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Developmental and Molecular Studies of Spruce Embryogenesis *in Vitro*

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ABSTRACT

Since first described in the mid-eighties somatic embryogenesis of spruce has been extensively utilized both as a tool for mass propagation and as a model system for the investigation of structural, physiological and molecular events occurring during embryo development in conifers. Despite the increased efforts to optimize media composition and culture environment, production of somatic embryos still poses problems, especially for recalcitrant genotypes. Low yield and/or poor quality embryos which are not able to regenerate viable plants are often observed in culture. Improvements to the system have been made possible through the combined applications of developmental, physiological and molecular studies. Cell tracking techniques have defined the different phases of embryo development with great precision and have allowed for comparative structural studies between somatic and zygotic embryos. Physiological work has revealed ways to improve embryo development in culture. Manipulating the embryonic environment during the early phases of development through changes in osmoticum and redox state, or during germination through applications of ascorbic acid, has improved embryo regeneration. Furthermore, recent molecular work has identified several genes involved in embryo development. Advances in spruce transformation have revealed the functional relevance of such genes in regulating the developmental fate of somatic embryos. The focus of this review is to provide an up-date on the current status of spruce embryogenesis and to emphasize developmental and molecular events characterizing the process.

Keywords: apical meristem, gene expression, physiology, *Picea glauca*, somatic embryogenesis

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INTRODUCTION

Somatic embryogenesis can be described as the formation of embryos from somatic cells, i.e. asexual cells, in culture. Embryos formed through this process appear as bipolar structures which are similar in structure and physiology to their zygotic counterparts. In combination with breeding programs and molecular techniques somatic embryogenesis represents an effective tool for accelerating the pace of genetic improvement of commercial crop species. This is especially true for trees, which compared to agronomic crops have a long vegetative period and consequently have fewer individuals which can be integrated into breeding and testing areas (reviewed by Timmis 1998). In addition, somatic embryogenesis is a low-cost technique for producing uniform plants, especially those difficult to propagate via cuttings. It is no surprise that over the last few years an increasing effort has been focused on improving the embryogenic process *in vitro* through the optimization of culture and media components.

Production of embryos in culture was first reported in flowering plants. Independent studies (Reinart 1958; Steward *et al*. 1958) documented the formation of embryogenic heads from carrot somatic cells. Since these first descriptions production of somatic embryos has been achieved in a variety of species (see Thorpe and Stasolla 2001). In

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gymnosperms the first indication of somatic embryogenesis occurred in the late 1970s and early 1980s in *Pinus banksiana* (Durzan and Chalupa 1976) and *Picea glauca* (Durzan 1980), although the embryos generated in these studies were not able to complete the developmental process and remained at an immature stage. It was only a few years later that morphologically and physiologically mature embryos able to regenerate viable plants were induced in Norway spruce (*Picea glauca*) (Chalupa 1985; Hakman *et al*. 1985). Since then spruce somatic embryogenesis has been used as a model system to investigate developmental, physiological, and molecular events occurring during embryogeny *in vitro* (reviewed by von Arnold *et al*. 1995, 1997; Thorpe and Harry 2000; Thorpe and Stasolla 2001; Hartmann *et al*. 2002; Yeung and Thorpe 2005). Although relatively easy to achieve compared to other species, somatic embryogenesis in spruce has not yet been fully optimized and several problems still persist. In many instances induction of embryogenic tissue is only restricted to juvenile tissue, i.e. immature embryos. Furthermore, the number and quality of embryos obtained in culture is genotype dependent and often varies from batch to batch. Based on the above the focus of this review is to outline recent advances in somatic embryogenesis in spruce and to describe the developmental and molecular processes occurring during the process.

ESTABLISHMENT OF SPRUCE SOMATIC EMBRYOS

The generation of spruce somatic embryogenesis is achieved through a series of steps requiring specific culture and media conditions (see Stasolla *et al*. 2002). Proper execution of each step affects the result of subsequent steps and ultimately the final outcome. Therefore it is imperative that each step be optimized through rational manipulations of culture conditions. In this review somatic embryogenesis is divided into three steps designated as (1) induction and proliferation of embryogenic tissue, (2) embryo development and maturation, and (3) embryo conversion.

