5 M NaOH + 1.5 M NaCl, followed by 3 rinses (10 min each) with 1 M ammonium acetate, and then blotted overnight onto Hybond-N nylon membrane (Amersham Corp., Arlington Heights, IL) using 1 M ammonium acetate. The blot was air dried and the DNA cross-linked to the membrane by UV exposure for 10 min. Gene copy reconstructions to approximate the number of vicilin gene copies were constructed using II5A.0

insert and assuming a haploid DNA content of 8.5 pg, which has been described for white spruce (Dhillon 1987).

3.14. RNA EXTRACTION, ELECTROPHORESIS AND BLOTTING

Solutions for RNA work were made using diethylpyrocarbonate-treated [0.1% (v/v)] deionized water. Total RNA was extracted using the protocol of Verwoerd *et al.* (1989). Tissues were isolated, frozen immediately in liquid nitrogen and stored at -80°C until needed. Frozen tissues were ground in liquid nitrogen to a fine powder in Eppendorf tubes using a glass rod (precooled in liquid nitrogen) that fitted precisely into the tubes. After grinding, 500 µl of hot (80°C) extraction buffer [phenol: 0.1 M LiCl, 100 mM Tris-HCl pH 8, 10 mM EDTA, 1% (w/v) SDS (1:1)] was added. The samples were mixed by vortexing for 30 sec, then chloroform (250 µl) was added, followed by another 30 sec vortex. After centrifugation (5 min; 14,000 rpm), the water phase was removed, an equal volume of extraction buffer (minus phenol) was added to each tube and the sample back-extracted. After centrifugation, the two water phases from each sample extraction were pooled and mixed with 1 vol of 4 M LiCl. RNAs were allowed to precipitate overnight at 4°C, then collected by centrifugation (15 min; 14,000 rpm). The pellets were dissolved in 250 µl water, 0.1 vol of 3 M sodium acetate pH 5.2 was added and the RNAs precipitated with 2 vol of 95% ethanol at -80°C for 30 min. After centrifugation, the pellets were washed with ethanol, dried and resuspended in TE buffer containing 0.1% (w/v) SDS.

The purity and integrity of samples was checked by agarose gel electrophoresis. Two μl of RNA sample was added to 8 μl of TE buffer containing 0.1% (w/v) SDS and 1 μl Ficoll dye mix. The samples were heated at 65°C for 5 min and then applied to a gel consisting of 1% (w/v) agarose in 1x TAE containing 0.1% (w/v) SDS. Electrophoresis buffer consisted of 1X TAE with 0.1% (w/v) SDS. For RNA quantification, 1 μl of sample was diluted to 100 μl with TE buffer and quantified spectrophotometrically at 260 nm.

For RNA electrophoresis and blotting, the protocol of Fournay *et al.* (1988) was used. RNA samples were adjusted to 5 μl with water, 25 μl of electrophoresis sample buffer [750 μl deionized formamide, 150 μl 10x MOPS/EDTA buffer (0.2 M 3-(N-morpholino) propanesulfonic acid, 50 mM sodium acetate, 10 mM EDTA, pH 7 and autoclaved), 240 μl 37% formaldehyde, 100 μl water, 100 μl glycerol, 80 μl 10% (w/v) bromophenol blue] added, and heated at 65°C for 15 min. Samples were fractionated on denaturing formaldehyde gels [225 ml water, 2.5 g agarose, 25 ml 10x MOPS/EDTA buffer, 4.1 ml 37% formaldehyde, 0.46 g iodoacetamide, 5 μl of 10 mg/ml EtBr] run overnight at 25 V, using 1x MOPS/EDTA as the electrophoresis buffer.

Following electrophoresis, gels were rinsed 20 min in 0.05 M NaOH in 1x SSC [20x SSC = 3 M NaCl, 0.3 M Na₃ citrate], followed by 2 rinses (20 min each) in 10x SSC. They were then blotted overnight onto Hybond-N nylon membrane using 10x SSC. Blots were allowed to air dry, and the RNA crosslinked to the membrane by exposure to UV light for 5 min.

3.15. cDNA PROBE PRODUCTION AND HYBRIDIZATION TO BLOTS

All probes were generated using the random oligonucleotide primer method. In a 1.5 μ l Eppendorf tube, 1 μ l of template cDNA (25-50 ng) was mixed with 9.5 μ l of deionized water, heated at 98°C for 10 min, then snap cooled on ice. To this was added 2 μ l 10x buffer [900 mM HEPES pH 6.6, 100 mM MgCl₂], 1 μ l 1 mg/ml BSA, 1 μ l 100 mM DTT, 2 μ l dNTP mix [10 mM each of dGTP and dTTP], 1 μ l random hexamer primer, 2 μ l each of α -(³²P-dATP) and α -(³²P-dCTP) (NEN-Du Pont Corp., Mississauga, ON; each with 3000 Ci/mmol) and 1 μ l Klenow DNA polymerase (5 units). This was incubated overnight at room temperature. The probe was precipitated by addition of 1 μ l of 0.5 M EDTA, 80 μ l deionized water, 3 μ l *E. coli* carrier tRNA (2 mg/ml), 50 μ l 7.5 M ammonium acetate and 375 μ l 95% ethanol to the tube and incubation at -80°C for 30 min, followed by centrifugation (15 min; 14,000 rpm) at 4°C. The pellet was washed with ethanol, vacuum dried and resuspended in 100 μ l TE buffer. One μ l of labelled probe was placed in a scintillation vial and the radioactivity determined. Probes were heated at 98°C for 5 min and then snap cooled on ice immediately prior to their use.

DNA blots were pre-hybridized overnight at 68°C in approximately 30 ml of pre-hyb/hyb solution [5x SSPE (20x SSPE = 3.6 M NaCl, 0.2 M Na₂HPO₄·7H₂O, 0.02 M EDTA), 100-200 μ g/ml calf thymus DNA, 1% (w/v) SDS, 5x Denhardt's solution (100x Denhardt's = 2% (w/v) BSA, 2% (w/v) polyvinylpyrrolidone, 2% (w/v) Ficoll), 0.05% (w/v) sodium pyrophosphate, 10% (w/v) dextran sulfate]. Following pre-hybridization, blots were hybridized at 68°C for 2

days with labelled probe ($1-2 \times 10^6$ cpm/ml of solution) in approximately 30 ml of hyb solution. Blots were washed 3 times (30 min each) at 68°C with $2\times$ SSC + 1% (w/v) SDS and allowed to expose at -80°C with an intensifying screen using Kodak X-Omat AR film.

RNA blots were pre-hybridized overnight at 68°C in pre-hyb/hyb solution [0.5 M sodium phosphate pH 7.2, 7% (w/v) SDS, 1 mM EDTA]. Following this, blots were hybridized for 22-24 hr at 68°C in approximately 30 ml of hyb solution containing $1-2 \times 10^6$ cpm of labelled probe/ml of solution. After hybridization, blots were washed twice (20 min each) with a solution of $1\times$ SSC + 0.1% (w/v) SDS at room temperature, followed by a 20 min wash at 68°C . Blots were then allowed to expose at -80°C with an intensifying screen using Kodak X-Omat AR film. For reprobing of blots, each blot was stripped with several 250 ml washes of boiling 0.1% (w/v) SDS and the membrane was checked by overnight exposure to ensure complete removal of probes. Autoradiographs of RNA blots were scanned at 595 nm in a DU-64 Spectrophotometer.

4. RESULTS

4.1. IDENTIFICATION AND CHARACTERIZATION OF ZYGOTIC EMBRYO STORAGE PROTEINS

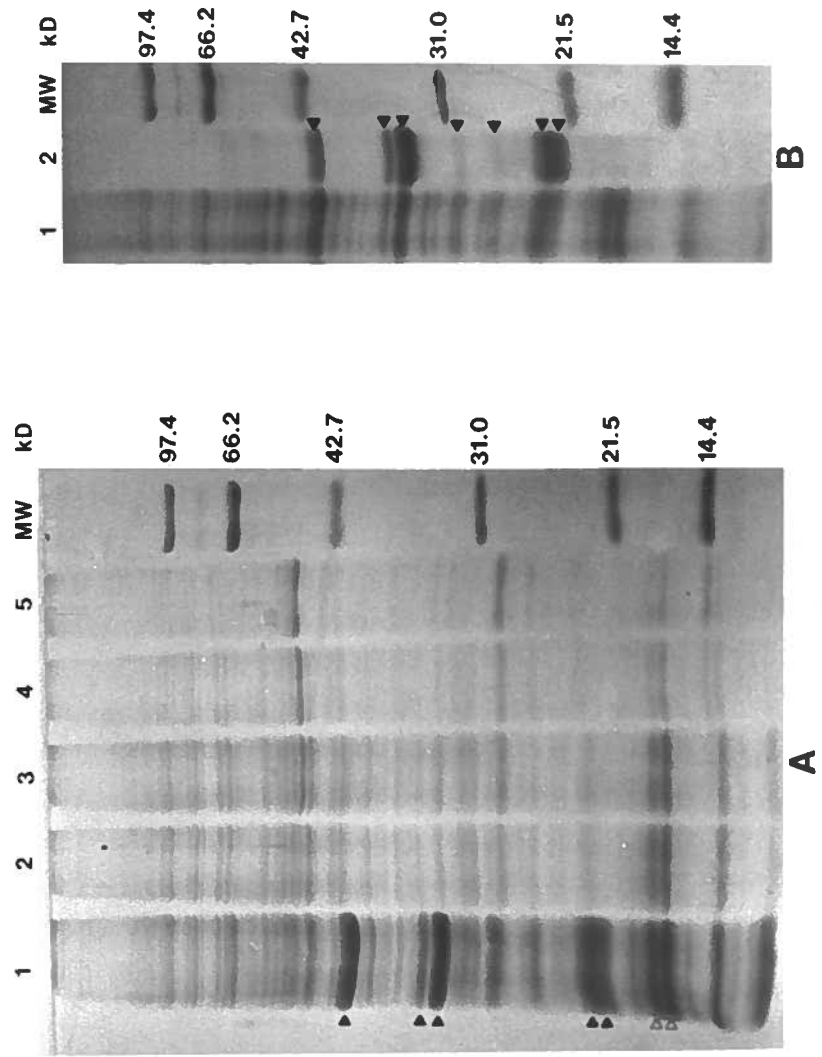
The storage proteins of interior spruce were identified by SDS-PAGE analysis of total proteins from mature seed embryos (Fig. 1). Prominent proteins with apparent molecular weights of 41, 35, 33, 24, 22, 17 and 16 kD were identified. Similar proteins, with the exception of the 17 and 16 kD proteins, were detected in megagametophyte tissue extracts (data not shown). To determine if any of the proteins were storage proteins, embryo protein profiles were examined during germination (Fig. 1A). By day 3 after sowing, at which time radicle emergence had occurred, the 41, 35, 33, 24 and 22 kD proteins were almost undetectable, indicating rapid degradation during germination, characteristic of storage protein mobilization. However, the 17 and 16 kD proteins were still detected on day 9 of germination (Fig. 1A), suggesting that they were not storage proteins. To test the assumption that the 5 proteins missing by day 3 were storage proteins, protein bodies were isolated from mature embryonic tissues, solubilized and analyzed electrophoretically (Fig. 1B). The 41, 35, 33, 24 and 22 kD bands were prominent in this profile. Minor bands of 30 and 27.5 kD were also observed (Fig. 1B). These proteins were also present in megagametophyte tissue (data not shown).

To determine if individual proteins were joined by disulfide linkages, extracts of isolated protein bodies made under non-reducing conditions were examined by SDS-PAGE under non-reducing,

FIGURE 1. Coomassie-stained SDS-PAGE of embryo proteins.

A. Total embryo protein changes during germination. Lane 1, mature embryos; Lane 2, 3 days after sowing; Lane 3, 6 days after sowing; Lane 4, 9 days after sowing; Lane 5, 12 days after sowing; MW, molecular weight standards. Fifteen μg protein was applied to Lane 1 and 10 μg protein to all others. The 41, 35, 33, 24 and 22 kD proteins (solid arrows), as well as the 17 and 16 kD proteins (open arrows) are indicated.

B. Proteins from mature embryos (Lane 1) and isolated protein bodies (Lane 2). MW, molecular weight standards. Fifteen μg protein was applied to each lane. Arrows denote the 41, 35, 33, 30, 27.5, 24 and 22 kD proteins.



and then reducing conditions (Fig. 2). The patterns observed under reducing conditions differed from those observed under non-reducing conditions. The 55-57 kD protein doublet present under non-reducing conditions (Fig. 2B) was absent after electrophoresis under reducing conditions and replaced by four bands of 35, 33, 24 and 22 kD (Figs. 2A and 2C), which were previously linked by disulfide bonds. It was difficult to determine the specific association of the proteins in the 55-57 kD doublet (Fig. 2C). The minor 34 kD protein observed under non-reducing conditions was also resolved into two component proteins of 14 and 22 kD when analysed under reducing conditions. The 14 kD protein was often not readily observed in the protein body fraction and was less prominent than the minor 30 and 27.5 kD proteins. Apparently, the disulfide linked 22 and 14 kD complex is a very minor component of the protein body fraction.

The storage proteins identified previously were classified according to their solubility characteristics. Isolated protein bodies that were extracted with phosphate buffer or water yielded only the major 41 kD and minor 30 and 27.5 kD bands (Figs. 3A and 3B). The remaining proteins were only soluble in SDS-containing buffer (Fig. 3A) or buffer containing 1 M NaCl (Fig. 3B). There were thus two major solubility classes of proteins in isolated protein bodies. Microscopic analysis of protein bodies from late cotyledonary zygotic embryos revealed two distinct protein-staining regions (Fig. 4), indicating the heterogenous nature of the proteins within these organelles.

FIGURE 2. Coomassie-stained SDS-PAGE of zygotic embryo protein body extracts under reduced (A), non-reduced (B), and two-dimensional SDS-PAGE of non-reduced extract under non-reducing conditions followed by electrophoresis under reducing conditions (C). The 41, 35, 33, 30, 27.5, 24 and 22 kD proteins are indicated by open arrows (A). The 55-57 kD doublet present under non-reduced conditions is indicated by solid arrows (B). The molecular weights of non-reduced proteins and the corresponding proteins obtained by second dimension SDS-PAGE are indicated (C). Each sample contains 4 μ g protein.

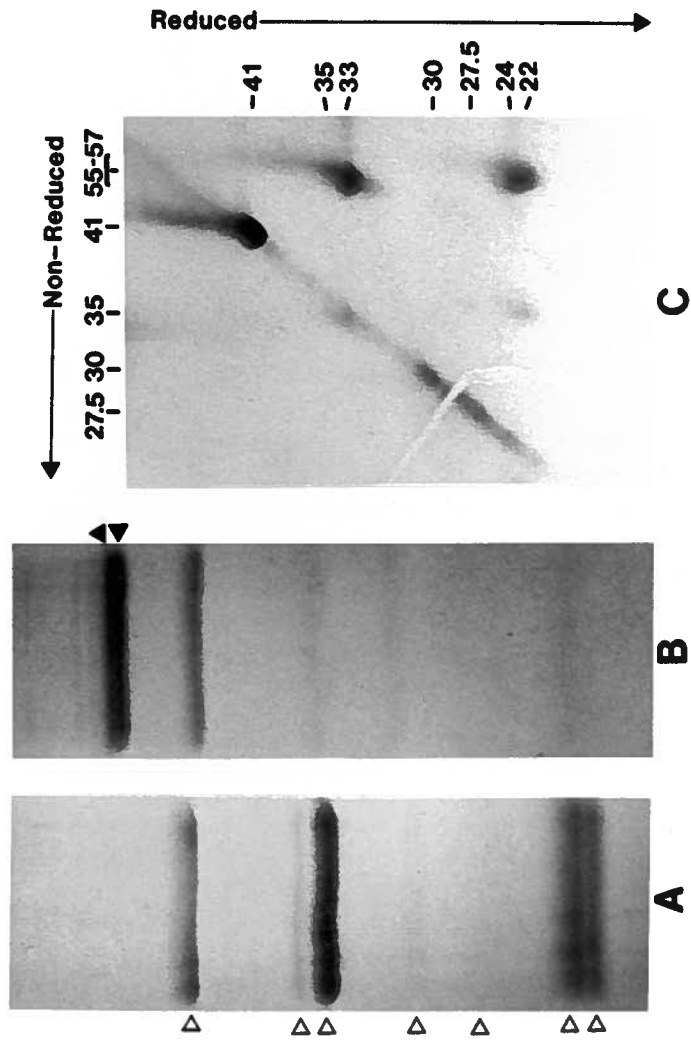


FIGURE 3. Coomassie-stained SDS-PAGE of protein body samples extracted under different conditions.

A. Buffer-soluble (Lane 1) and SDS-soluble (Lane 2) proteins extracted from isolated protein bodies. A 10 μ l sample was applied to each lane. MW, molecular weight standards.

B. Water-soluble (Lane 1), buffer-soluble (Lane 2), low salt-soluble (Lane 3) and high salt-soluble (Lane 4) proteins extracted from isolated protein bodies. A 10 μ l sample was applied to each lane. MW, molecular weight standards.