Induction and proliferation of embryogenic tissue

In spruce the choice of the explant greatly affects the induction process. Different tissue types within the same plant or the same tissue at different stages of development induce different responses *in vitro* (Roberts *et al*. 1989). The optimal explant consists of an immature zygotic embryo at the pre-cotyledonary stage of development dissectted from a maturing seed (**Fig. 1A**). In some instances mature cotyledonary embryos can also be used (Verhagen *et al*. 1989). Embryogenic tissue which is translucent in color (**Fig. 1B**) usually originates from the hypocotyl region of the embryos (Ramarosandratanam and Stadem 2005). Other types of explants, including female gametophytes and cotyledons can also be employed with a high rate of success (Lelu and Bornman 1990; Krogstrup 1986). The utilization of more mature explants, including seedlings (Attree *et al*. 1990) and 1-year-old somatic embryos and needles of 7-year old tree (Ruaud *et al*. 1992; Westcott 1992) have been documented, although with reduced success. The requirement of juvenile explants for the induction process clearly indicates that the re-direction of the developmental program is difficult to achieve in spruce and in conifers. This is different from flowering plants where embryogenic tissue can often be initiated from mature explants. The induction process requires the presence of auxins (2,4-dichlorophenoxyacetic acid, 2,4-D) and cytokinins (benzyladenine, BA) and can last from 4 to 6 weeks. If zygotic embryos are used as explants, embryogenic tissue originates from the hypocotyl region and mainly consists of a translucent mass of imamture somatic embryos, often referred to as filamentous embryos. Such embryos are composed of an embryogenic head of densely cytoplasmic cells and a long suspensor-like tail of elongated and vacuolated cells. This is in contrast to non-embryogenic tissue which is dark in color and has no defined cellular structures. How the initial embryogenic tissue divides is unclear, although several mechanisms have been proposed. These include cleavage polyembryony (through multiplication of the embryogenic heads), division of those meristematic cells occasionally located within the suspensor region, and division of single cells (see von Arnold and Hakmann 1988).

Once formed, the embryogenic tissue can proliferate on both solid or liquid LV medium (Litvay *et al*. 1995) and requires both 2,4-D and BA although at lower concentrations to those used for the induction process. Using cell tracking experiments Filonova *et al*. (2000) have documented that when cultured in liquid medium Norway spruce (*Picea abies*) embryogenic tissue passes through three distinct developmental stages. These stages are characterized by defined cellular aggregates denoted as pro-embryogenic masses (PEMs) I, PEMs II, and PEMs III (**Figs. 1C, 2**). Pro-embryogenic masses I have a very simple structure and are composed of a small cluster of cytoplasmic cells subtended by one elongated cell. Upon further growth, PEMs I develop into PEMs II in which the embryogenic head has increased in size and the tail region is composed of several elongated cells. The final developmental stage observed in the proliferation medium is represented by PEMs III in which the vacuolated cells radiate in all directions from the central cluster of cytoplasmic cells (**Figs. 1C, 2**). The percentage composition of PEMs varies during the culture period with PEMs III predominating over the other cellular aggregates after day 3, when auxin (2,4-D) and cytokinin (BA) become depleted in the medium. When subcultured into fresh proliferation medium PEMs III differentiate into PEMs I and the cycle is repeated.

Prolonged subcultures, especially in liquid medium, have been often associated with changes in embryogenic potential of white spruce tissue, resulting in a lower production of embryos (Dunstan *et al*. 1993). Alternative methods of tissue maintenance, reported in white spruce, involve cryopreservation (Kartha *et al*. 1988), long-term storage in gas impermeable serum-capped flasks at room temperatures (Joy *et al*. 1991a), and storage at low temperatures, i.e. 0-10°C (Attree and Fowke 1993).

Embryo development and maturation

In Norway spruce embryo development is initiated by removing auxin and cytokinin from the medium. Removal of these plant growth regulators inhibits cell proliferation and induces the transdifferentiation of PEMs III into immature somatic embryos. Such embryos are characterized by a well developed embryo proper subtended by a large suspensor tail composed of elongated cells. After this treatment, lasting 7 days, embryos are transferred to development LV medium (Litvay *et al*. 1995) containing abscisic acid (ABA). In the presence of ABA embryo development continues and the process culminates in the generation of fully cotyledonary embryos (**Figs. 1D, 1E, 2**). Responsiveness of the embryogenic tissue to ABA treatments varies among species; high levels of ABA (40 μM) were necessary to promote embryogenesis in *Picea rubens* (Harry and Thorpe 1991), whereas a similar effect was achieved in *P. glauca* and *P. mariana* (12 μM) (Attree *et al*. 1990). The developmental process generally lasts from 4 to 6 weeks depending on genotype and the number of embryos produced is variable. Although morphologically similar to their zygotic counterparts, cotyledonary spruce somatic embryos are not physiologically ready to germinate and regenerate viable plants. In order to do so they required a maturation period in which the embryos experience a mild water stress which redirects the developmental program towards germination. Maturation of somatic embryos can be achieved in two different ways; through imposition of high osmolarity in the development medium, or through a desiccation period following development. Restriction of water uptake in the development medium can be achieved by using either permeating osmoticum agents, such as sucrose, mannitol,

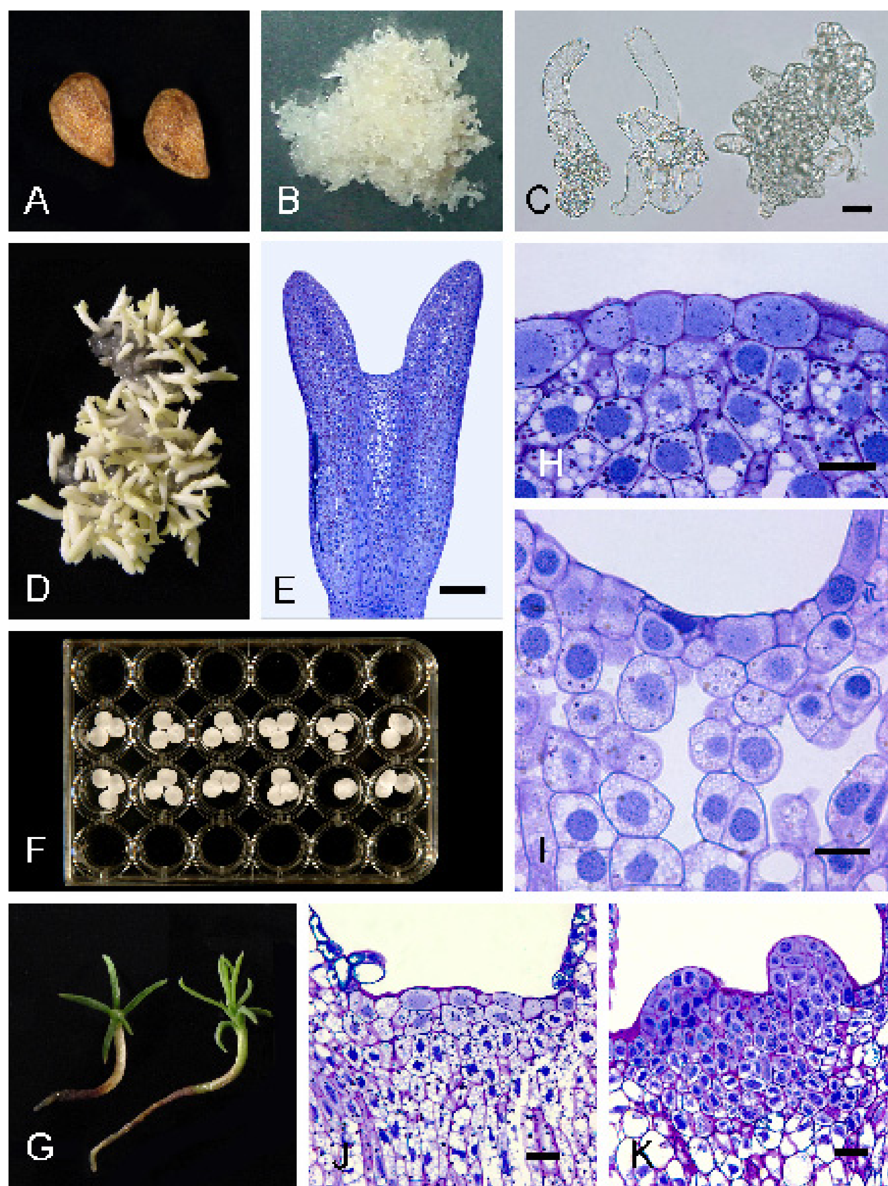

Fig. 1 Overview of initiation and development of somatic embryogenesis in spruce. Somatic embryogenic tissue is often initiated from immature zygotic embryos found within developing spruce seeds (**A**). Following an induction period and in the presence of plant growth regulators, auxin and cytokinin, white embryogenic tissue forms and continues to proliferate (**B**). In Norway spruce, embryogenic tissue passes through three distinct developmental stages. These stages include PEMI, PEMII and PEMIII (left to right) and are characterized by densely cytoplasmic embryogenic heads subtended by large vacuolated suspensor-like tails (scale bar = 100 µm) (**C**). Population of mature spruce somatic embryos produced in the presence of abscisic acid (**D**). Median longitudinal section through a mature cotyledonary stage spruce somatic embryo (scale bar = 200 µm) (**E**). During the partial drying treatment (PDT) embryos are placed onto sterilized filter paper in the center wells of a 24-well plate while the outer wells are filled with water. This process improved conversion frequency (see text for details) (**F**). After the PDT embryos are germinated; some embryos are not able to regenerate a viable shoot and root (left), whereas other convert into viable plants (right) (**G**). The shoot apical meristem of fully developed embryos varies in shape and morphology. Some are dome-shaped (scale bar = 25 µm) (**H**), whereas other are flat and disrupted by the presence of intercellular spaces (scale bar = 25 µm) (**I**). The organization of a well developed meristem is retained after the PDT (scale bar = 25 µm) (**J**) and cellular reactivation is observed upon transfer onto the germination medium (scale bar = 25 µm) (**K**).

amino acids, etc. although they can be toxic in culture (Attree and Fowke 1993), or by using non-permeating osmoticum agents, such as polyethylene glycol (PEG). Quantitative and qualitative improvements during spruce somatic embryogenesis have been reported when PEG is added in the development medium (Attree *et al.* 1991; Kong and Yeung 1995). However conflicting reports exist on the effects of this compound during post-embryonic growth, as in some genotypes PEG-treated embryos fail to regenerate viable plants at high frequency (Bozhkov and von Arnold 1998). The second approach used to mature fully developed somatic embryos is through the imposition of a partial drying treatment (PDT), which involves a gradual and limited loss of moisture content over a 10 day period. This can be achieved by plating the embryos in the central wells of tissue culture plates in which the outer wells have been filled with water (**Fig. 1F**) (Roberts *et al.* 1990). During the PDT the moisture content declines only 20% (Kong 1994). Partially drying embryos of white spruce germinate synchronously and convert at high frequency (Kong 1994).