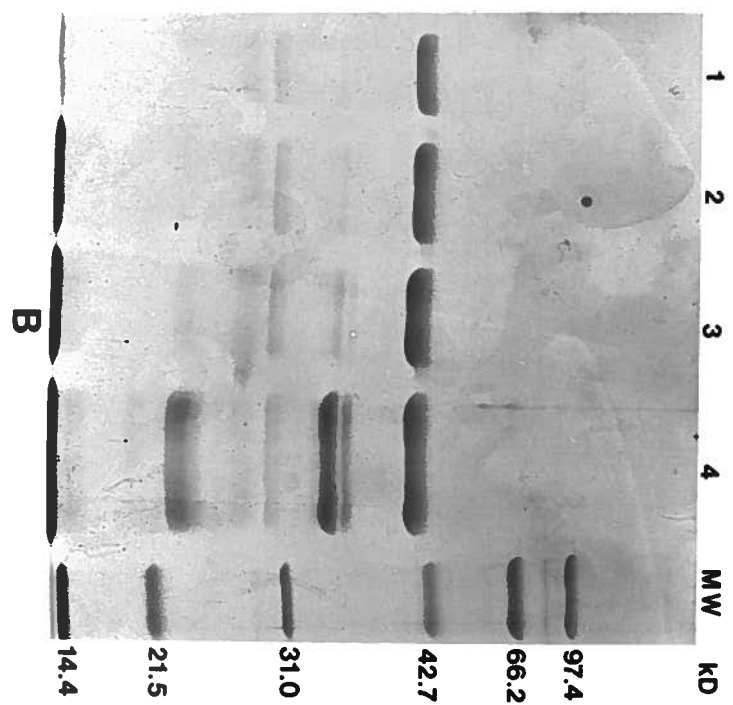
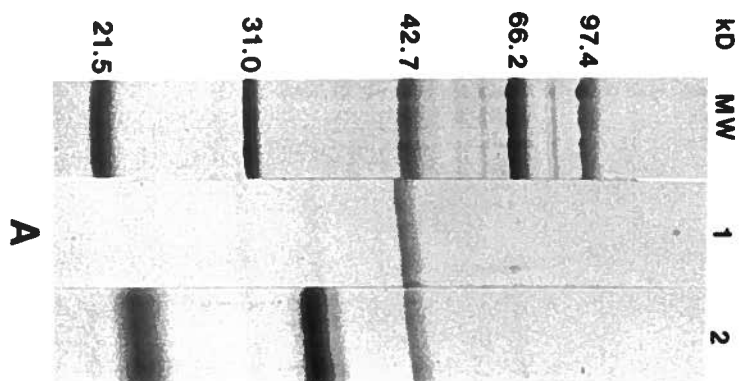
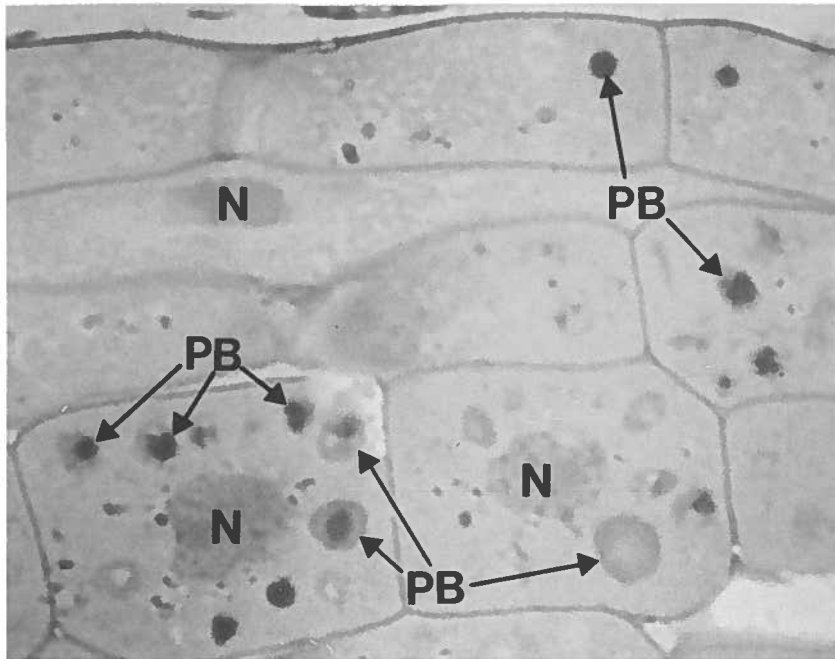


FIGURE 4. Light micrograph of a longitudinal cotyledon section from a late maturation stage zygotic embryo, stained by the periodic acid-Schiff's technique and counter-stained with aniline blue black. Protein bodies (PB) containing light and dark staining zones are visible. N, nucleus. x 1100.



Protein body samples were subjected to two-dimensional electrophoresis using both narrow pH (5-8 ampholytes) and broad pH (3-10 ampholytes) ranges (Fig. 5). The 41 kD protein contained the largest number of isoelectric variants, with approximately 10 or more proteins found across the range of the narrow pH gradient gel. It was often difficult to resolve distinct spots for the 35, 33, 24 and 22 kD proteins and although different gradients and running conditions were tried, these proteins were usually seen as smears. The 35 and 33 kD protein variants were located primarily at the basic end of the narrow gradient gel. However, the 24 and 22 kD proteins which were also basic variants were more readily observed in the broader pH gradient gel (Fig. 5B). The minor 30 and 27.5 kD proteins were located towards the acidic region of the gels and were more readily visualised in the narrow pH gradient gel (Fig. 5A).

4.2. STORAGE PROTEIN ACCUMULATION DURING ZYGOTIC EMBRYO DEVELOPMENT

To follow the appearance of the major storage proteins over time, embryos from Stage 2 to maturity were analyzed by one- and two-dimensional electrophoresis. Embryos collected during the summer of 1988 were used for two-dimensional analysis (Fig. 6), and a more complete developmental sequence was obtained from the one-dimensional analysis of embryos collected during 1989 (Fig. 7). Major accumulations of storage proteins, especially the 41 kD protein, occurred in the Stage 4 embryo, when cotyledons were already well developed (Figs. 6 and 7). The other storage proteins were detectable, albeit at low levels, at much earlier

FIGURE 5. Silver-stained two-dimensional electrophoretograms of zygotic embryo protein body extract examined using pH 5-8 ampholytes (A) or pH 3-10 ampholytes (B). The acidic (+) and basic (-) ends of the gel are indicated. A total of 12 μ g protein was analyzed for each pH gradient.

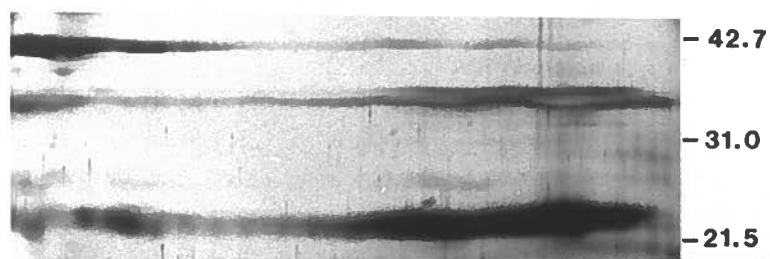
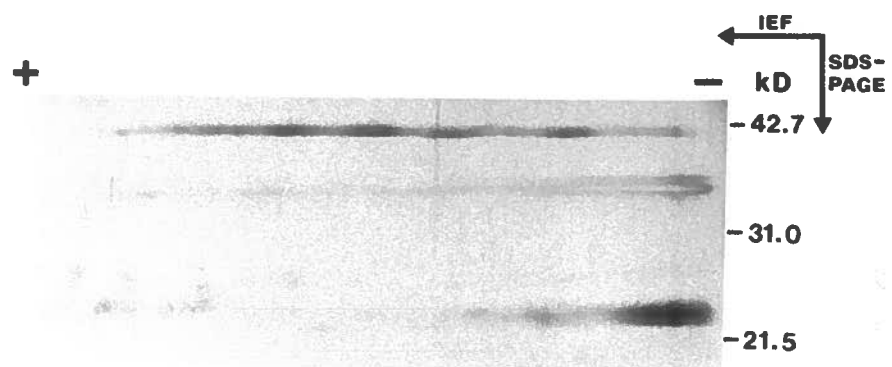


FIGURE 6. Silver-stained two-dimensional electrophoretograms of 3 representative zygotic embryo stages collected during the summer of 1988. Proteins were separated using pH 5-8 ampholytes, and the representative portions of the gels containing the major storage proteins (boxed regions) are shown. Acidic (+) and basic (-) ends of the gels are indicated.

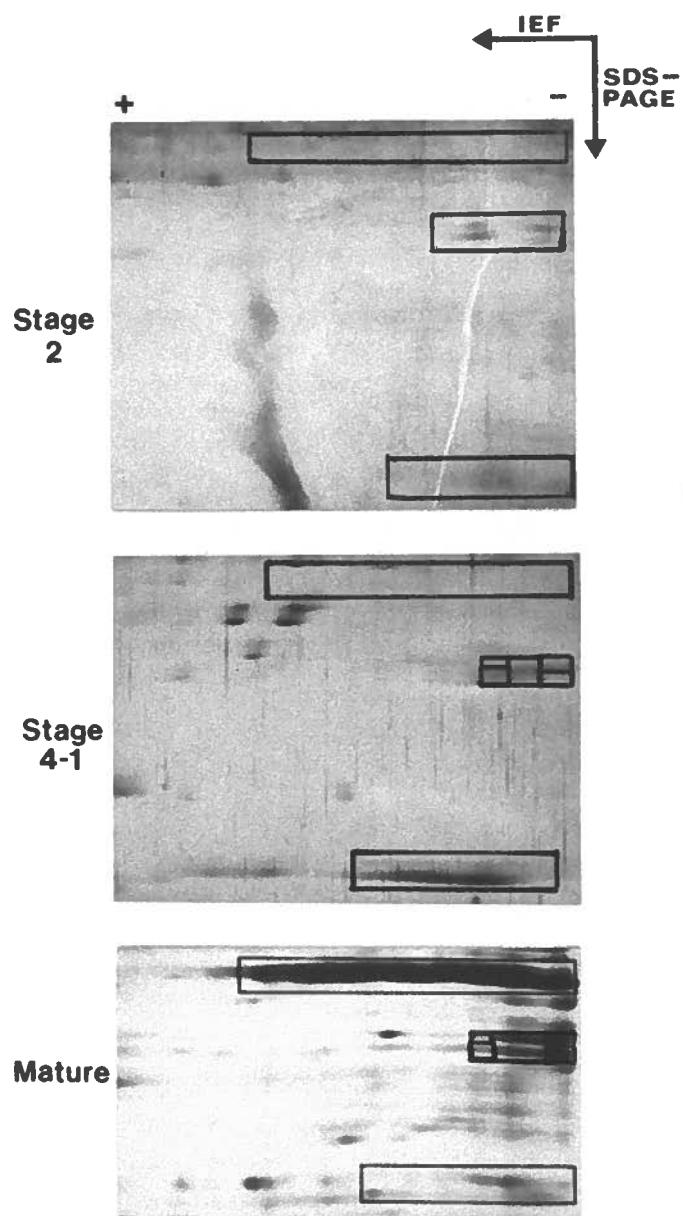
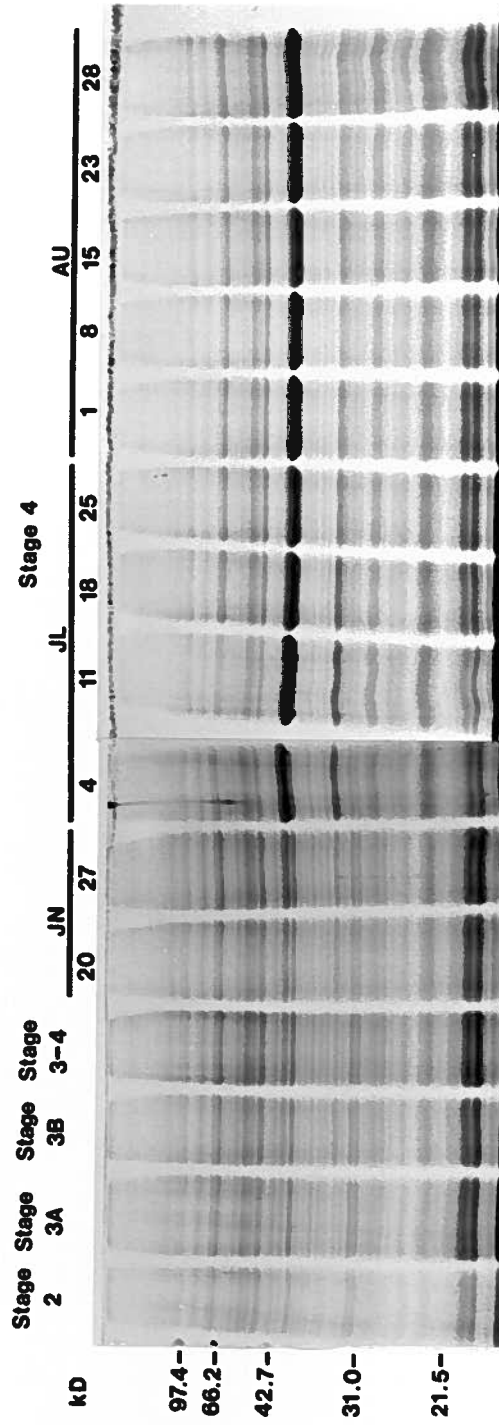


FIGURE 7. Coomassie-stained SDS-PAGE of developmental stage changes in total protein during EK10 zygotic embryogenesis. Zygotic embryos collected during the summer of 1989, ranging in development from Stage 2 to mature seed embryos (Stage 4, August 28 collection) are shown. Molecular weight standard locations are shown on the left. Twelve μg total protein was loaded in each lane.



stages of development, suggesting the differential regulation of storage proteins. Although the 35, 33, 24 and 22 kD proteins did not show major accumulations until cotyledonary development, they were detected at low levels in Stage 2 embryos (Fig. 6). A summary of the developmental appearance of the major storage proteins in 1988 collection embryos is presented in Table 2.

4.3. IDENTIFICATION AND CHARACTERIZATION OF SOMATIC EMBRYO STORAGE PROTEINS AND COMPARISON WITH ZYGOTIC EMBRYO STORAGE PROTEINS

Somatic embryos were matured in the presence of various levels of ABA (0-60 μM) and an ABA-dependent developmental profile was obtained (Fig. 8). The level of ABA affected the number and type of structures that developed. Low ABA levels favoured production of shooty structures or precocious germinants with extensive chlorophyll development. Higher ABA concentrations stimulated the production of late cotyledonary somatic embryos that did not germinate precociously during the experimental period. The optimal level of ABA for line W29 was between 40-60 μM . Based on morphological characteristics described by Buchholz and Stiemert (1945), somatic embryos differentiated on 40 μM ABA proceeded through embryogenesis in a manner similar to that of conifer zygotic embryos (Fig. 9). The late cotyledonary somatic embryos were larger in girth but similar in length and overall appearance to zygotic embryos after 9 weeks of maturation on ABA (Fig. 9). These embryos were used for the identification and characterization of somatic embryo storage proteins.

TABLE 2. The presence (+) or absence (-) of various storage proteins during zygotic embryo development, 1988 collection.

| Storage protein (kD) | Embryo developmental stage | | | | | | Mature |
|-------------------------|----------------------------|---|-----|---------------|---------------|---------------|--------|
| | 2 | 3 | 3-4 | 4-1 (JL13) | 4-2 (JL27) | 4-3 (AU24) | |
| 41 | - | - | - | - | + | + | + |
| 35 | + | + | + | + | + | + | + |
| 33 | + | + | + | + | + | + | + |
| 24 | + | + | + | + | + | + | + |
| 22 | + | + | + | + | + | + | + |

FIGURE 8. Absciscic acid-dependent developmental profile of genotype W29, showing the types and numbers of structures obtained with maturation using different ABA concentrations. ME, late cotyledonary somatic embryo; PE, precociously germinating somatic embryo; SH, shooty structure. The bar graphs represent quantitative data for the cultures depicted below.

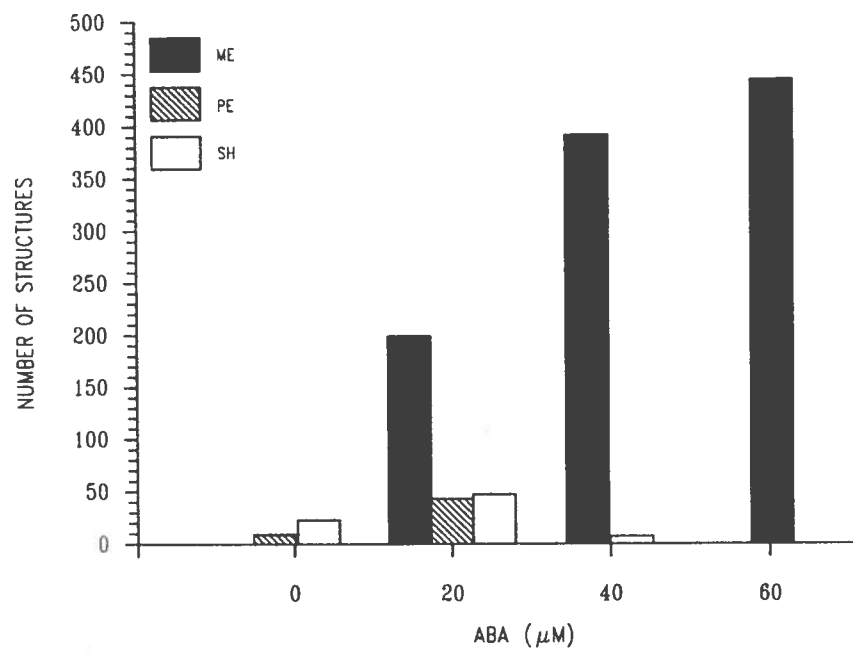
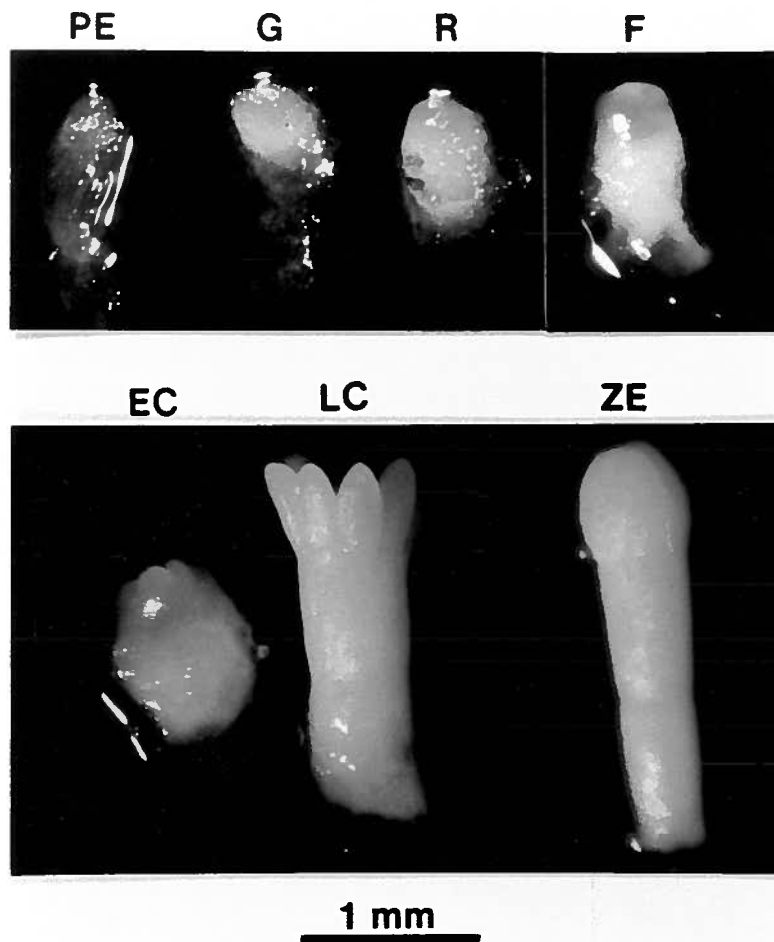


FIGURE 9. Examples of somatic embryo developmental stages. PE, proembryo; G, globular embryo; R, round head torpedo embryo; F, flat head torpedo embryo; EC, early cotyledonary embryo; LC, late cotyledonary embryo; ZE, zygotic embryo.



SDS-PAGE analysis of total embryo proteins revealed that proteins were similar in zygotic and somatic embryos (Fig. 10), as well as in the isolated protein bodies of both embryo types. Total protein gels suggested that there were differences between storage protein levels of the two embryo types (Fig. 10). However, the relative levels of 35, 33, 24 and 22 kD proteins in isolated protein bodies were similar from both sources, although the level of 41 kD protein was greater in somatic embryo protein bodies (Fig. 10).

An analysis of the somatic embryo protein body fraction under reducing and non-reducing conditions revealed distinct differences between the two treatments (Fig. 11), similar to those observed for zygotic embryo storage proteins (Fig. 2).

The somatic embryo storage proteins were characterized further using solubility criteria. Isolated somatic embryo protein body preparations contained phosphate buffer-soluble 41, 30 and 27.5 kD proteins, with the remaining proteins requiring SDS for solubilization (Fig. 12). These results were similar to those obtained for zygotic embryo storage proteins (Fig. 3).

Somatic embryo protein body samples which were analysed by two-dimensional electrophoresis using narrow and broad pH gradients (Fig. 13) were similar to those observed for zygotic embryo storage proteins.

A comparison of the steady state storage protein levels between zygotic and W29 somatic embryos by scanning densitometry of total protein profiles revealed some differences between the proportion of major storage proteins in the two embryo types

FIGURE 10. Coomassie-stained SDS-PAGE of zygotic embryo (Lane 1), zygotic protein body (Lane 2), 9-week ABA somatic embryo (Lane 3) and somatic embryo protein body (Lane 4) extracts. Arrows indicate the 41, 35, 33, 30, 27.5, 24 and 22 kD proteins. Lanes 1 and 3 contain 15 μ g protein and lanes 2 and 4 contain 5 μ g protein. MW, molecular weight standards.

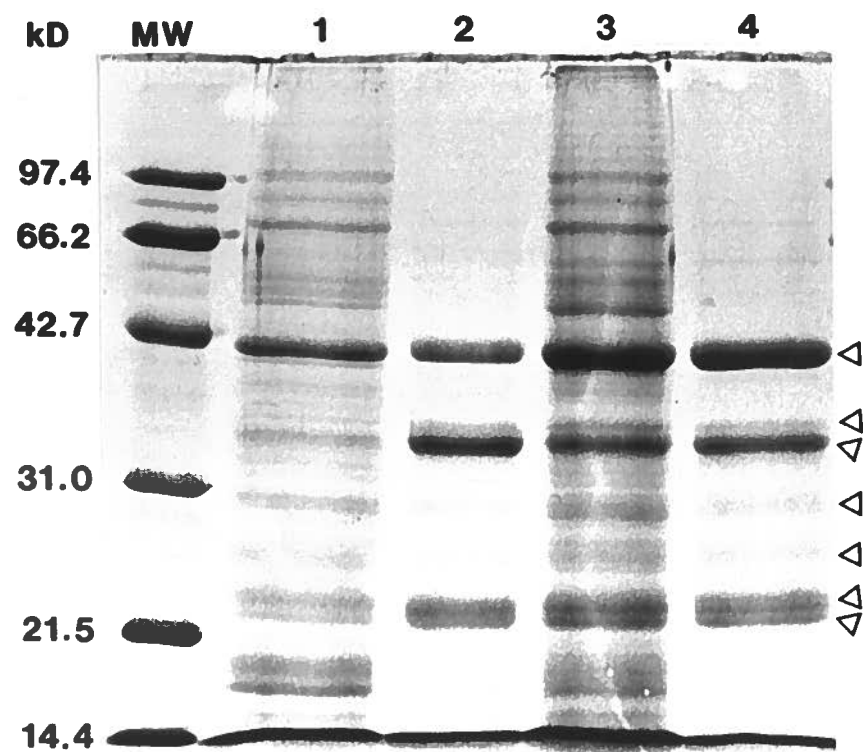


FIGURE 11. Coomassie-stained SDS-PAGE of somatic embryo protein body extracts under reduced (A), non-reduced (B), and two-dimensional SDS-PAGE of non-reduced extract under non-reducing conditions followed by electrophoresis under reducing conditions (C). The 41, 35, 33, 30, 27.5, 24 and 22 kD proteins are indicated by open arrows (A). The 55-57 kD doublet present under non-reduced conditions is indicated by the solid arrow (B). The molecular weights of non-reduced proteins and the corresponding proteins obtained by second dimension SDS-PAGE are indicated (C). Each sample contains 5 μ g protein.

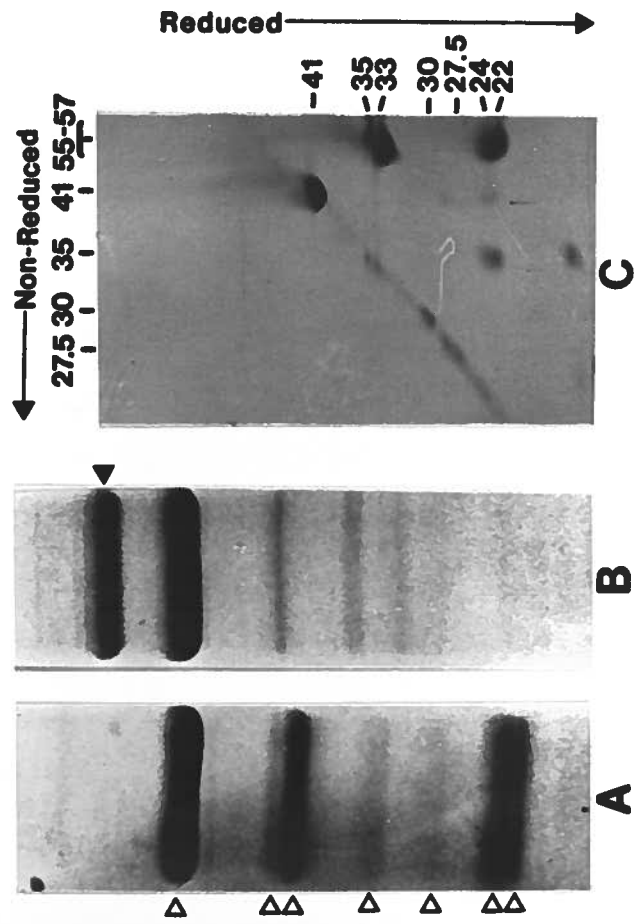


FIGURE 12. Coomassie-stained SDS-PAGE of somatic embryo protein body samples extracted under different conditions. Buffer-soluble (Lane 1) and SDS-soluble (Lane 2) proteins extracted from isolated protein body samples are shown. A 10 μ l sample was applied to each lane. MW, molecular weight standards.

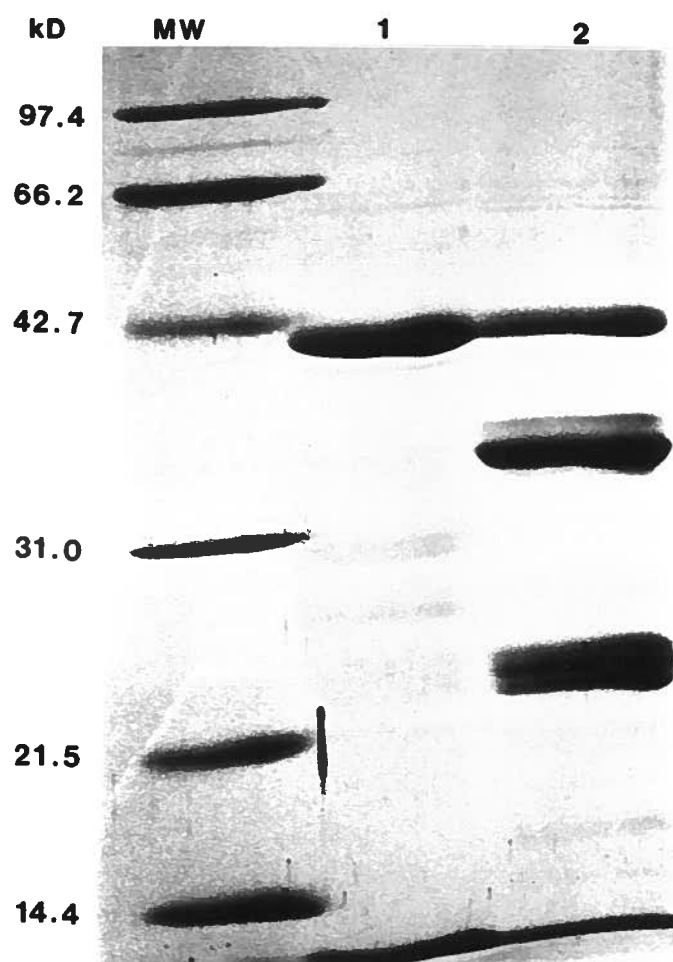
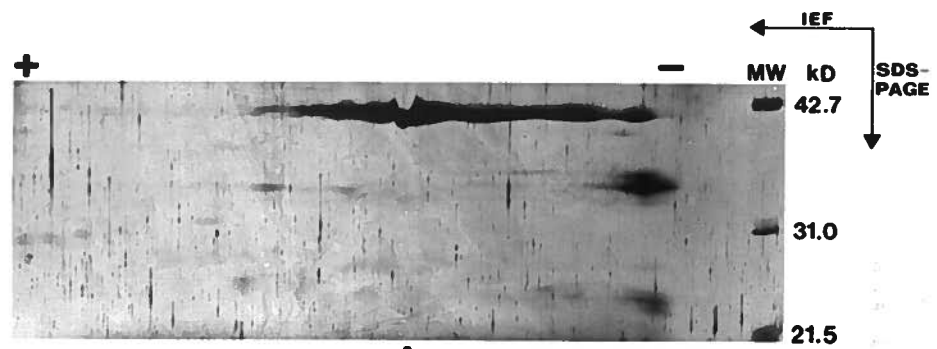


FIGURE 13. Silver-stained two-dimensional electrophoretogram of somatic embryo protein body extract examined using pH 5-8 ampholytes (A) or pH 3-10 ampholytes (B). The acidic (+) and basic (-) ends of the gel are indicated. A total of 20 μ g protein was analyzed for each pH gradient. MW, molecular weight standards.



(Table 3). Somatic embryos of genotype W29, after 9 weeks on ABA, contained more of the major storage proteins as a percentage of total protein than did mature zygotic embryos (Table 3).

To determine the extent to which storage proteins accumulated in other somatic embryo genotypes, the protein profiles of late cotyledonary (7 weeks on ABA) somatic embryos of uniform length were compared with those from genetically related seed (PG118 or EK10) (Fig. 14). Somatic embryos of several genotypes derived from EK10 seed showed variable levels of storage proteins, with W29 embryos containing the highest levels (Fig. 14A). Embryos from two different genotypes derived from PG118 seed had storage protein levels similar to each other, which were similar to or slightly higher than the levels in PG118 zygotic embryos (Fig. 14B). However, storage proteins accumulated to a significant degree in late cotyledonary embryos of each genotype.

4.4. STORAGE PROTEIN ACCUMULATION IN SOMATIC EMBRYOS AND COMPARISON WITH ZYGOTIC EMBRYOS

A comparison of early cotyledonary (Stage 3-2) and late cotyledonary (Stage 4-9) W29 somatic embryos by two-dimensional electrophoresis revealed a differential appearance of the major storage proteins during development. In late cotyledonary somatic embryos, the 41 kD protein was abundant (Fig. 15). However, during earlier embryogenic stages, the 35, 33, 24 and 22 kD proteins were detected at low levels, but the 41 kD protein was not. In addition, the major accumulation of all storage proteins occurred during the later stages of embryo maturation (Fig. 16). The differential appearance of the 41 kD versus the

TABLE 3. Major storage protein distribution in EK10 zygotic and W29 somatic embryos as determined by densitometry. Mean \pm SE.

| Storage protein (kD) | Zygotic embryo | Somatic embryo |
|----------------------------|--------------------|--------------------|
| | % of total protein | % of total protein |
| 41 | 24 \pm 3 | 32 \pm 1 |
| 35 + 33 | 5 \pm 3 | 13 \pm 1 |
| 24 + 22 | 12 \pm 2 | 13 \pm 2 |
| Total protein | 41 \pm 4 | 58 \pm 1 |

FIGURE 14. Coomassie-stained SDS-PAGE of total proteins for two zygotic embryo genotypes and different somatic embryo genotypes derived from them. Protein profiles of the different somatic embryo genotypes derived from (A) EK10 (W29, W74, W76, W77) and (B) PG118 (W46, W70) seed are indicated. Each lane contains 7.5 μ g protein. MW, molecular weight standards.

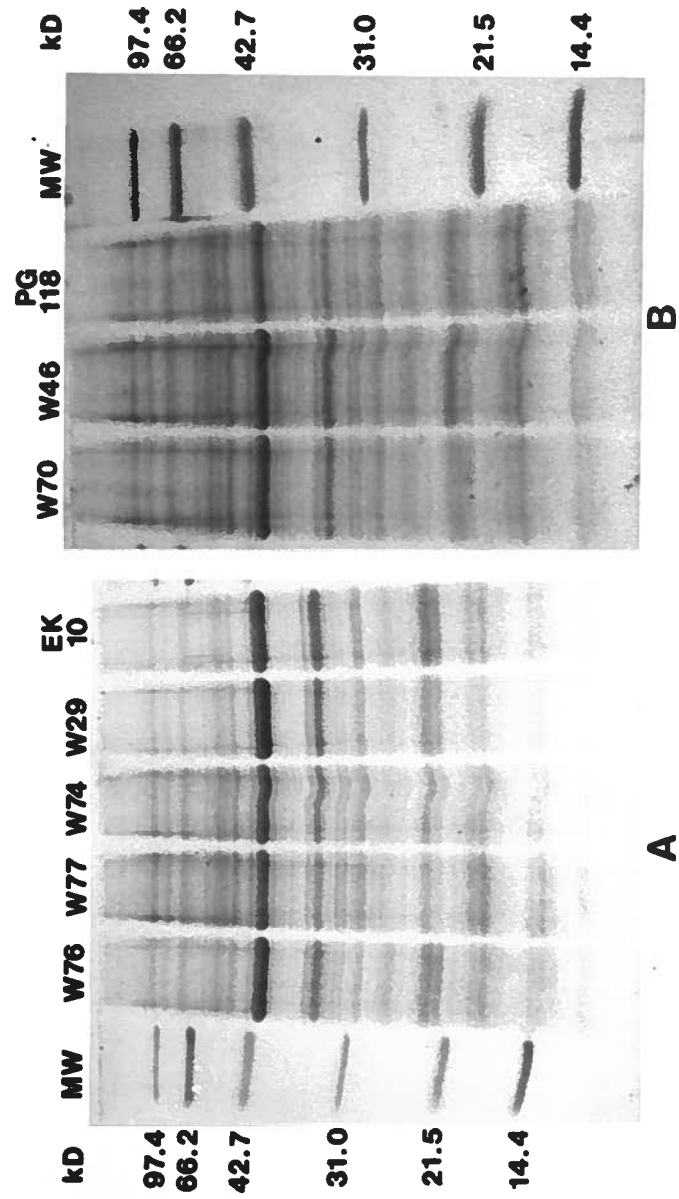


FIGURE 15. Silver-stained two dimensional electrophoretograms of total proteins from stage 4-9 (A) and stage 3-2 (B) somatic embryos differentiated on 40 μ M ABA. Proteins were separated using pH 3-10 ampholytes, and the representative portions of the gels containing the major storage proteins (boxed regions) are shown. Acidic (+) and basic (-) regions of the gels are indicated.