Embryo conversion

Conversion is defined as the ability of mature cotyledonary embryos to produce viable shoots and healthy root systems when transferred onto germination medium (**Fig. 1G**), and is therefore a test used to estimate the quality of embryos produced in culture. Conversion frequency is genotype dependent and is not related to embryo yield, as some lines produce many embryos which are however unable to regenerate plants. Embryo conversion in spruce is achieved by plating mature embryos on hormone-free medium with low levels of sucrose. After reaching a desirable size the embryo-derived plantlets are transplanted into soil. Uniformity of growth habit and form are typically observed in cloned trees (Timmis 1998; Hogberg *et al.* 2003).

STRUCTURAL AND DEVELOPMENTAL PROCESSES

Structural studies have revealed that the first signs of histodifferentiation in white spruce somatic embryos occur at the early-filamentous stage of development with the formation of the protoderm, the outermost layer of cells of the embryo proper (Kong *et al.* 1999). Cells within the protoderm predominantly divide anticlinally making this layer easily distinguishable from adjacent subapical layers, where both anticlinal and periclinal divisions occur. The formation of the protoderm is an important event during somatic embryo development, as it may regulate further embryo growth (Yeung 1994). Anatomical observations of early-filamentous embryos of white spruce demonstrate that mitotic figures are mostly visible within the cells of the embryo proper, but not in the suspensor cells, except in small cells, from which new somatic embryos originate (Hakman *et al.* 1987). Differentiation of meristems, the shoot apical meristem (SAM) and the root apical meristem (RAM) starts at the filamentous stage of embryo development and is com-

pleted in early cotyledonary embryos. During the late phases of development, a ring of 5-8 cotyledons emerges from the proximal portion of the embryo proper, and this delineates the beginning of the cotyledonary phase of embryo development. At this stage, growth of the suspensor is reduced and in fully mature embryos the suspensor region consists of a few cells connecting the embryos to the embryogenic tissue.

The differentiation of the SAM and RAM represents a critical step during the embryogenic process, as embryo quality is closely related to the structure of the apical meristems. In white spruce, differentiation of the SAM occurs at the filamentous stage of embryo development, when a dome-shaped projection emerges from the proximal portion of the embryo (Kong *et al.* 1999). Within the differentiating shoot meristem, the apical layer is composed of large, densely cytoplasmic cells, whereas the subapical layers are formed by vacuolated cells, characterized by heavy accumulation of starch and protein. Upon further development, vacuoles in the subapical layers decrease in size (Kong 1994). The role played by the subapical cells, especially in creating a special environment for the differentiation of the overlying cells has been well documented (Kong and Yeung 1992; Kong *et al.* 1999). Differences in shape of the SAMs are often observed among embryos of different cell lines, as flat- or dome-shaped meristems are observed (**Fig. 1H, 1I**). Compared to their zygotic counterparts (Yeung *et al.* 1998), the structure of the SAMs in cotyledonary stage somatic embryos (Kong and Yeung 1992) is less organized. Such meristems are often poorly developed and their cellular integrity is disrupted by the presence of intercellular air spaces (**Fig. 1I**) (Kong and Yeung 1992). It has also been demonstrated that the structure and organization of the SAM in developing embryos affect post-embryonic growth. Well organized meristems are able to reactivate germination and produce viable shoots (**Fig. 1J, 1K**) whereas poor germination and conversion frequentcy are often observed in embryos characterized by poorly developed meristems (Kong and Yeung 1992). Detailed studies on the formation of the RAM during spruce embryogenesis *in vitro* are not available. In general, the organization of the RAM in white spruce somatic embryos closely resembles that of their zygotic counterparts; the only exception being a reduced number of initials occupying the central region of the root (Kong 1994; Yeung *et al.* 1998).

In developing embryos the accumulation of storage products follow a precise pattern. Starch usually accumulates first, followed by proteins and lipids (Joy *et al.* 1991). In early filamentous embryos a preferential accumulation of starch is generally observed in proximity of the suspensor whereas in more mature embryos starch is observed mainly in the cortex and within the sub-apical cells of the SAM (Joy *et al.* 1991; Kong *et al.* 1999). Lipids and protein bodies tend to accumulate acropetally in more mature embryos. This pattern, which closely matches that of zygotic embryos, may represent a "physiological cue" for proper tissue pattern differentiation and meristem formation (Yeung *et al.* 1998).

PHYSIOLOGICAL PROCESSES

While our knowledge on the morphogenic aspects of white spruce somatic embryogenesis is increasing, investigations dealing with the physiological processes are scarce, especially in relation to the early stages of development. This results from the fact that spruce cell lines often respond differently in culture and discrepancies in their physiological responses can be observed. In this review physiological and biochemical studies are classified into early, middle and late embryogeny.

Early embryogeny: formation of immature embryos

Transdifferentiation of PEMs III into immature somatic embryos is a key step during the early stages of embryogenesis in Norway spruce. Recent physiological studies conducted in von Arnold's group have revealed that an important event regulating this transition is represented by programmed cell death (PCD). By using *in situ* detection of DNA fragmentation during several developmental phases Filonova *et al.* (2000b) have shown that cells within PEMs III undergo massive PCD prior to their differentiation into somatic embryos. This process is executed by the cysteine protease mcII-Pa (Bozkhov *et al.* 2005) and is accompanied by a pronounced dismantling of several cellular components, characteristics of PCD (Filonova *et al.* 2000b). New evidence has shown that PCD is a required step for somatic embryo formation. The number of cell undergoing PCD is positively correlated to the number of somatic embryos produced by the PEMsIII (Filonova *et al.* 2000b). Furthermore, inhibition of PCD by buffering the culture medium decreases the number of embryos produced in response to ABA (Bozhkov *et al.* 2002). Execution of PCD by embryonic cells does not only occur *in vitro*, but is also observed during zygotic development, where it eliminates additional embryos formed in spruce seeds (Filonova *et al.* 2002). Overall these studies suggest that PCD may fulfill similar mechanisms during plant (Drury and Gallois 2006) and animal embryogenesis. As in spruce, *Drosophila* embryos also fail to develop if PCD is experimentally blocked through mutagenesis (White *et al.* 1994).

Middle embryogeny: completion of embryo development

In this review middle embryogeny will be referred as to that

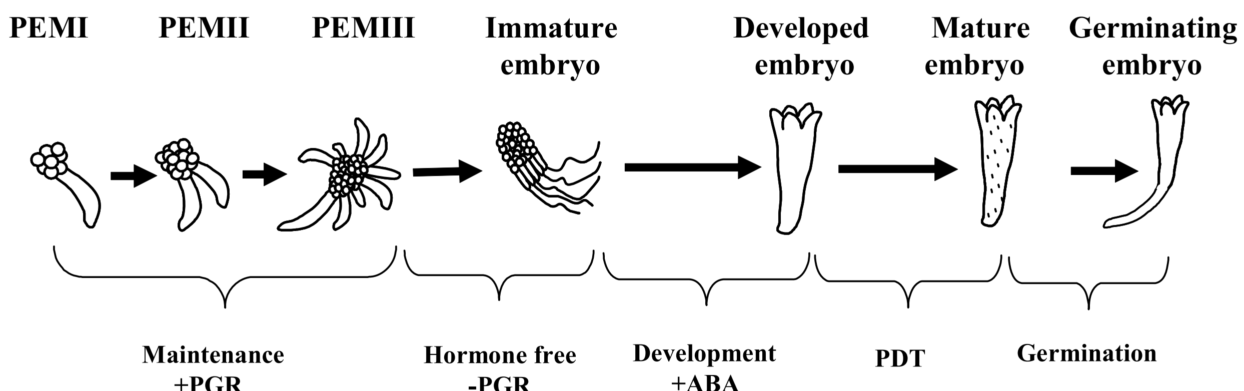

Fig. 2 The process of somatic embryo formation in *Picea abies* (Norway spruce). In media containing the plant growth regulators auxin and cytokinin (+PGR), proembryogenic masses (PEMs) proliferate. PEMs I are continually formed and develop into PEMs II and III. PEMs III can divide to produce more PEMs I after transfer onto hormone-free media (-PGR), immature somatic embryos are formed from PEMs III. Once transferred onto development medium containing ABA, the immature embryos developed into cotyledonary embryos. Such embryos must experience a water stress through the imposition of a partial drying treatment (PDT) prior to germination. Adapted from Filonova *et al.* (2000a).

phase starting with the transdifferentiation of immature somatic embryos from PEMs III and culminating with the formation of fully developed cotyledonary embryos (**Fig. 2**). This process which lasts several weeks has been extensively investigated by several laboratories over the past few years especially in relation to hormonal requirement, nitrogen metabolism, and cellular redox state.

Hormones and embryo development

The major factor promoting the development of somatic embryos from PEMs is represented by the inclusions of ABA in the culture medium (**Fig. 2**). Studies conducted by Kong *et al*. (1997) have shown that endogenous ABA levels in developing seeds are low during the initial stages of development, reaching maximum levels at the midpoint of embryo maturation, and then declining again during the late stages, as the seed dries (Kong *et al*. 1997). Abscisic acid seems to promote storage product deposition while preventing precocious germination of developing embryos (Bewely and Black 1994). Differences in ABA content are often observed between zygotic and somatic embryos, with higher levels observed in the former. Such differences are due to the presence of the megagametophytic tissue in the seed which is the major source of ABA for the developing zygotic embryos (Kong *et al*. 1997). It is therefore obvious that the high levels of exogenous ABA, needed to promote somatic embryo development in white spruce, replace the ABA supplied by the megagametophytic tissue during embryogenesis *in vivo*.