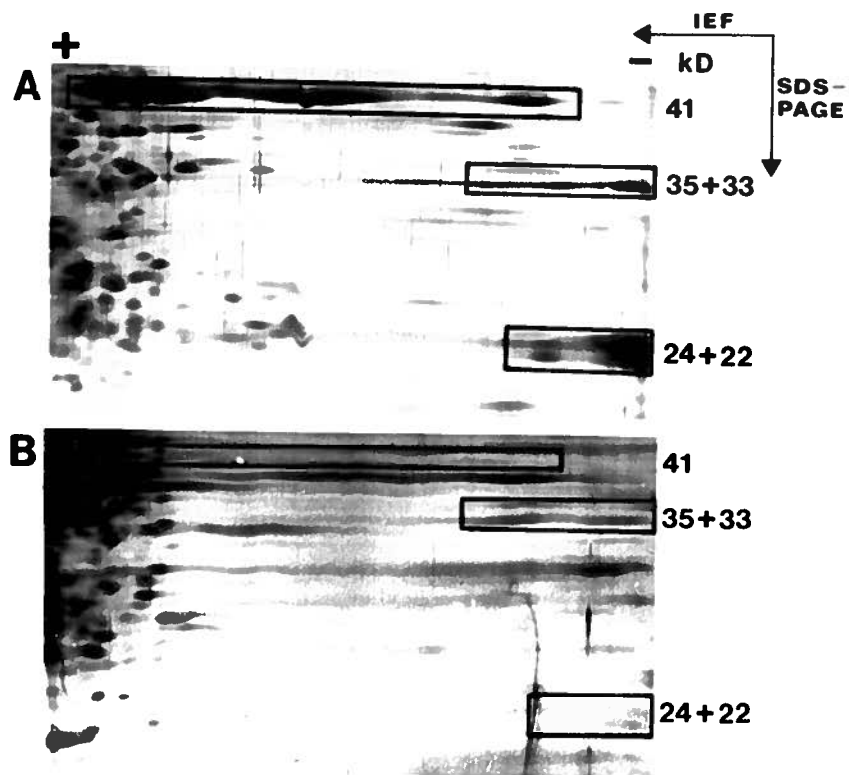
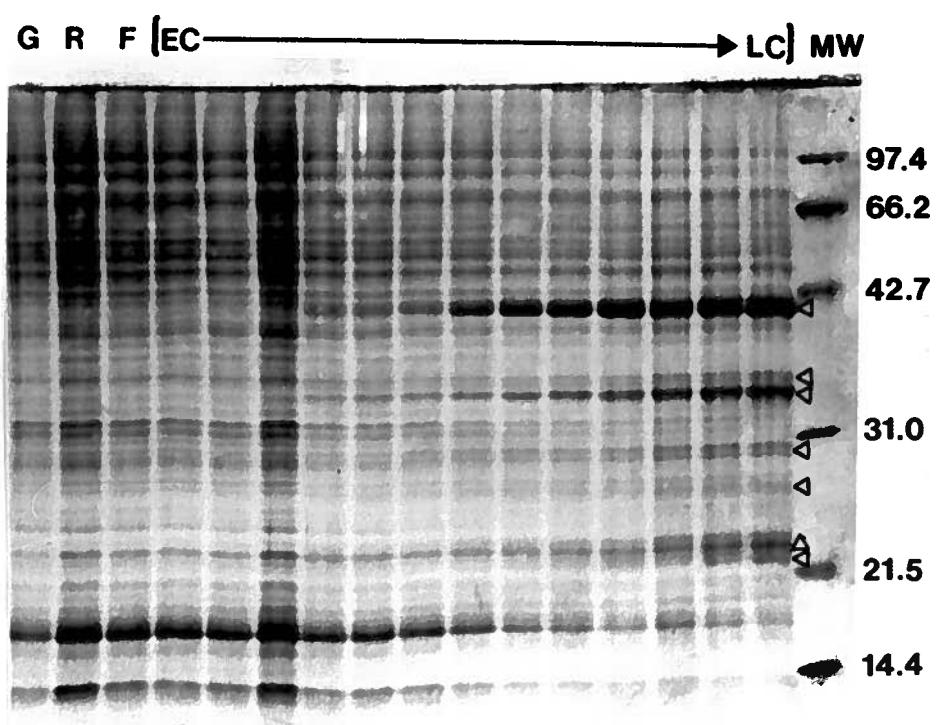


FIGURE 16. Coomassie-stained SDS-PAGE of developmental stage changes in total somatic embryo protein of genotype W29 matured on 40 μ M ABA. Storage proteins are indicated by arrows. G, globular embryos; R, round head torpedo embryos; F, flat head torpedo embryos; EC, early cotyledonary embryos; LC, late cotyledonary embryos. The range from early cotyledonary to late cotyledonary includes the range of embryos from the onset of Stage 3 development to 9 weeks on ABA as described in Table 1. Each lane contains 15 μ g protein. MW, molecular weight standards.



35, 33, 24 and 22 kD proteins during the early stages of embryo maturation was similar in zygotic and somatic embryos.

Polyclonal antibodies were made against the 41 and 24 + 22 kD storage proteins, and analysis by immunoblotting (Figs. 17 and 18) showed that these proteins accumulated in both somatic and zygotic embryos, although there were differences between the accumulation patterns of the two embryo types. Quantification of immunoblots by densitometry indicated that the 41 kD protein in somatic embryos showed an initial rapid accumulation which continued during the 6-week period of cotyledon/embryo maturation in our differentiation protocol (Fig. 17). In zygotic embryos, there was also an initial, rapid accumulation of the 41 kD protein over a 3-week period, but thereafter the protein levels remained relatively constant or increased only slightly (Fig. 17). The accumulation of the 24 + 22 kD proteins displayed similar differences between somatic and zygotic embryos. There was a prolonged period of accumulation in the somatic embryos, and a more rapid, shorter accumulation period in the zygotic embryos (Fig. 18). The relative abundance of the 24 + 22 kD proteins actually declined during the later stages of embryo maturation in the zygotic samples.

4.5. IDENTIFICATION AND CHARACTERIZATION OF A cDNA ENCODING THE 41 kD STORAGE PROTEIN

Comparison of storage protein gene expression at the molecular level in zygotic and somatic embryos required the use of storage protein cDNA probes. A cDNA library was constructed with the expression vector pUC 18, using mRNA extracted from late

FIGURE 17. Relative quantification of 41 kD protein immunoblots during zygotic and somatic embryo development.

UPPER PANEL: Immunoblot of zygotic embryo cotyledonary stages collected during 1989. ND, not detected on scan.

LOWER PANEL: Immunoblot of somatic embryo developmental stages on 40 μ M ABA. The developmental stages from early to late cotyledonary are described in Table 1. ND, not detected on scan.

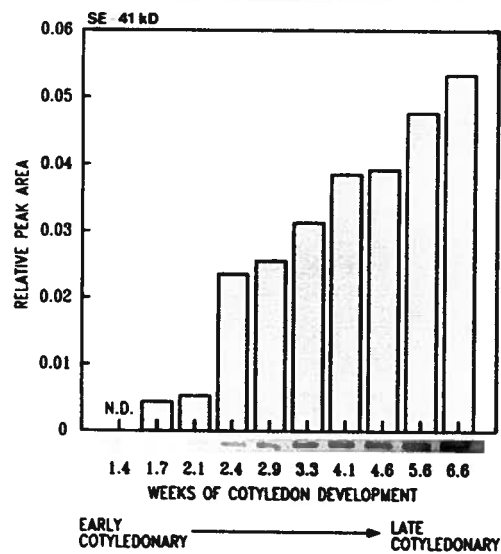
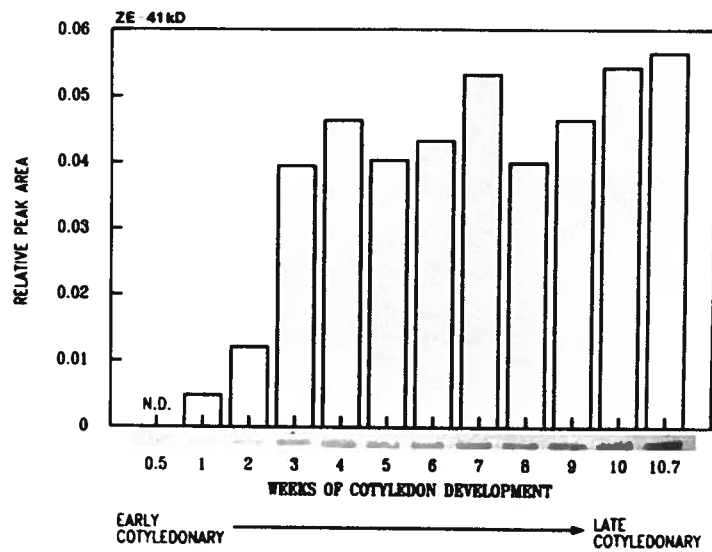
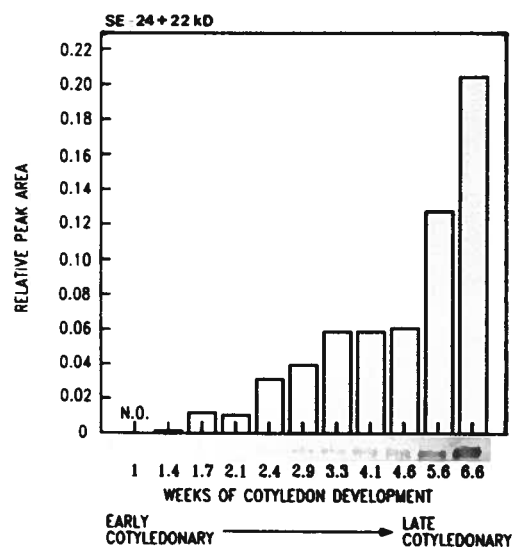
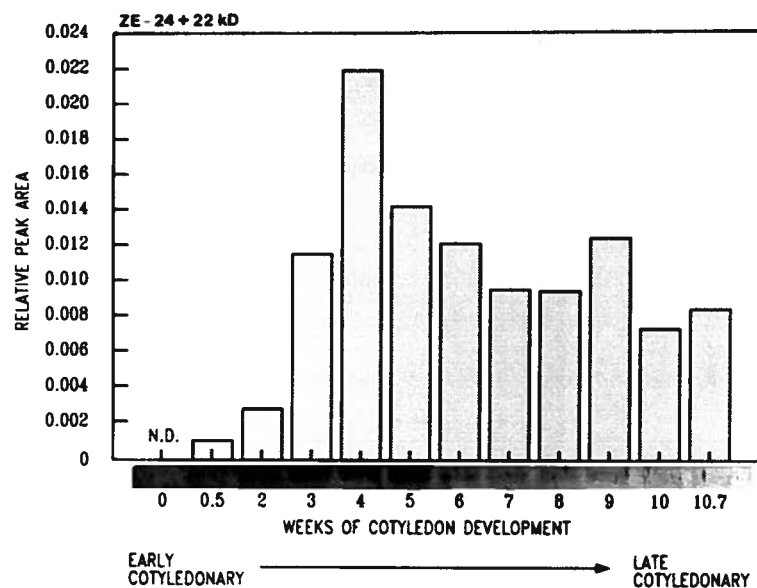


FIGURE 18. Relative quantification of 24 + 22 kD protein immunoblots during zygotic and somatic embryo development.

UPPER PANEL: Immunoblot of zygotic embryo cotyledonary stages collected during 1989. ND, not detected on scan.

LOWER PANEL: Immunoblot of somatic embryo developmental stages on 40 μ M ABA. The developmental stages from early to late cotyledonary are described in Table 1. ND, not detected on scan.



cotyledonary somatic embryos. A portion of the library was screened using polyclonal antibodies against purified 41 kD storage protein. A positive clone, designated II5A.0 was identified and characterized further. This clone contained an insert of approximately 1.7 kb, making it large enough to code for the 41 kD protein class. Other clones have been identified by cross hybridization with II5A.0 and are of similar size (Dr. Craig Newton, personal communication). Sequencing of the II5A.0 clone revealed a 1633 bp insert (Fig. 19), with a reading frame of 1406 bp, encoding a putative precursor protein of 466 amino acids which ran from the ATG start codon at base 9 to the stop codon at base 1406. This protein had a predicted molecular mass of 51,835. The putative polypeptide was compared with known protein sequences in the EMBL data bank and showed a high degree of similarity to several angiosperm vicilin-type storage protein sequences (Fig. 20). It was concluded that the II5A.0 cDNA was a spruce storage protein cDNA.

The predicted molecular mass of the putative storage protein was slightly higher than the apparent molecular mass of the mature protein, as determined by SDS-PAGE. Amino-terminal sequencing of the protein suggested that the mature protein started at the glycine residue which corresponded to amino acid 48 (Dr. Craig Newton, personal communication), with the preceeding amino acid region serving as a signal sequence which was co-translationally cleaved. Computer analysis to identify the potential hydrophobic signal sequence indicated that the first 18 amino acid residues could serve as the signal sequence

FIGURE 19. Sequence analysis of the spruce II5A.0 cDNA clone. The nucleotide sequence of the spruce II5A.0 cDNA described in the Results is shown. The deduced amino acid sequence is given in single-letter code. The N-terminal amino acid sequence displaying almost complete duplication is underlined, and the potential N-terminal amino acid residue of the mature protein is indicated by the solid triangle.

GCATCATCATGGTTTTTCGCTTCTTTACTTATGATTCTTCTTGCAATCTCCTCCTCGGCTGCCCTCACCGAG 74
M V F A S L L M I L L A I S S S S A A L T E

CCACTAGCCAGCACGGCCAATCCAACCTCCTCCTCGGCTGCCCTCACTGAGCCACTATCCAGCACGGCCAAT
P L A S T A N P T S S S S A A L T E P L S S T A N

CCAGGAGTTTTTCTGAATATCTCGGCCGAGGCCGAGGAGACGAGAAGAAGAGCGAGAGGAGAATCCATACGTA 224
P G V F P E Y L G R G R G R R E E E R E E N P Y V
▲

TTCCACAGTGACAGCTTCAGGACCAGAGCATCATCTGAAGCTGGTGAAATCAGAGCTCTGCCGAACCTTGGGGAG
F H S D S F R T R A S S E A G E I R A L P N F G E

GTCTCTGAACTTCTGAAGGGATTAGAAAATTCAGAGTTACCTGCATTGAAATGAAACCCAATACAGTGATGCTC 374
V S E L L E G I R K F R V T C I E M K P N T V M L

CCTCACTATATTGATGCGACATGGATCTTATATGTTACTAGAGGAAGAGGCTACATAGCCTATGTGCACCAGAAT
P H Y I D A T W I L Y V T R G R G Y I A Y V H Q N

GAGCTGGTTAAAGAAAGTTGGAGGAAGGAGATGTATTCGGTGTCCAAGTGGTCATACATTTTATCTCGTTAAC 524
E L V K R K L E E G D V F G V P S G H T F Y L V N

AACGATGACCATAGCACCTTCGCATTGCCAGTCTCCTCGCTCCCGTGTCTACGATCCCAGGAGAATATCAGCCC
N D D H S T L R I A S L L R P V S T I P G E Y Q P

TTCTACGTTGCGGGAGGTTCGAATCCTCAGAGTGTCTTACTCTGCCTTTAGCGATGATGTTCTCGAGGCTGCATTC 674
F Y V A G G R N P Q S V Y S A F S D D V L E A A F

AATACGAACGTACAGCAGCTTGAACGTATTTTCGGTGGACACAAAAGCGGAGTCATAATCCACGCAATGAAGAA
N T N V Q Q L E R I F G G H K S G V I I H A N E E

CAGATTAGAGAAATGATGAGGAAACGGGGATTATCAGCAGGATCCATGTCTGCACCTGAGCACCCCAAGCCTTTC 824
Q I R E M M R K R G L S A G S M S A P E H P K P F

AACCTTCGGAACCAAGCCAGATTTTCGAGAACGAAAATGGCAGGTTTACTATTGCTGGTCCCAAAAATTATCCT
N L R N Q K P D F E N E N G R F T I A G P K N Y P

TTTCTAGACGCGCTCGACGTTTCTGTTGGGCTTGCCGATTTGAATCCTGGATCCATGACAGCCCCATCTCTCAAC 974
F L D A L D V S V G L A D L N P G S M T A P S L N

TCGAAATCAACGTCAATCGGCATTGTTACGAATGGGGAAGGAAGGATTGAGATGGCATGCCCGCACCTTGGTCAA
S K S T S I G I V T N G E G R I E M A C P H L G Q

CATAGCTGGTCTAGTCCGCGTGAGAGAGGCGACCAAGATATTACTTACCAGAGAGTCTGGGCAAAGCTGAGGACC 1124
H S W S S P R E R G D Q D I T Y Q R V W A K L R T

GGCAGCGTTTATATTGTTCTGCTGGTCATCCAATCACGGAGATAGCTTCAACAAAACAGCCGCTGCAAATCTTG
G S V Y I V P A G H P I T E I A S T N S R L Q I L

TGGTTTGATCTTAATACCCGCGGCAATGAGAGACAATTCTGGCAGGAAAGACAATGTGCTTAACACGTTGGAG 1274
W F D L N T R G N E R Q F L A G K N N V L N T L E

AGGGAGATCAGGCAGATATCCTTCAACGTACCACGTGGGGAAGAGATTGAAGAAGTGTGTCAGGCACAAAAGGAT
R E I R Q I S F N V P R G E E I E E V L Q A Q K D

CAAGTGATCCTCAGAGGCCCAACGACGAAGCCGGGACGAGGCGAGGAGCTCTTCTTAGATCCATGTCATCATT 1424
Q V I L R G P Q R R S R D E A R S S S *

GCAGATCGCATTATGGACGACATGACAAGAGTTTCTCCACGTTCACTCTTAATATGTAGTTAAGAATAAGCTATC
CATAAATGTGTTGCAAGATGAACCTCTTCTGTTTAAATGAATTATGTATGAGTCTAACAAAGCTATCGTTGGGCT 1574
CCTCTTCTACTTCAATGCAATGAAACGCGAGGTCTCTCTTAAAAA