Tissue response to ABA, usually applied as a racemic ($\pm$) mixture, is very rapid (Dong and Dunstan 1996a; Dong *et al*. 1997) and results in the reduction of cell proliferation and the initiation of embryo development. Studies on purine and pyrimidine nucleotide biosynthesis have characterized this transition, as nucleotide availability affects nucleic acid synthesis and ultimately cell division (see Ross 1981). Tracer experiments conducted during white spruce somatic embryogenesis have shown that all pathways of pyrimidine metabolism, i.e. *de novo*, salvage, and degradation, are affected by ABA (Ashihara *et al*. 2000). In the presence of ABA orotic acid and uridine are actively utilized for nucleotide and nucleic acid synthesis, whereas a large fraction of uracil is degraded to CO_2 (Ashihara *et al*. 2000). Salvage and degradation of purine nucleotides are also affected by ABA (Ashihara *et al*. 2001). In the embryogenic tissue adenine and adenosine are extensively salvaged for nucleotide and nucleic acid synthesis. A reduction of this salvage activity occurs upon transfer onto the ABA-maturation medium, in conjunction with a decline in specific activity of the major salvage enzymes: adenine phosphoribosyltransferase (APRT) and adenosine kinase (AK) (Ashihara *et al*. 2001). It was suggested that ABA regulates the transition from PEMs III to somatic embryos by altering nucleotide metabolism.

Ethylene, a gaseous phytohormone which often accumulates during *in vitro* culture (Kong *et al*. 1999) has also been shown to affect the developmental process. It varies from ABA however as ethylene accumulation is deleterious to proper embryonic growth as it induces structural aberrations resulting in reduced embryo yield and poor emryo quality. Several independent studies have confirmed the negative effects of ethylene in culture. One has shown that non-embryogenic lines of white spruce accumulates more ethylene than embryogenic lines (Kumar *et al*. 1989). Another that accumulation of ethylene during maturation of white spruce somatic embryos inhibits embryo development and disrupts the architecture of the SAMs by promoting separation of meristematic cells and formation of intercellular spaces (Kong and Yeung 1995). Disrupted SAMs are unable to reactivate at germination and fail to produce viable shoots, leading to stunted growth and in extreme cases to embryo abortion. An experimental decrease of ethylene levels, effected by applications of aminoethoxyvinyl-glycine (AVG) which inhibits ethylene biosynthesis, improves embryo yield and produces embryos with normal SAMs (Kong and Yeung 1995). A similar correlation between ethylene levels and embryo growth has also been reported by El Maskaoui *et al*. (2000). An increase in cellular ethylene levels through addition of 1-aminocyclopropane-1-carboxylic acid (ACC) or pure ethylene inhibited embryo maturation. Conversely, applications of the ethylene biosynthetic inhibitor, N-aminooxyacetic acid (AOA), and the ethylene action inhibitor silver nitrate ($AgNO_3$), increased the production of cotyledonary embryos (El Meskaoui *et al*. 2000). Although the influence on ethylene production *in vitro* by other hormones, especially ABA, remains controversial (Tan and Thimman 1989; Riov *et al*. 1990; Biddington *et al*. 1993), a negative feedback has been observed as high levels of exogenously supplied ABA decrease the levels of ethylene during the first two weeks of white spruce somatic embryo maturation (Kong 1994).

Nitrogen metabolism

Pronounced changes in nitrogen metabolism and ultimately in storage product accumulation are also observed upon application of ABA (Joy *et al*. 1997). Spruce embryogenic tissue is able to take up and incorporate both inorganic nitrogen species (NO_3^- and NH_4^+) (Joy *et al*. 1997). The most notable changes in the amino acid profile as a result of ABA treatments are the increased concentrations of glutamic acid, glutamine, and arginine (Joy 1994). ^{15}N NMR spectroscopic studies confirmed this trend, as applications of ABA increased resonances for arginine, glutamine and glutamate, as well as for aliphatic amines (Joy *et al*. 1997). Accumulation of these three amino acids was ascribed to the activity of the glutamine synthase/glutamate synthase (GS/GOGAT) pathway, which also increases in the presence of ABA (Joy *et al*. 1997). These changes in nitrogen metabolism are soon followed by a pronounced accumulation of protein bodies enriched in alanine and arginine. Although storage protein deposition is a common event observed during both in vivo and *in vitro* embryo maturation, differences in matrix and crystalloid polypeptides occur between the two systems (Joy *et al*. 1991b; Misra *et al*. 1993). Studies conducted by Misra and Green (1991) and Misra *et al*. (1993) have shown that some major crystalloid polypeptides are not detected in ABA-matured somatic cotyledonary embryos, which show an overall crystalloid protein profile similar to that of immature zygotic embryos. This observation indicates that fully developed somatic embryos are not physiologically mature.

Redox status and embryo development

Another important factor regulating embryo development is the cellular redox status affected by the presence of two major redox pairs: ascorbic acid (AA)-dehydroascorbate (DHA) and reduced glutathione (GSH)-oxidized glutathione (GSSG). Studies conducted by Stasolla and Yeung (2001) revealed that changes in the redox state of the total ascorbate pool delineate specific stages in embryo development. An increase in AA synthesis via the de novo pathway and a switch of the total ascorbate pool towards its reduced state are induced by ABA. These alterations, not observed in non-embryogenic lines, may represent a “metabolic switch” which induces embryo formation. Embryo development becomes precluded if cellular AA is experimentally depleted (Stasolla and Yeung, unpublished data).

Glutathione is recognized to orchestrate organized development in both *in vivo* and *in vitro* systems. In white spruce a high glutathione redox state (high GSH-GSSG ratio) promotes cell division and proliferation through the activation of nucleotide synthesis (Belmote *et al*. 2003, 2005), while a more oxidized state (low GSH-GSSG) induces organized embryonic growth. Embryos developed in an oxidized state have a zygotic-like structure and perform better at germination (Belmonte and Yeung 2004).

Based on these observations, Belmonte *et al*. (2005)

have optimized culture conditions in which embryogenic tissue is first cultured in the presence of GSH to promote growth, and then transferred onto GSSG-containing media to favor proper embryo development. These conditions enhance both embryo number and quality. Compared to control, treated embryos have a more organized SAM devoid of intercellular spaces and able to reactivate at germination. In addition SAMs of glutathione-treated embryos have a larger expression pattern of HBK1, a gene preferentially expressed in the meristematic cells of the apical pole (discussed in the next section). Overall these observations indicate that glutathione improves shoot apical meristem structure and identity. The beneficial effects of glutathione on embryo development is not restricted to conifers but also to flowering plants (Belmonte *et al*. 2006).

Late embryogeny: embryo maturation

As indicated previously, fully developed cotyledonary embryos are morphologically mature, closely resembling their zygotic counterparts, but are not physiologically prepared to germinate. In order to do so they must undergo a desiccation process effected by either applications of PEG in the development medium, or by a partial drying treatment (PDT) at the end of the developmental process. Both these treatments mimic the *in vivo* desiccation period occurring during the late phases of seed development. As documented by Yeung and Brown (1982), the liquid endosperm of flowering plants has more negative osmotic values than those of the embryo. In white spruce, applications of PEG (ranging from 5-10% depending on the genotype) in the development medium result in a three-fold increase in maturation frequency and produce somatic embryos with superior appearance to those matured with ABA alone (Attree *et al*. 1991). Such embryos have an increased tolerance to drying (Attree *et al*. 1995), a nine fold increase in the amount of storage lipid triacylglycerol with a fatty acid composition resembling that of zygotic embryos (Attree *et al*. 1992), and a three-fold higher protein content than somatic embryos matured in the absence of PEG (Misra *et al*. 1993). Misra *et al*. (1993) showed that the physiological maturity of PEG-treated embryos is reflected by the appearance of some of the major matrix and crystalloid polypeptides which are absent in somatic embryos developed in ABA and low osmoticum, but present in mature seed embryos (Misra *et al*. 1993). The effects of ABA and osmoticum in the regulation of somatic embryo development, especially protein synthesis, appear to be additive. *In vitro* translation studies have shown that crystalloid protein synthesis is first initiated by ABA alone, but sequentially regulated by PEG at a translational or post-translational level (Misra *et al*. 1993).

As well as through applications of PEG, physiological maturation can be achieved through the PDT. It is surprising that despite the importance of this treatment there are very studies describing the physiological changes of the embryos occurring during the PDT. Kong and Yeung (1995) have shown that compared to fully developed embryos, partially dried white spruce somatic embryos have a lower endogenous level of ABA, as well as a reduced sensitivity to ABA. Reduced levels and sensitivity to this hormone are beneficial for successful germination and conversion. Supporting this notion is the observation that applications of ABA in the germination medium have a negative effect on embryo conversion and plant regeneration.

Besides affecting hormonal levels, the imposition of a PDT may be required to increase the ability of white spruce somatic embryos to generate purine and pyrimidine nucleotides in preparation for the resumption of growth at germination. Both uridine and adenine salvage enzymes, uridine kinase (UK) and adenine phosphoribosyltransferase (APRT) were found to increase in partially dried embryos (Stasolla *et al*. 2001a). High activities of these enzymes are required for the extensive salvage of both purine and pyrimidine precursors occurring at the inception of germination, before the restoration of the de novo nucleotide biosynthesis (Stasolla *et al*. 2001b, 2001c).