FIGURE 20. Amino acid sequence comparison of II5A.0 with other angiosperm vicilin-type storage protein sequences. The figure compares the amino acid sequence of spruce II5A.0 with those of the cotton vicilin (alpha-globulin A) precursor, the broad bean vicilin precursor (BRDBN) and the soybean β -conglycinin alpha-chain precursor (SOYBN). Sequences were aligned to maximize identities. Positions in the alignment that are perfectly conserved are boxed. Positions that are well conserved are indicated by asterisks.

| | |
|---|--------|
| M---V---FASLLMILTA-----IS-----SSS-----AALTEP----- | II5A.0 |
| M---VRNKSVMFVLLFSLHLS-----FGLLCSAKDFPGR-----RSEDDPQQRY- | COTTON |
| MAATTLKDSFPLLTLGIAHLASV-----CLSS----- | BRDBN |
| M---MRARFPLL-LLGVVFLASVSFSGIAYWEKQNP SHNKL RSCNSEKDSYR | SOYBN |
| * * * | |
| -----LASTANPT-----S-----SSSAALTE----- | II5A.0 |
| -EDCRKRCQLETRGQTEQDKCED-----RSETQLKEEQQRDGEDPQR- | COTTON |
| ----- | BRDBN |
| NQACHARCNLLKVEE--EEECEEGQIPRPRPQHPERERQQHGEKEEDEGEQPRPF | SOYBN |
| -----PLSSTANPGVFPEYL----- | II5A.0 |
| RYQDCRQHCQEE-----RRLRPHCEQSCREQYEQQQQQPDKQFKECQQ | COTTON |
| ----- | BRDBN |
| PFP RPRQPHQEEHEQKEHEWHRKEEKHGGKGS EEEQDEREHP RPHQPHQKEE | SOYBN |
| -----GRGRGRREEEREE-----N | II5A.0 |
| RCQWQEQRP ERKQQCVKECREQYQEDPWKGERENKWREEEEEESD-EGEQQRNN | COTTON |
| -----RSDQDN | BRDBN |
| KHEWQ--HKQEKHQ--GKESEEEEEEDQDEDEEQDKESQSEGESQREPRRHKNKN | SOYBN |
| PYVFHSDSFRTASSEAGEIRALPNFGVEVSELLEGIRKERVTCIEMKPNTVMPLPH | II5A.0 |
| PYYFHRRSFOERFREEHGNRMVLORFADKHHLIRGINEHRIAILEANPNTFVLP | COTTON |
| PFVFNENFOTLFENENGHIRLLQKFDQHSKILLENLQNYRLLEYKSKPHITIFLPQ | BRDBN |
| PFHFNKSKFOTLFKNQYGHVRMLORFNKRSQQLQNLRDYRILEFNKSKPHITIFLP | SOYBN |
| * * * * * | |
| YIDATWILYVTRGRGYIAYVHQNELVKKRLEEQDVFGVPSCHTFY----- | II5A.0 |
| HCDAEKIYVVVTNGRGTVTFVTHENKESYNVVPGVVVVRIFAGSTVY----- | COTTON |
| QTDADFILVVLSGKAILTVLLPNDNRNFSLSERGDTIKIFAGT----- | BRDBN |
| HADADYLIVILNGTAILTVNDDDRSYNLOSQDALRVHAGTTFFVVPNDNDENL | SOYBN |
| * * * * * | |
| -----LVNNDHSTLRIASLLRPVSTIPGEYQPFVAGGRNPQSVYSAFSD | II5A.0 |
| -----LANQDNREKLTIAVLRHPVNN-PGQFQKFFPAGQENPQSYLRIFSR | COTTON |
| --IG-----YLVNRDDEEDLRVLDLVIPVNR-PGEPOSFLLSGNQNP SILSGFSK | BRDBN |
| RMIAGTTFYVVPNDNDENLRMITLAIIPVNK-PGRFESFLLSSTQAQCSYLQGF SK | SOYBN |
| * * * * * | |
| DVLEA AFNINVOQLERIF-----GSHKS-----GVIIHANEEDIRE | II5A.0 |
| EILEA FNMTRSEQLDEL P-----GGRQSHRRQQQGGMFRKASQEQIRA | COTTON |
| NILEASFNDIYKEIEKVLLEEKGKEYHRRLKDRRQRQGEENVIVKISRQIEE | BRDBN |
| NILEASYDIKFEEINKVLF--GREGQQQG--EERLQ-----ESVIVEISKQIRE | SOYBN |
| * * * * * | |
| MMRKRGLSAGSMSAPEHPKPFNLNRQKPD FENENGRFTIAGPKNYPF-LDALDVS | II5A.0 |
| LSQG---ATSPRGKSGEYAFNLLSQTPRYSNQNGRFYEACPRNFQQQLREVDSS | COTTON |
| L-NKNAKSSSKKSTSSSEFFNLRSREPIYSNKFGKFFETPKRNP-QLQDLNIF | BRDBN |
| L-SKHAKSSSRKTISSEDKFNLGSRDPIYSNKLGLFEIT-QRNP-QLRDLDFV | SOYBN |
| * * * * * | |
| VGLADINPESMTAPSLNSKSTSIGIVTNGEGRIEMACPHLGQHS--WSSPRE--R | II5A.0 |
| VVAFEINKGSI FVPHYNSKATFVVLVTEGNHVMVCPHLSRQSSDWSSREEEEQ | COTTON |
| VNYVEINEGSLLPHYNSRAIVIVTVNECKGDFELVGQRNENQQGLREEYDEEKE | BRDBN |
| LSVVDMMNEGALFILPHFNSKAI VVLVINEGEANIELVGIKEQQQR----- | SOYBN |
| * * * * * | |
| GDQDIT-----YQRVWAKLRTGSVYIVPAGHPITEIASTNSRLQILWFDL-NTRG | II5A.0 |
| EEQEVERSSGQYKRVRAQLSTGNLFVVPAGHPVTFVASQNEDLGLLGFGLYNGQD | COTTON |
| QGEIEIRK--QVQNYKAKLSPGDVLVIPAGMPV--AIKASSNINLVGFGI-NAEN | BRDBN |
| QQQEEQPL--EVRKYRAELSEQDIFVVPAGMPV--MVNATSDLNFFAFGI-NAEN | SOYBN |
| * * * * * | |
| NERQFLAG-KNNVLNTLEREIRQISFNVPRGEEIEEVLQAKQDV-ILRGPPRRS | II5A.0 |
| NKRIFFVAG-KTNNVRQWDRQAKELAFGVE-SRLVDEVFNNNPQESYFVSGRDRRG | COTTON |
| NORYFLAGEEDNVISQIHKPVKELAFPGS-AQEVDTLLENQKQ-SHFANAQPRER | BRDBN |
| NORNFLAGSKINVISQIPSQVQELAFPRS-AKDIENLIKQSE-SYFVDAQPPQK | SOYBN |
| * * * * * | |
| RDEARSSS----- | II5A.0 |
| FDERRGSNNPLSPFLDFARLF | COTTON |
| ERGSQEI KDHLYSILG--SF- | BRDBN |
| EENKGRKGPLSSILR--AFY | SOYBN |
| * * * | |

(MVFASLLMILLAISSSSA/ALT). Interestingly, part of the potential signal sequence was contained in an almost complete sequence duplication at the amino terminus (Fig. 19). The predicted molecular weight of the protein encoded by amino acids 48 through 466 was approximately 47 kD, still slightly larger than the mature protein, suggesting that further post-translational processing might occur. To test this assumption, late cotyledonary somatic embryos were labelled for 4 hours with ^{35}S -methionine, and then chased for up to 24 hours. Total protein profiles (Fig. 21, upper panel) and immunoprecipitation of labelled proteins with 41 kD polyclonal antibodies (Fig. 21, lower panel) revealed a precursor protein that was approximately 3-4 kD larger than the mature protein. Processing of the precursor was not evident until at least 4 hours of chase, and was almost complete by 8 hours of chase (Fig. 21). Another protein was detected by immunoprecipitation that did not change during the chase period (Fig. 21). Since no major proteins of this size were observed in total labelled protein profiles, it was concluded that this is a cross-reacting protein.

Two-dimensional protein analysis suggested that the 41 kD protein class consisted of several isoelectric variants, possibly encoded by a multi-gene family (Figs. 5 and 13). Spruce genomic DNA was digested with several different restriction enzymes, blotted and probed with II5A.0 cDNA under moderately-stringent conditions (Fig. 22). Numerous bands were observed in each digest and copy reconstruction standards suggested the presence of approximately 10-15 copies per genome (Fig. 22).

FIGURE 21. Pulse:chase-labelling of late cotyledonary somatic embryos differentiated on 40 μ M ABA. Embryos were labelled for 4 hours with 35 S-methionine (0 time), then chased with cold methionine for up to 24 hours.

UPPER PANEL: Total labelled protein profile. Open arrow denotes the potential precursor to the mature 41 kD protein (closed arrow).

LOWER PANEL: Labelled proteins from the above treatments immunoprecipitated with 41 kD antibody. The disappearance of the precursor protein (open arrow) and the appearance of the mature protein (closed arrow) is visible.

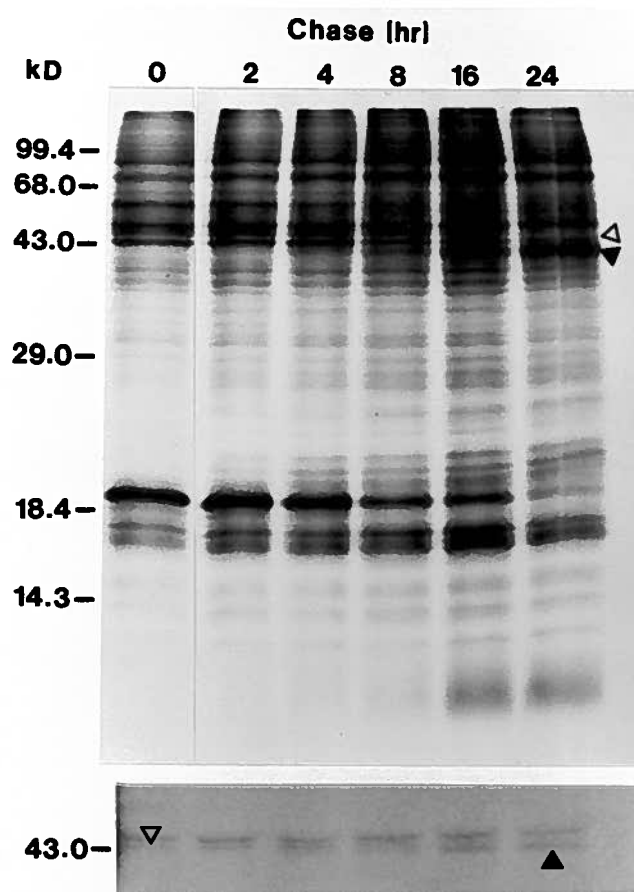
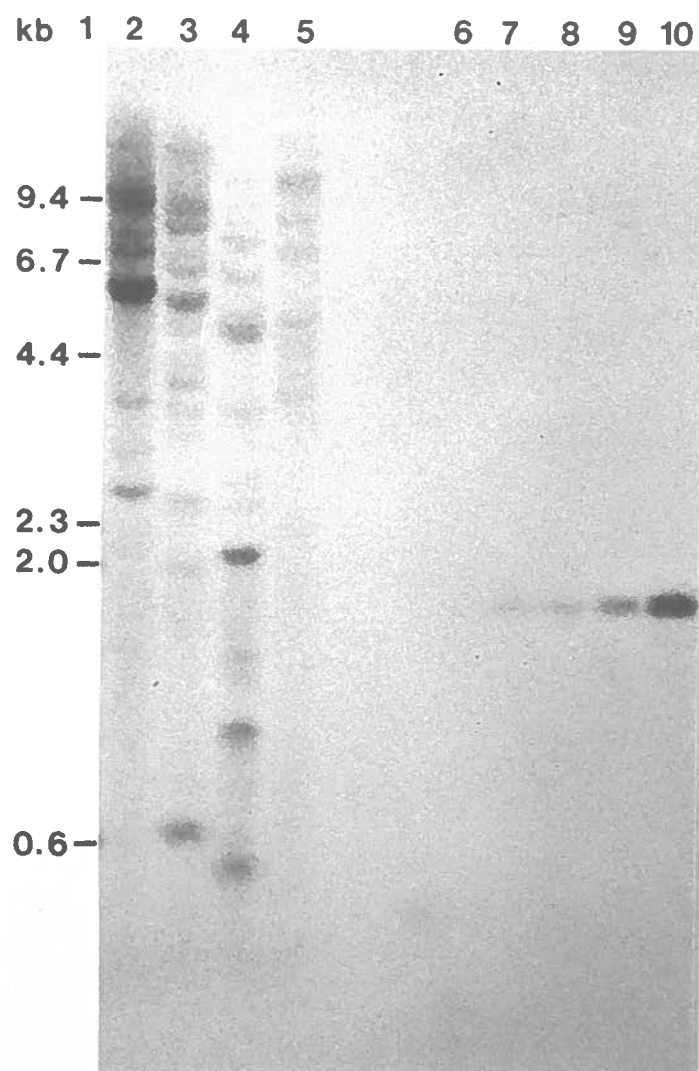


FIGURE 22. DNA gel blot analysis of spruce DNA probed with II5A.0 cDNA. Lane 1 contains lambda *Hind* III molecular weight standards. Lanes 2 through 5 contain 5 μ g of spruce DNA digested with *Hind* III (Lane 2), *Bam* HI (Lane 3), *Eco* RI (Lane 4) or *Xba* I (Lane 5). Lanes 6 through 10 contain gene copy number reconstructions at 0.8 copy per haploid genome (Lane 6), 1.6 copies (Lane 7), 2.4 copies (Lane 8), 4 copies (Lane 9) or 8 copies (Lane 10). Gene copy reconstructions were made using II5A.0 cDNA, and a haploid DNA content of 8.5 pg.



During the course of the above work, other cDNAs were also isolated and sequenced. The results revealed similarity to angiosperm legumin-type storage proteins (Dr. Craig Newton, personal communication). The spruce legumin-type sequences, designated XI5H, encoded precursor proteins with conserved proteolytic processing sites which would yield disulfide-linked 35-33 and 24-22 kD proteins (Dr. Craig Newton, personal communication), similar to the storage proteins identified in this study. Thus, probes were available to study the expression of the spruce vicilin-type (41 kD) and legumin-type (35-33, 24-22 kD) storage proteins.

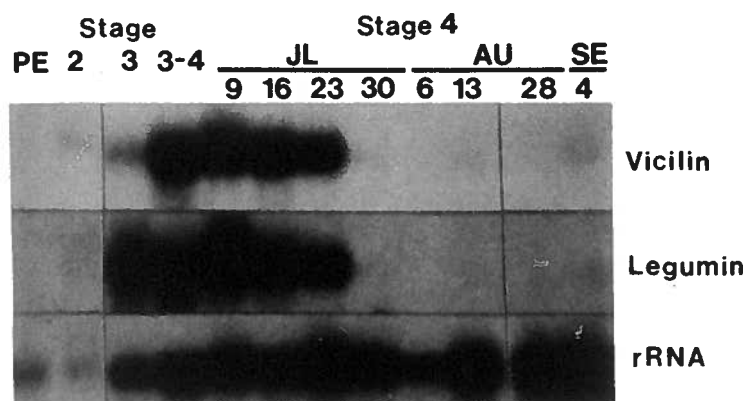
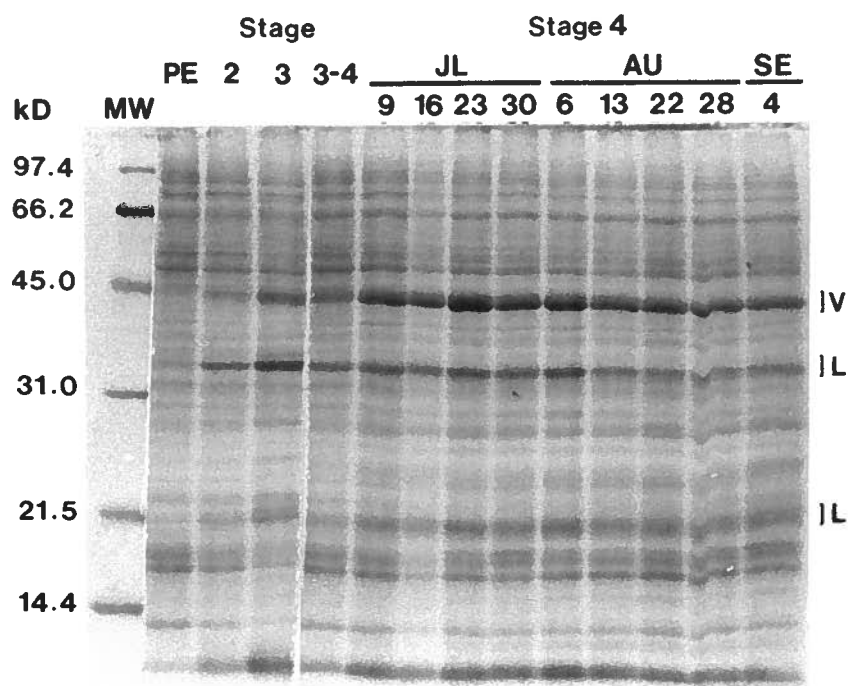
4.6. RNA GEL BLOT ANALYSIS OF LEGUMIN AND VICILIN STORAGE PROTEIN TRANSCRIPTS IN ZYGOTIC EMBRYOS AND SOMATIC EMBRYOS DIFFERENTIATED ON 40 μ M AND 10 μ M ABA

Analysis of zygotic embryos collected during 1991 revealed, as described previously, that the storage proteins displayed differential accumulation patterns, accumulated to high levels during cotyledon development, and reached steady state levels 1-1½ months prior to mature seed shed (Fig. 23). Analysis of the mRNA levels for the spruce vicilin and legumin proteins revealed that, although the different protein classes displayed differential accumulation patterns, their messages were detectable at the same time, torpedo stage (Stage 2), of development (Fig. 23). The messages accumulated rapidly, reaching high levels during cotyledon development, and then declined rapidly to low levels. Scanning densitometry of RNA gel blots (using values normalized to constant rRNA) was used to

FIGURE 23. Changes in total proteins and storage protein mRNA during zygotic embryo development.

UPPER PANEL: Coomassie-stained SDS-PAGE of total proteins from zygotic embryos collected during 1991, ranging in development from proembryos (PE) to mature seed embryos (Stage 4, September 4). The spruce vicilin (V) and legumin (L) proteins are indicated. Each lane contains 12 μ g protein. MW, molecular weight standards.

LOWER PANEL: RNA gel blot analysis of total RNA from the same zygotic embryo developmental stages described above, probed with spruce vicilin (II5A.0), legumin (XI5H) or yeast 18S rRNA cDNA.



compare changes in levels of the mRNAs during development. The low loading in the proembryo and Stage 2 lanes, caused by inadequate supply of zygotic material from these early stages, required use of correction values (on the order of 68x) to normalize these values. This increased the error probability for the normalized values, so only samples from Stage 3 onwards were normalized. Densitometry revealed that the legumin mRNA reached high levels earlier in cotyledon development than the vicilin mRNA (Table 4). The vicilin mRNA remained slightly higher in Stage 4 embryos during the period between July 16 to July 30, but then both mRNA classes declined to around 1% or less of maximal levels (Table 4).

A different genotype (W70) was used during this phase of the research because of a loss in vigour of genotype W29. Somatic embryos of W70 were similar in general appearance to those of W29 although, by 9 weeks on 40 μ M ABA, those of genotype W70 had a higher chlorophyll content (Table 5), but had not precociously germinated.

The W70 embryos which were matured on 40 μ M ABA displayed a similar trend in storage protein accumulation to that observed for genotype W29. Legumin protein accumulations were detectable by SDS-PAGE in early cotyledonary stages, prior to the vicilin (Fig. 24). All proteins accumulated during cotyledonary development, and accumulation was still evident after 9 weeks on ABA (Fig. 24). RNA gel blots revealed that both vicilin and legumin mRNAs were detectable at the same time in round torpedo stage embryos (Fig. 24), similar to the observations with zygotic

TABLE 4. Developmental changes in storage protein mRNAs in zygotic embryos as determined by scanning densitometry of RNA gel blots.

| Developmental Stage | Storage protein mRNA level (% of maximal hybridization) | |
|---------------------|--|---------|
| | Vicilin | Legumin |
| Stage 3 | 72 % | 100 % |
| Stage 3-4 | 96 % | 50 % |
| Stage 4 (JL 9) | 100 % | 59 % |
| Stage 4 (JL 16) | 93 % | 39 % |
| Stage 4 (JL 23) | 32 % | 11 % |
| Stage 4 (JL 30) | 7 % | 1 % |
| Stage 4 (AU 6) | 1 % | < 1 % |
| Stage 4 (AU 13) | 1 % | < 1 % |
| Stage 4 (AU 28) | 1 % | < 1 % |
| Mature | 2 % | < 1 % |

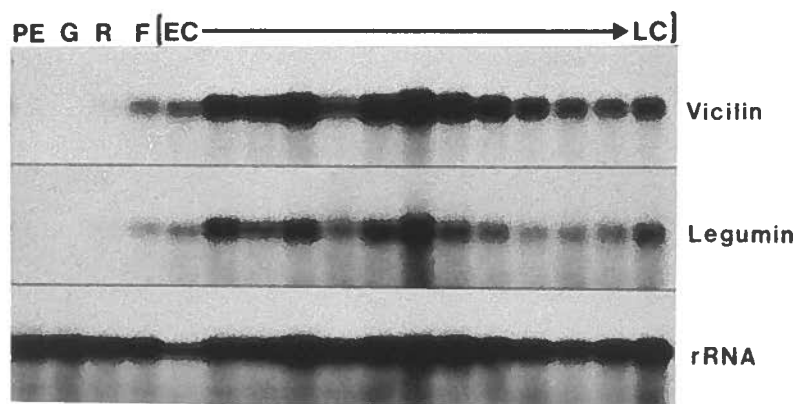
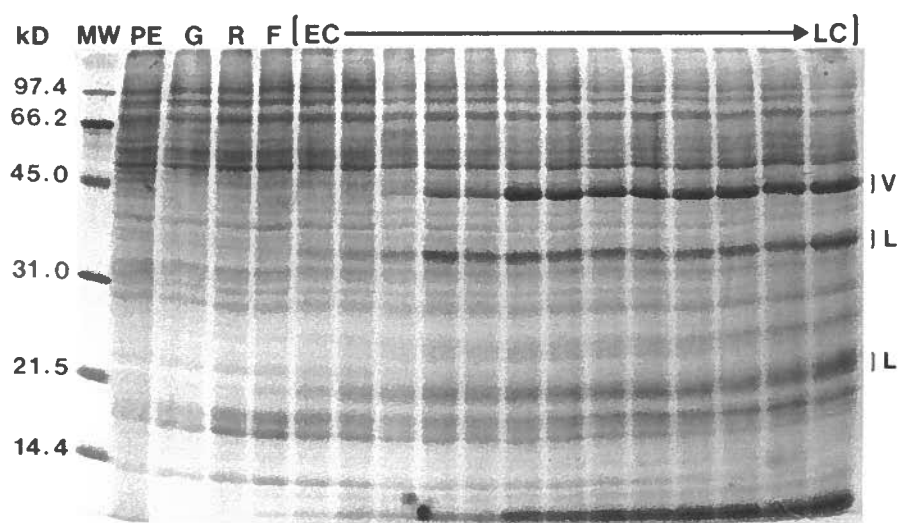
TABLE 5. Chlorophyll content (mg/g FW) of Stage 4-6 and 9 week ABA somatic embryos of genotypes W29 and W70. Mean \pm SE.

| Genotype | Stage of embryo development | |
|----------|-----------------------------|-------------------|
| | 4-6 | 9 weeks ABA |
| W29 | 0.014 \pm 0.006 | 0.011 \pm 0.001 |
| W70 | 0.019 \pm 0.001 | 0.098 \pm 0.030 |

FIGURE 24. Changes in total proteins and storage protein mRNA during somatic embryo development on 40 μ M ABA.

UPPER PANEL: Coomassie-stained SDS-PAGE of total proteins from somatic embryos matured up to 9 weeks on ABA. Developmental stages are as described for Fig. 16. The vicilin (V) and legumin (L) proteins are indicated. Each lane contains 12 μ g protein. MW, molecular weight standards.

LOWER PANEL: RNA gel blot analysis of total RNA from the same somatic embryo developmental stages described above, probed with spruce vicilin (II5A.0), legumin (XI5H) or yeast 18S rRNA cDNA.



embryos. Both mRNA classes increased during cotyledon development, but declined during the later stages. RNA gel blots were analyzed by densitometry as described previously. The legumin mRNA reached high levels earlier in cotyledon development than the vicilin mRNA (Table 6), similar to observations made with zygotic embryos. While both mRNA classes declined after their peak levels (Fig. 24, Table 6), their decline was not as large as observed for zygotic embryos (Fig. 23, Table 4) and, after 9 weeks on ABA, both mRNA classes were present at approximately 50% of their maximal levels (Table 6).

Somatic embryos which matured on 10 μ M ABA developed in a similar manner to those on 40 μ M ABA up to the mid-cotyledon stage. However, once the cotyledons had overgrown the shoot apex (after Stage 4-2), the embryos accumulated chlorophyll and exhibited hypocotyl/cotyledon elongation, characteristic of precocious germination. These differences prohibited the classification of later stage embryos on 10 μ M ABA based on the length/morphological standards used for non-germinating embryos on 40 μ M ABA, and they were thus classified as germinants (Germ 1-6). Embryos from both 10 μ M and 40 μ M ABA were collected for analysis after the same duration of ABA exposure. Germ 1 samples were precociously germinating embryos collected at the same time as Stage 4-3 embryos on 40 μ M ABA and Germ 6 were more advanced precocious germinants collected at the same time as Stage 4-8 embryos (8 weeks). The intermediate stages formed a developmental size continuum of precocious embryos.

TABLE 6. Developmental changes in storage protein mRNAs in somatic embryos differentiated on 40 μ M ABA as determined by scanning densitometry of RNA gel blots.

| Developmental Stage | Storage protein mRNA level (% of maximal hybridization) | |
|---------------------|--|---------|
| | Vicilin | Legumin |
| Proembryo | 0 % | 0 % |
| Globular | 0 % | 0 % |
| Round head | 8 % | 7 % |
| Flat head | 36 % | 29 % |
| Stage 3-1 | 62 % | 66 % |
| Stage 3-2 | 84 % | 100 % |
| Stage 3-3 | 76 % | 63 % |
| Stage 3-4 | 80 % | 55 % |
| Stage 4-1 | 50 % | 50 % |
| Stage 4-2 | 86 % | 64 % |
| Stage 4-3 | 100 % | 67 % |
| Stage 4-4 | 69 % | 40 % |
| Stage 4-5 | 73 % | 46 % |
| Stage 4-6 | 60 % | 41 % |
| Stage 4-7 | 61 % | 47 % |
| Stage 4-8 | 46 % | 41 % |
| Stage 4-9 | 51 % | 50 % |

Somatic embryos on 10 μM ABA also displayed differential storage protein accumulation during the early stages of cotyledon development. Legumin accumulation was detected prior to vicilin (Fig. 25), although these proteins occurred at a slightly later stage in cotyledon development than in 40 μM ABA embryos. However, the storage protein levels declined as precocious germination commenced (Fig. 25) and did not reach the levels found in 40 μM ABA somatic embryos. RNA gel blots revealed that both vicilin and legumin mRNAs were detectable in flat torpedo stage embryos (Fig. 25). Both mRNA classes increased during cotyledon development and declined as precocious germination commenced. However, very low levels were still detectable at the Germ 6 stage, several weeks after precocious germination had started (Fig. 25). Densitometric analysis of RNA gel blots revealed that the legumin mRNA increased earlier than the vicilin mRNA (Table 7), similar to the pattern observed for zygotic embryos and somatic embryos on 40 μM ABA. Comparison of 10 μM ABA samples to a control sample from 40 μM ABA somatic embryos on the same blots revealed that the maximum vicilin and legumin mRNA levels attained in the 10 μM ABA samples were approximately 70% of the maximum levels found in 40 μM ABA somatic embryos.

4.7. ANALYSIS OF STORAGE PROTEIN EXPRESSION IN SOMATIC EMBRYOS IN RESPONSE TO OSMOTIC STRESS

Early cotyledonary (Stage 3-4) embryos differentiated on 40 μM ABA were cultured for 2 weeks on medium containing no growth regulators, 40 μM ABA, 15% mannitol or mannitol plus the ABA biosynthetic inhibitor fluridone, to determine if osmotic stress-

FIGURE 25. Changes in total proteins and storage protein mRNA during somatic embryo development on 10 μ M ABA.

UPPER PANEL: Coomassie-stained SDS-PAGE of total proteins from somatic embryos matured up to 8 weeks on ABA. Somatic embryos were collected after the same duration of total ABA exposure as those on 40 μ M ABA. Embryos were classified by the same developmental stages as those on 40 μ M ABA until after mid-cotyledon development (Stage 4-2), after which they began to precociously germinate (Germ 1). Precocious germinants were collected at increasing stages of development (Germ 1-Germ 6; see text). The vicilin (V) and legumin (L) proteins are indicated. Each lane contains 12 μ g protein. MW, molecular weight standards.

LOWER PANEL: RNA gel blot analysis of total RNA from the same somatic embryo developmental stages described above, probed with spruce vicilin (II5A.0), legumin (XI5H) or yeast 18S rRNA cDNA.

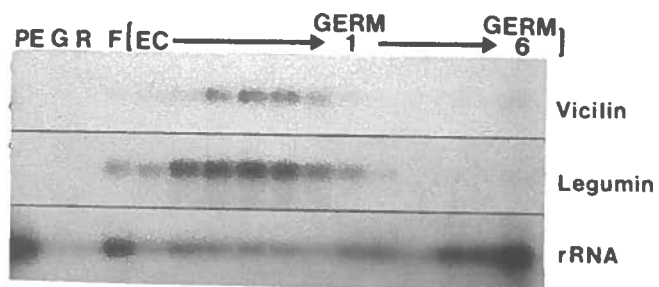
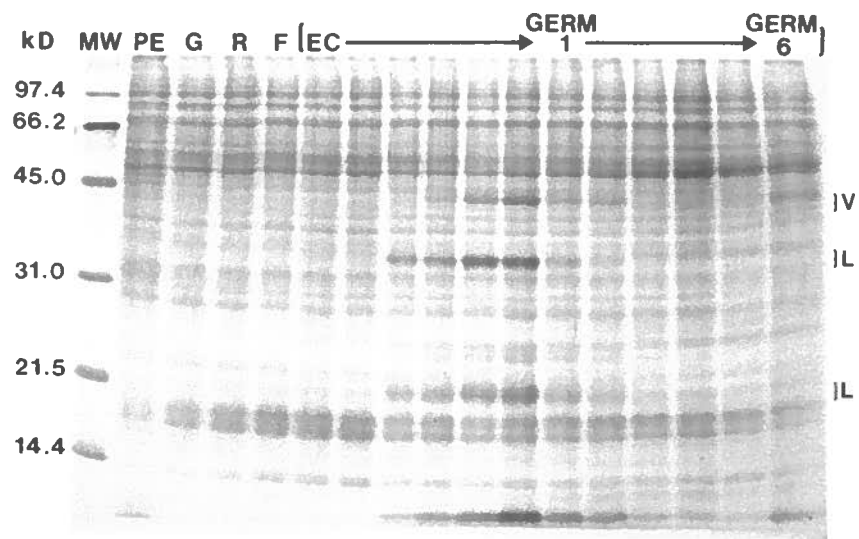
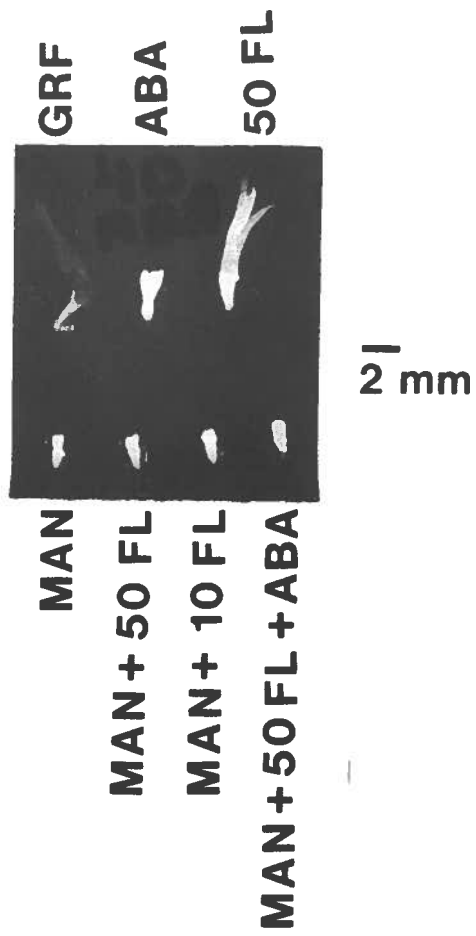


TABLE 7. Developmental changes in storage protein mRNAs in somatic embryos differentiated on 10 μ M ABA as determined by scanning densitometry of RNA gel blots.

| Developmental Stage | Storage protein mRNA level (% of maximal hybridization) | |
|---------------------|--|---------|
| | Vicilin | Legumin |
| Proembryo | 0 % | 0 % |
| Globular | 0 % | 0 % |
| Round head | 0 % | 0 % |
| Flat head | 5 % | 12 % |
| Stage 3-1 | 15 % | 47 % |
| Stage 3-2 | 23 % | 60 % |
| Stage 3-3 | 82 % | 81 % |
| Stage 3-4 | 100 % | 95 % |
| Stage 4-1 | 88 % | 100 % |
| Stage 4-2 | 75 % | 68 % |
| Germ 1 | 41 % | 67 % |
| Germ 2 | 9 % | 14 % |
| Germ 3 | 1 % | 2 % |
| Germ 4 | 1 % | 2 % |
| Germ 5 | 1 % | 2 % |
| Germ 6 | 3 % | 2 % |

FIGURE 26. Effects of culture on media containing no growth regulators, 40 μ M ABA, 15% mannitol or fluridone on somatic embryo development. Somatic embryos were matured on 40 μ M ABA until early cotyledonary stage (EC; Stage 3-4), after which they were cultured for a further 2 weeks on media containing no growth regulators (GRF), 40 μ M ABA (ABA), 50 mg/L fluridone (50 FL), 15% mannitol (MAN), mannitol + 50 mg/L fluridone (MAN + 50 FL), mannitol + 10 mg/L fluridone (MAN + 10 FL) or mannitol + 50 mg/L fluridone + 40 μ M ABA (MAN + 50 FL + ABA).



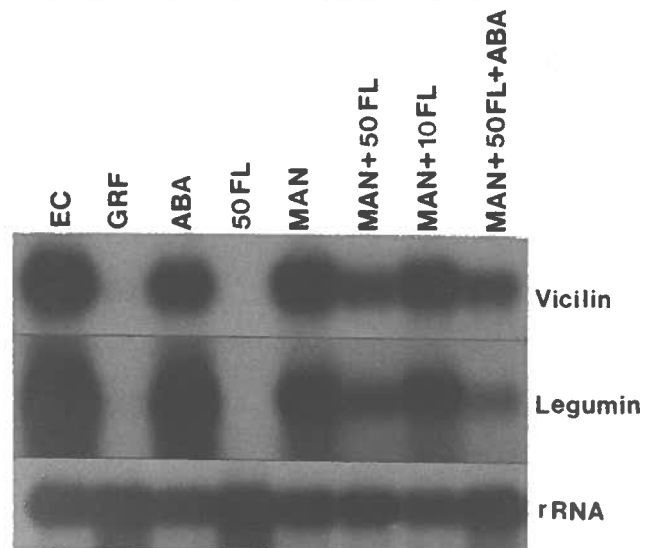
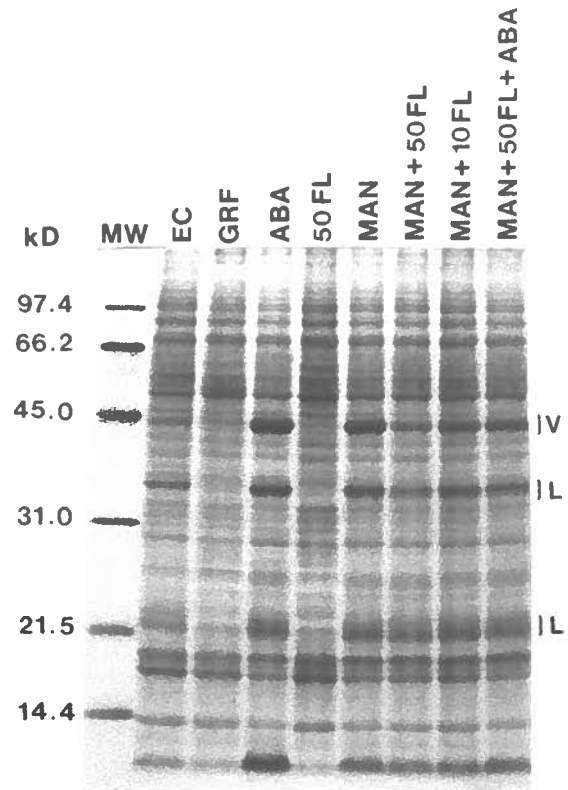
induced storage protein accumulation was mediated via ABA. After 2 weeks on growth regulator-free medium (with or without fluridone), embryos had precociously germinated (Fig. 26), although those cultured with fluridone were bleached in appearance. These results indicated that fluridone was not inhibitory to embryo survival or growth. Embryos cultured on 40 μ M ABA or 15% mannitol did not germinate precociously, and embryos cultured on mannitol were smaller in size than those on 40 μ M ABA (Fig. 26). Embryos cultured on mannitol, mannitol plus fluridone or mannitol plus fluridone and ABA were similar in size, although those exposed to fluridone were bleached in appearance (Fig. 26).

Protein profile differences between the various treatments after the 2 week culture period were analyzed by SDS-PAGE (Fig. 27). Early cotyledonary embryos collected prior to the start of the treatments contained low levels of storage proteins (Fig. 27). After 2 weeks on growth regulator-free medium (with or without fluridone), no storage protein accumulation was observed, and the low levels initially present in the embryos had declined (Fig. 27). Embryos on 40 μ M ABA accumulated storage protein, as did those exposed to 15% mannitol, although accumulation was slightly less in the mannitol-treated embryos (Fig. 27). Embryos cultured on mannitol plus 50 mg/L fluridone contained lower levels of storage proteins compared to the mannitol treatment, while embryos on mannitol plus 10 mg/L fluridone or mannitol plus 50 mg/L fluridone and 40 μ M ABA displayed higher storage protein

FIGURE 27. Changes in total proteins and storage protein mRNA in somatic embryos exposed to no growth regulators, 40 μ M ABA, 15% mannitol or fluridone.

UPPER PANEL: Coomassie-stained SDS-PAGE of total proteins from somatic embryos. Embryos were treated as described for Fig. 26. The vicilin (V) and legumin (L) proteins are indicated. Each lane contains 12 μ g protein.

LOWER PANEL: RNA gel blot analysis of total RNA from the same somatic embryo treatments as above, probed with spruce vicilin (II5A.0), legumin (XI5H) or yeast 18S rRNA cDNA.



levels than those in the mannitol plus 50 mg/L fluridone treatment (Fig. 27).

Analysis of storage protein mRNAs were carried out for the above treatments. Vicilin and legumin mRNAs were present in initial explants, declined to very low levels in the growth regulator-free treatment, and were undetectable in embryos exposed to growth regulator-free conditions with 50 mg/L fluridone. Storage protein mRNAs were present at high levels in embryos cultured on 40 μ M ABA or 15% mannitol (Fig. 27). Embryos exposed to high levels of fluridone (50 mg/L) while on mannitol had reduced storage protein mRNA levels, but embryos exposed to low fluridone levels (10 mg/L) contained higher storage protein mRNA levels, similar to the mannitol-only and ABA treatments (Fig. 27). However, embryos cultured on medium containing mannitol plus 50 mg/L fluridone and ABA contained vicilin and legumin mRNA levels similar to the mannitol plus 50 mg/L fluridone treatment. The addition of ABA to this treatment did not enhance storage protein transcript levels.

5. DISCUSSION

5.1. INTERIOR SPRUCE STORAGE PROTEINS

This study showed that the major storage proteins of interior spruce were a buffer-soluble 41 kD matrix protein and high salt-soluble disulfide-linked crystalloid proteins of 35-33 kD and 24-22 kD. Minor proteins of 30 and 27.5 kD were also identified. Storage proteins of similar molecular weight, solubility and disulfide-linkage characteristics have been described from other conifers such as white spruce (Gifford and Tolley 1989, Misra and Green 1990), *Pinus* species (Gifford 1988) and Douglas fir (Green et al. 1991), and similar disulfide-linked proteins were found in the non-coniferous gymnosperm *Ginkgo biloba* (Jensen and Berthold 1989). In addition to these proteins, there were 2 minor disulfide-linked proteins of 22 kD and 14 kD, similar to the "small-dimer" protein found in *Ginkgo biloba* (Jensen and Berthold 1989).

The results of this present study differ somewhat from those of Misra and Green (1990, 1991), who described a 42 kD crystalloid storage protein. However, this may have been an artifact arising from incomplete extraction procedures. The fact that antibodies raised against the 42 kD crystalloid protein cross-reacted with the matrix 42 kD protein (Misra and Green 1991) supports this conclusion.

The solubility and disulfide-linkage characteristics of the spruce 35-33 kD and 24-22 kD proteins were similar to those of angiosperm 11-12S globulin legumin-type storage proteins, whereas the 41 kD protein was similar to angiosperm albumin or 7S

globulin vicilin-type proteins (Higgins 1984, Shotwell and Larkins 1989). The identification and characterization of 2 cDNA classes that are highly expressed during spruce embryo development revealed further similarity to angiosperm vicilin-type and legumin-type storage proteins (this study and Dr. Craig Newton, personal communication), suggesting that the major spruce storage proteins are homologous to these two angiosperm storage protein classes.

Further similarities between interior spruce storage proteins and angiosperm storage proteins included isoelectric heterogeneity for each protein class, revealed by two-dimensional electrophoresis, suggesting that both protein groups were members of multi-gene families. This was substantiated for the spruce vicilin using DNA gel blots which indicated the presence of approximately 10-15 gene copies, a similar result to reports that angiosperm storage proteins are encoded by multi-gene families (Harada *et al.* 1989, Higgins 1984, Nielsen *et al.* 1989, Shotwell and Larkins 1989).

The relatively simple mixture of storage proteins in spruce protein body samples and embryos suggested that complex proteolytic processing patterns did not occur during their synthesis. In angiosperms, both 7S (vicilin-type) and 11S (legumin-type) storage proteins can show post-translational processing, apart from co-translational signal sequence cleavage, to yield mature proteins (Müntz 1989, Shotwell and Larkins 1989). Angiosperm 11S proteins are synthesized as large precursor proteins. The precursor protein forms an intrachain disulfide

linkage and is proteolytically processed at either a single point to yield two disulfide-linked proteins, or the precursor is proteolytically processed at two sites to yield two disulfide-linked proteins and a short, free linker polypeptide (Shotwell and Larkins 1989). Further processing of the disulfide-linked proteins may also occur (Dure 1989, Raynal et al. 1987). The spruce legumin cDNA sequence revealed a similar single conserved proteolytic cleavage site (Dr. Craig Newton, personal communication).

Pulse:chase labelling revealed that the mature interior spruce vicilin protein arose from the processing of a precursor protein 3-4 kD larger, suggesting that extensive proteolytic processing of the spruce vicilin did not occur. Some angiosperm vicilin-type proteins, such as β -conglycinin (Meinke et al. 1981) and phaseolin (Shotwell and Larkins 1989) also do not undergo extensive proteolytic processing apart from signal sequence removal. In contrast, some angiosperm vicilins, such as those from cotton (Dure 1989) and pea (Spencer et al. 1983) undergo extensive post-translational processing to form complex protein patterns.

5.2. ZYGOTIC EMBRYO STORAGE PROTEIN EXPRESSION

The results of this present study indicate that the spruce legumin-type proteins were detectable before the vicilin-type, thus indicating differential accumulation patterns. Low levels of legumin proteins were detected in pre-cotyledonary torpedo-stage embryos, whereas the vicilin protein was only detected in cotyledonary embryos, although both classes displayed major

accumulations during cotyledon development and reached peak levels well before mature seed shed, as water loss associated with maturation drying began (data not shown). This pattern was similar to that commonly described for angiosperms, where different storage protein classes within the same embryo displayed different accumulation patterns. In pea, vicilin synthesis and accumulation precedes that of legumin (Boulter *et al.* 1987), while in rapeseed napin accumulates earlier than cruciferin (Crouch and Sussex 1981, Murphy *et al.* 1989) and in soybean, β -conglycinin proteins are detectable prior to glycinin (Meinke *et al.* 1981).

Storage protein accumulation occurs during the cell expansion phase, after cell division and prior to drying and desiccation (Bewley and Black 1985). The major accumulation of spruce storage proteins during cotyledon development and prior to maturation drying is similar to these observations with angiosperms. The presence of low levels of spruce legumin storage proteins in torpedo-stage embryos indicated that some storage protein expression occurred prior to cotyledon development/expansion. Domoney *et al.* (1980) also reported that some pea legumin accumulated prior to the expansion phase. Recent work has shown that storage protein mRNAs only accumulate in cells lacking mitotic activity (Hauxwell *et al.* 1990), so spruce storage protein expression in pre-cotyledonary embryos may reflect expression in non-dividing, expanding cells of these early stage embryos. It is also possible that the differential appearance of spruce legumins in pre-cotyledonary embryos

reflected differences in storage protein expression patterns between the embryonic axis and cotyledons, with the vicilins primarily expressed in cotyledonary cells. Organ-specific storage protein expression patterns in soybean showed little glycinin accumulation compared to β -conglycinin in embryonic axes, but prominent levels of both storage proteins in cotyledons (Meinke et al. 1981).

The pattern of storage protein accumulation described in this study does not agree with most of the results reported recently for white spruce (Misra and Green 1991). While these workers also reported accumulations of some crystalloid proteins prior to the accumulation of a 42 kD protein, they found that the 35-34 kD crystalloids accumulated before 24-23 kD crystalloids. In contrast, the present results indicated that the 35-33 kD and 24-22 kD crystalloids displayed the same temporal pattern. Based on solubility, disulfide-linkage and cDNA sequence characteristics, the spruce 35-33 kD and 24-22 kD proteins appear homologous to the angiosperm legumins and, since the disulfide-linked angiosperm legumin proteins show concurrent accumulation patterns (Higgins 1984, Meinke et al. 1981, Müntz 1989, Shotwell and Larkins 1989), the results of this present study agree with the angiosperm data. Misra and Green (1991) also reported that the major accumulation of the 42 kD protein occurred between the last two collection dates (Aug. 29 and mature seed), well after the attainment of maximum dry weight and during the period of seed development in which maturation drying occurs. These results contrasted with those of this present study, where greatest

protein levels were attained at least 1 month prior to mature seed shed. This maximum coincided with steady state dry weight and the onset of maturation drying (data not shown), similar to results with angiosperms. Some of the discrepancies between Misra and Green (1991) and this work arise from the methods used by these workers to carry out their protein extractions and antibody production. It was also difficult to compare the present results with those of Misra and Green (1991) because they did not include data on morphological development, so that the protein samples analyzed were related only to collection date.

Analysis of interior spruce zygotic embryos revealed that both vicilin and legumin messages were detectable in torpedo stage embryos, even though the storage proteins displayed different accumulation patterns. Both the mRNAs increased to peak levels during cotyledon development, with legumin mRNA peaking and declining prior to that of vicilin. However, both dropped to low levels during the last month or so of zygotic seed development, coincident with the drying phase. The simultaneous appearance of both storage protein mRNAs may have been indicative of coordinate regulation. Most angiosperm embryos which show differential storage protein accumulation also show differential appearance of storage protein mRNAs, with the different mRNA classes displaying temporal differences in appearance by a few days (Boulter *et al.* 1987, Finkelstein *et al.* 1985, Walling *et al.* 1986, Yang *et al.* 1990), although Meinke *et al.* (1981) noted that soybean 7S and 11S storage protein mRNAs were detectable at

the same time during development. It is possible that the spruce vicilin and legumin mRNAs do display different appearance patterns, but these were not detected due to the time frame of the zygotic embryo collections. More frequent, meticulous collections during the early stages of zygotic embryo development are required to determine if different appearance patterns exist for the various spruce storage protein mRNAs.

Angiosperm storage protein mRNAs appear well after fertilization and during early embryo development (Boulter *et al.* 1987, Finkelstein *et al.* 1985, Harada *et al.* 1989, Nielsen *et al.* 1989), as did those in spruce (this study). However, recent work by Kamalay *et al.* (1991) described the appearance of legumin-like mRNAs in megagametophyte tissue of eastern white pine just following fertilization, and Misra and Green (1991) reported low levels of crystalloid protein in white spruce megagametophytes just following fertilization, well before their visible accumulation in this study. These results suggest that low levels of transient storage protein expression in megagametophytes may be associated with fertilization or the induction of embryo development. Recent work with rapeseed microspore-derived embryos revealed that the heat shock treatment used to induce embryogenesis also caused the transient appearance of storage protein (napin) mRNA (Boutilier *et al.* 1991), providing further support for this hypothesis.

The different accumulation patterns for the spruce storage protein mRNAs were similar to results described for angiosperms. In pea, vicilin mRNA and protein showed peak accumulations prior

to those for legumin (Boulter *et al.* 1987, Yang *et al.* 1990) and, in rapeseed, napin mRNA and protein showed peak accumulations prior to those of cruciferin (Crouch and Sussex 1981, Finkelstein *et al.* 1985). The accumulation of angiosperm storage protein transcripts during development is primarily regulated at the transcriptional level, although differences in mRNA stability also play a role (Delisle and Crouch 1989, Evans *et al.* 1984, Harada *et al.* 1989, Nielsen *et al.* 1989). While analyses of transcriptional rates were not performed, the different storage protein accumulation patterns, and the accumulation patterns of their mRNAs suggest, by analogy with other systems, that regulation in spruce may also be primarily at the transcriptional level, although other factors may also be involved. Both storage protein mRNAs are present at the same early stage, but the vicilin protein accumulates later than the legumin proteins, suggesting that translational or post-translational regulation may also be involved. In soybean, vicilin mRNA is detectable well before protein accumulation and the proteins are synthesized, but then degraded rapidly (Shuttuck-Eidens and Beachy 1985). As embryo development proceeds, the protein becomes more stable and accumulates, indicating that developmental changes in post-translational regulation for soybean vicilin exist. A similar type of mechanism may be associated with spruce vicilin accumulation.

5.3. SOMATIC EMBRYO STORAGE PROTEINS

This study revealed that interior spruce somatic embryos accumulated the same major storage proteins as zygotic embryos,

based on molecular weight, solubility and disulfide-linkage characteristics, and migration in two-dimensional gels. Hakman *et al.* (1990) also reported that Norway spruce somatic and zygotic embryos contained similar proteins, while Joy IV *et al.* (1991) reported that white spruce somatic and zygotic embryo total protein profiles were similar, although some of the major proteins were absent or were not highly expressed. Work with angiosperm systems has also shown that non-zygotic (somatic and microspore-derived) embryos contain the same proteins as their zygotic counterparts (Crouch 1982, Krochko *et al.* 1989, Shoemaker *et al.* 1987, Stuart *et al.* 1988, Tewes *et al.* 1991). These results suggest that the tissue culture process does not alter the regions of storage protein genes encoding structural information, leading to the expression of the same storage proteins as found in zygotic embryos. This is important if somatic embryo-derived material is to be used for biotechnological applications.

The levels of storage proteins that accumulated in spruce somatic embryos in the presence of 40 μ M ABA were similar to or higher than those found in mature zygotic embryos. Recent work in our lab (Cyr *et al.* 1991) has confirmed that somatic embryo storage protein levels may differ from those of zygotic embryos. These results are in contrast to the angiosperm data, where storage proteins were significantly lower in non-zygotic embryos (Crouch 1982, Krochko *et al.* 1989, Shoemaker *et al.* 1987, Stuart *et al.* 1988, Taylor *et al.* 1990). Also, storage protein proportions are altered in alfalfa somatic embryos (Krochko *et*

al. 1989, Stuart *et al.* 1988). The alteration of storage protein levels in alfalfa somatic embryos is believed to be due to low translation efficiency of storage protein mRNAs during early development, a reflection of their inability to be incorporated into polysomes (Pramanik *et al.* 1991). However, none of these angiosperm studies utilized ABA during somatic embryo differentiation, with the exception of Taylor *et al.* (1990), who used a short pulse of low ABA level during embryo maturation. Since ABA induces storage protein accumulation in cultured zygotic embryo tissues (Barratt 1986, Bray and Beachy 1985, Croissant-Sych and Bopp 1988, Eisenberg and Mascarenhas 1985, Finkelstein *et al.* 1985) and may enhance storage protein translation (Finkelstein *et al.* 1985), the fact that spruce somatic embryos were differentiated continuously on 40 μ M ABA during maturation could explain the high levels of storage proteins observed in somatic embryos in this study. Hakman *et al.* (1990) also noted that Norway spruce somatic embryos differentiated on ABA contained abundant levels of storage proteins, although quantitative comparisons were not made. Recently, Joy IV *et al.* (1991) reported that white spruce somatic embryos contained significantly lower levels of total proteins and some major proteins as identified by SDS-PAGE. However, these workers used low levels of ABA (10 μ M) compared to the 40 μ M level used here.

The analysis of different somatic embryo genotypes indicated that differences in storage protein levels occurred among cotyledonary somatic embryos after 7 weeks of maturation on 40 μ M

ABA, although all genotypes accumulated significant levels. The variations observed between the different genotypes could have reflected effects of the differentiation protocol, which may have to be optimized for each embryogenic line in order to obtain similar storage protein levels in each line. On the other hand, the variations may have reflected genetic differences arising from the individual seed embryos used for embryogenic tissue induction, since it is known that genotype-dependent differences in storage protein accumulation can occur (Higgins 1984).

5.4. DEVELOPMENTAL EXPRESSION OF STORAGE PROTEINS IN SOMATIC EMBRYOS ON 40 μ M AND 10 μ M ABA

Spruce somatic embryos cultured on 40 μ M ABA displayed differential storage protein accumulation patterns similar to those described for zygotic embryos. The legumin-type proteins appeared earlier in development, but both types accumulated primarily during cotyledon development. The appearance of vicilin and legumin mRNAs in torpedo stage somatic embryos on 40 μ M ABA was also similar to their developmental appearance in zygotic embryos. Also, the developmental accumulation pattern of storage protein mRNAs in somatic embryos on 40 μ M ABA was similar to that in zygotic embryos, with legumin mRNA peaking and starting to decline prior to vicilin mRNA. All of these results suggest that the patterns of storage protein gene induction and accumulation were similar between the two embryo types, although total storage protein mRNA levels were not compared between them. These results are different from those commonly reported for angiosperm non-zygotic embryos, which show temporally-altered

storage protein expression, with accumulations earlier in development than in their zygotic counterparts (Crouch 1982, Shoemaker *et al.* 1987). However, these studies did not use ABA during differentiation. Since ABA promotes more normal embryo maturation in excised zygotic embryos (Ackerson 1984ab) and developing somatic embryos (this study, Ammirato 1974, Kamada and Harada 1981, Roberts *et al.* 1990a), as well as induces storage protein gene expression in angiosperms, these altered developmental expression patterns may be due to the lack of exogenous ABA, which, if it were supplied would help to promote more normal patterns of gene expression in these embryos. Support for this proposal comes from a recent study by Wilen *et al.* (1990) using microspore-derived rapeseed embryos. These workers found that exposure of embryos to a 48 hour pulse of ABA at different stages of development promoted the correct developmental induction of napin and cruciferin transcripts. They also obtained levels of storage protein mRNA similar to that observed in equivalent stage zygotic embryos, although the developmental accumulation patterns of the storage proteins themselves were not characterized.

Apart from the similarities described above for spruce zygotic and somatic embryos, there were also differences in storage protein gene expression. Storage proteins accumulated gradually and continuously for a prolonged period in somatic embryos, and were still increasing after 9 weeks of maturation on 40 μ M ABA. High levels of storage protein mRNAs were also observed during this period, such that after 9 weeks on ABA, they were still

present at 50% of their maximal levels. This pattern was in marked contrast to that observed for zygotic embryos, which displayed a more rapid and transient period of storage protein and storage protein mRNA accumulation, after which protein levels did not increase and mRNA levels declined rapidly, to approximately 1% or less of maximal levels. The continuous accumulation of storage proteins and the high levels of their transcripts may reflect the constant exposure of somatic embryos to high levels of ABA throughout maturation. Also, somatic embryos remain fully hydrated during maturation and, based on storage protein accumulation, metabolically active. In contrast, zygotic embryos desiccate and enter a period of metabolic quiescence during later stages of seed development (Bewley and Black 1985). Storage protein synthesis and message levels generally decline during the maturation drying and desiccation stage of embryo development (Dure and Galau 1981, Finkelstein et al. 1985, Galau et al. 1987, Kermode et al. 1989). Recent results in our lab have indicated that storage protein transcript levels also decline rapidly in spruce somatic embryos exposed to an artificial drying regime (Dr. David Cyr, personal communication) that is used to mimic normal seed/embryo drying.

Spruce somatic embryos differentiated on low levels (10 μ M) of ABA also accumulated some storage protein. The differential storage protein accumulation patterns observed in zygotic embryos and somatic embryos exposed to 40 μ M ABA were also observed on 10 μ M ABA. Furthermore, both vicilin and legumin mRNAs were detected by torpedo stage in these embryos, as were the

differential accumulation patterns, with legumin mRNA increasing prior to vicilin. This was similar to the pattern observed with zygotic embryos and somatic embryos on 40 μ M ABA, although the maximal storage protein mRNA levels attained on 10 μ M ABA were less than those for 40 μ M ABA somatic embryos. This is attributed to the lower ABA level used. Similar ABA dose-dependence has been shown for storage protein gene expression in cultured zygotic (Finkelstein et al. 1985, Delisle and Crouch 1989) and non-zygotic (Wilén et al. 1990) embryos, with higher ABA treatments resulting in higher storage protein transcript levels. Interestingly, somatic embryos on high or low ABA displayed similar developmental induction and differential regulation of storage proteins, suggesting that ABA could maintain the normal developmental pattern of gene expression regardless of the level used, although ABA did affect the quantitative expression of storage protein genes in similar ways to its action in microspore-derived embryos (Wilén et al. 1990). The fact that both high and low ABA-treated somatic embryos did not exhibit storage protein transcripts until torpedo stage of development, although they had been exposed to ABA since the proembryo stage, suggested that the competence to respond to ABA, as manifested by storage protein mRNA induction, did not occur until torpedo stage. The differences between the relative levels of vicilin and legumin transcripts during their accumulation in early cotyledonary embryos were not as great in somatic embryos on high levels of ABA. This may be attributed to a more stimulatory effect of high levels of ABA on vicilin transcript

levels. The preferential enhancement by ABA of vicilin proteins and transcripts over other storage proteins has been documented (Bray and Beachy 1985, Schroeder 1984).

In contrast to the pattern observed in somatic embryos on 40 μM ABA, embryos on 10 μM ABA began to germinate precociously once they had reached mid-cotyledon stage of development. This was paralleled by a decline in storage protein mRNAs and storage protein levels, so that storage proteins did not accumulate to the high levels observed in 40 μM ABA somatic embryos. Recently, Joy IV *et al.* (1991) also reported that white spruce somatic embryos that were differentiated on low (10 μM) ABA did not contain storage proteins at the level of their zygotic counterparts. While low ABA could maintain embryo development and prevent precocious germination initially, it was no longer effective by the mid-cotyledonary stage, suggesting that a loss of sensitivity to ABA occurred as the embryo matured. Loss of ABA sensitivity has been reported to occur during zygotic embryo maturation (Eisenberg and Mascarenhas 1985, Finkelstein *et al.* 1985, Kermode *et al.* 1989, Rivin and Grudt 1991), and this change in sensitivity has been attributed to changes caused by maturation drying and desiccation (Kermode *et al.* 1989). The decline in storage protein transcripts commonly observed during late zygotic embryogeny (this study, Finkelstein *et al.* 1985, Harada *et al.* 1989, Nielsen *et al.* 1989, Walling *et al.* 1986) may partially reflect changes due to lowered ABA levels and reduced ABA sensitivity.

While changes in ABA sensitivity occur in response to desiccation, work with maize *viviparous* mutants, which do not undergo desiccation, has shown that embryos also undergo a loss of ABA sensitivity during maturation without desiccation (Rivin and Grudt 1991). These results and the present results with spruce somatic embryos on 10 μ M ABA indicate that there may be developmental changes in ABA sensitivity that occur during maturation, regardless of drying, allowing precocious germination.

Spruce somatic embryos that germinated precociously on 10 μ M ABA still contained low levels of storage protein transcripts, even after several weeks of germination. Both zygotic embryos and partially-dried somatic embryos have undetectable levels of storage protein transcripts within days of germination (Dr. David Cyr, personal communication). It has been suggested that drying or desiccation is required to switch from an embryo maturation to a germinative program (Kermode and Bewley 1989, Kermode *et al.* 1989). Furthermore, spruce somatic embryos given a partial drying treatment prior to germination exhibit much more normal germinative and post-germinative growth (Roberts *et al.* 1990b, 1991). The role of embryo developmental stage on gene expression during precocious germination was explored by Finkelstein and Crouch (1984). Mid-cotyledon stage rapeseed zygotic embryos placed on germination media germinated abnormally, with root development but little hypocotyl elongation and secondary cotyledon development formation as opposed to leaf formation. These embryos continued to express storage protein genes and

accumulate storage proteins during the 4 weeks of precocious germination. Older, maturation stage embryos germinated into normal-looking seedlings, but storage protein transcripts were still detectable and storage protein degradation took several weeks. This was attributed to the continued synthesis and turnover of storage proteins, indicating concurrent expression of embryo developmental and germinative programs. Only mature, dry, seed embryos responded normally during germination, with no storage protein transcript retention and rapid degradation of storage proteins. These results suggested that drying was required to switch developmental programs, and that embryos were capable of expressing both embryo maturation and germination programs simultaneously. Kriz *et al.* (1990) also reported that precociously germinating embryos of *viviparous* mutants, which did not undergo desiccation, exhibited prolonged expression of storage protein transcripts and an incomplete switch from embryo development to germination, further indicating the importance of drying for the change from embryo developmental to germinative gene expression. These results are similar to the observations with spruce somatic embryos on 10 μ M ABA, which appear have germinated, but still contain low levels of storage protein transcripts, suggesting simultaneous expression of embryo and germination programs, in contrast to germinants of normal embryos or partially-dried somatic embryos (Dr. David Cyr, personal communication).

It was suggested that somatic embryos on 10 μ M ABA lost their sensitivity to ABA and germinated precociously. However, somatic

embryos on 40 μ M ABA did not display such an evident change. After 9 weeks on ABA, these embryos had not yet precociously germinated, although prolonged culture on these high levels of ABA will eventually lead to their germination (data not shown, Dunstan *et al.* 1991), suggesting that they also exhibit a developmental change in ABA sensitivity. Somatic embryos on 40 μ M ABA displayed a decline in storage protein transcript levels by 9 weeks of maturation. This decline may be indicative of a change in ABA sensitivity, suggesting that the embryos are, at least at the molecular level, starting to switch into a precocious germination mode even though protein levels are still high. Further studies using germination-specific probes are required to determine if this is the case. The use of molecular probes to determine the state of embryo development/germination has important implications in determining the appropriate developmental stage at which maturing somatic embryos should be removed for partial drying and artificial seed production.

5.5. OSMOTIC STRESS AND STORAGE PROTEIN GENE EXPRESSION

Early cotyledonary spruce somatic embryos cultured on 40 μ M ABA or 15% mannitol for 2 weeks matured into well developed cotyledonary stage embryos which did not precociously germinate, although embryos on 15% mannitol were smaller in size. This promotion and/or enhancement of somatic embryo differentiation has been reported for angiosperm (Litz 1986, Nadel *et al.* 1989) and conifer (Lu and Thorpe 1987, Roberts 1991, Tremblay and Tremblay 1991a) systems. In this study, somatic embryos matured on ABA or mannitol contained high levels of storage protein

transcripts and storage proteins, although storage protein levels in the mannitol-treated embryos were not as high as in the ABA-treated embryos. This confirms previous results that indicated an osmotic stress-induced accumulation of storage proteins in spruce somatic embryos (Roberts 1991) and agrees with angiosperm data showing the same pattern in cultured embryo tissues (Barratt 1986, Finkelstein and Crouch 1986, Goffner *et al.* 1990, Xu *et al.* 1990) and developing microspore-derived embryos (Wilén *et al.* 1990).

Osmotic stress-induced storage protein accumulations have been attributed to a stimulation of endogenous ABA levels by the stress (Barratt 1986, Rivin and Grudt 1991, Wilén *et al.* 1990), although others have suggested that endogenous ABA is not directly associated with the response (Barratt *et al.* 1989, Finkelstein and Crouch 1986, Goffner *et al.* 1990, Xu *et al.* 1990). Since both ABA and osmoticum inhibit water uptake (Finkelstein and Crouch 1986, Schopfer and Plachy 1984), it has been suggested that alteration in embryo cell osmotic potential, and not ABA, is the primary effector of the storage protein response. To study the potential role of ABA in osmotic stress-induced storage protein gene expression in spruce, developing somatic embryos were exposed to the ABA biosynthetic inhibitor, fluridone, during osmotic stress. This study indicated that somatic embryos exposed to high levels of fluridone contained low levels of storage protein transcripts and did not accumulate high levels of storage proteins. Furthermore, lower fluridone levels in combination with osmotic stress, which should have allowed

more endogenous ABA biosynthesis, displayed higher levels of storage protein transcripts and storage protein accumulation. These results suggested that osmotic stress-induced storage protein gene expression in spruce somatic embryos was mediated via ABA. However, while embryos matured on mannitol-containing medium plus high levels of fluridone and ABA contained higher storage protein levels compared to those on mannitol plus fluridone, the storage protein transcript levels did not increase substantially. This suggested that exogenous ABA could not stimulate the fluridone-induced inhibition of storage protein transcript levels under the conditions used here. The inability of exogenous ABA to stimulate storage protein transcript levels may have been due to little uptake of applied ABA, due to the highly negative osmotic potential of the mannitol-containing medium. The possible inhibition of ABA uptake by osmotic stress was suggested by the results of Bray and Beachy (1985). These workers noted that low levels of osmoticum (0.5-3% sucrose) stimulated endogenous ABA levels and exogenous ABA applications enhanced these levels. However, at the highest sucrose concentration tested (10%), endogenous ABA levels were lower than those from the 0.5-3% sucrose treatments, and exogenous ABA application did not enhance these levels.

If ABA uptake was low due to the high osmoticum used in this study, this could potentially account for the discrepancy observed between storage protein and transcript levels. Finkelstein *et al.* (1985) reported that excised zygotic embryos cultured on low ABA did not accumulate storage protein

transcripts to the levels found in seed embryos, although storage proteins did reach their normal levels, suggesting that ABA enhanced translation. It is possible that the ABA level to which the spruce somatic embryos were exposed during the mannitol plus fluridone and ABA culture was not sufficient to induce a major increase in steady state storage protein transcript levels, but enhanced translation of the message to allow storage protein accumulation.

It is also possible that the inability of ABA to completely restore storage protein transcript levels in the presence of high levels of fluridone may have been due to some other indirect effect of fluridone, although fluridone exposure did not inhibit embryo survival or growth. Interestingly, Fong *et al.* (1983) noted that exogenous ABA could only partially reverse fluridone-induced vivipary in maize embryos.

While this present study did not measure endogenous ABA levels during development, other studies have shown that fluridone does inhibit ABA biosynthesis and reduces endogenous ABA levels (Barratt *et al.* 1989, Bray and Beachy 1985). ABA is synthesized via the carotenoid pathway (Parry and Horgan 1991, Rock and Zeevaart 1991), and fluridone inhibits phytoene desaturation in this pathway, thereby inhibiting ABA biosynthesis (Zeevaart and Creelman 1988). The bleached appearance of the somatic embryos exposed to fluridone was indicative of carotenoid biosynthetic suppression (Fong *et al.* 1983), and therefore, ABA biosynthesis. It is possible that alterations in endogenous ABA pools in response to the osmotic stress could have occurred, but other

studies using fluridone have shown that endogenous ABA levels do decline, and the two week period of culture during this experiment should have allowed depletion of endogenous ABA pools without replenishment via biosynthesis.

5.6. CONCLUDING STATEMENT

This study showed that both zygotic and somatic embryos of spruce expressed the same storage proteins. These proteins appeared homologous to known angiosperm storage protein classes, based on solubility, disulfide linkage and cDNA sequence characteristics. The two different storage protein classes displayed differential accumulation patterns. This was similar to the differential accumulation patterns often reported for angiosperm storage proteins. Spruce storage protein gene expression was regulated by ABA and while somatic embryos matured on high or low levels of ABA displayed a similar developmental induction of storage protein transcripts and their proteins to zygotic embryos, only those embryos cultured on high levels of ABA were capable of prolonged development without precocious germination. This allowed the accumulation of storage proteins to levels similar to or higher than levels found in zygotic embryos, although the period of storage protein transcript and protein accumulation was more prolonged than that observed in zygotic embryos. These results suggested that, using storage protein gene expression as a marker, somatic embryos that matured on high levels of ABA were developmentally similar to zygotic embryos. This finding bodes well for their utilization in biotechnological applications.

Somatic embryos must exhibit vigorous germination and post-germinative growth if they are to be useful. It is known that somatic embryos subject to an artificial drying regime, which mimics normal seed/embryo desiccation and switches gene expression from an embryo developmental to germinative mode, perform much better than if not dried (Parrott *et al.* 1988, Roberts *et al.* 1990b). Therefore, the removal and drying of somatic embryos at the appropriate time, prior to precocious germination, is critical to optimize plantlet recovery. While somatic embryos on high ABA levels did not germinate precociously during the culture period used here, they eventually did, indicating an apparent loss of ABA sensitivity during the later stages of somatic embryo maturation. Although the embryos had a prolonged period of storage protein accumulation that indicated a delay in precocious germination, the decline of storage protein mRNAs may have indicated the onset of loss of ABA sensitivity and precocious germination. Further studies, using embryo developmental and germination-specific probes are required to confirm this, but molecular markers clearly have the potential to gauge the maturity of somatic embryos and could indicate the onset of precocious germination well before protein changes become apparent. This would facilitate the removal of somatic embryos for partial drying and artificial seed production.

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