Embryo conversion

Information on the physiological events occurring during embryo conversion are quite scarce in the literature. This is due mainly to the fact that the ability of the embryos to regenerate viable plants at germination is strictly dependent upon their maturation conditions. This notion, however, has been partially disproved by the observation that treatments during germination may "rescue" poorly developed embryos and induce them to convert at a high frequency. In general, conditions for root germination are less stringent than those required for shoot growth (reviewed by Yeung and Stasolla 2000). As a result a lot of attention has been focused on improving shoot reactivation by encouraging organized cell division in the shoot poles of germinating embryos. The "rescue" of structurally disorganized SAMs has been documented in white spruce when ascorbic acid (AA) is included in the germination medium (Stasolla and Yeung 1999). Applications of AA stimulate cell division and meristemoid formation within SAMs developed under suboptimal conditions and disrupted by the presence of intercellular spaces. These AA-induced meristemoids, often forming at the base of the cotyledons are able to develop further and form vigorous shoots through a process resembling shoot organogenesis (Stasolla and Yeung 2006). The formation of vascular connections between these shoots and the hypocotyls of the embryos leads to the production of viable plants. Although not thoroughly investigated, AA may reactivate poorly organized SAMs through the activation of both purine and pyrimidine nucleotide synthesis. In germinating embryos, applications of AA increase the purine nucleotide pool, especially ATP which is required for energetic processes and for the synthesis of nucleic acids (Stasolla *et al*. 2001). Changes in pyrimidine metabolism were also observed in AA-treated embryos. Incorporation of thymidine for nucleic acid synthesis is promoted by inclusions of AA in the germination medium (Stasolla and Yeung 2006). In the same study it was shown that an experimental reduction of AA levels, effected by applications of lycorine which inhibits the *de novo* synthesis of AA, decreases thymidine and uridine salvage through the inhibition of thymidine kinase and uridine kinase. Therefore it appears that AA may promotes SAM reactivation and embryo conversion through the activation of nucleotide metabolism.

MOLECULAR PROCESSES

Unlike for flowering plants, there are very few studies dealing with the molecular mechanisms operating during conifer embryogenesis. As suggested by Pullman *et al*. (1999), this paucity of information has prevented the identification of embryo-stage specific molecular markers which would provide new tools for improving somatic embryogenesis. Early molecular work conducted in Dunstan's laboratory has identified three major clusters of genes expressed during embryo growth. The first group includes genes constitutively expressed during all stages of embryo development, the second group is composed by genes expressed during the middle and late stages of development, whereas the third cluster includes genes expressed not only at all stages of development, but also in other plant tissues (Dong and Dunstan 1996a, 1996b; Dong and Dunstan 2000). Among genes of the first groups there are several encoding for pathogenesis-related proteins, including a chitinase (PgChi-1) and a glucanase (PgGlu-1). The importance of chitinases during spruce embryogenesis has been revealed by Egertsdotter and von Arnold (1998) and Dyachok *et al*. (2002) who showed that early embryo development can be improved through the addition of chitinases from *Streptomyces griseus* in the culture medium.

Members of the second class of genes include late embryogenic abundant (LEA) proteins and heat shock proteins

(HSPs). The expression of LEA proteins generally increases during development where they are implicated in water stress mechanisms. Bewley and Black (1994) have suggested that this class of protein can be used as a marker for completion of embryo maturation in spruce, as well as in other coniferous species.

Our understanding of the molecular mechanisms operating during embryo development in conifers has been greatly improved through the utilization of novel techniques, including differential display and cDNA microarray. Studies conducted in Cairney's lab have employed differential display to monitor the activity of several hundreds of genes during embryo development (Xu *et al.* 1997; Cairney *et al.* 1999, 2000). Further improvements in the field have been rendered possible by the production of expressed sequence tags (ETSs) from several pine libraries conducted at North Carolina State University by Dr. Sederoff and at Georgia Tech by Dr. Cairney. This effort has allowed the use of cDNA microarray technique to analyze the transcript levels during different phases of embryo development (van Zyl *et al.* 2002; Stasolla *et al.* 2003). Stasolla *et al.* (2003) reported the existence of almost 200 genes which were differentially expressed during the early phases of spruce embryogenesis. As this number declined during the successive stages of development it was suggested that a reprogramming of gene expression is critical during early embryogeny. This observation also supports the notion that improvement of embryo quality can be achieved through manipulations of culture conditions early in the embryogenic process. Form the same study it also emerged that several conifer genes, homologous to *Arabidopsis* genes involved in embryo development, including *ZWILLE* and *SCARECROW*, are expressed during embryo development in spruce (Stasolla *et al.* 2003). Similarities in gene expression between conifers and flowering plants were also revealed by Cairney *et al.* (2006). Through the analysis of ESTs generated from pine embryo libraries the authors observed that of 108 embryo-related genes in angiosperms, 83 homologs were also observed in pine embryos. These observations suggest that the molecular mechanisms controlling embryogenesis are similar in angiosperms and gymnosperms.

Several spruce genes have been isolated and characterized over the past few years (Elfstrand *et al.* 2001; Ingouff *et al.* 2001; Hjortswang *et al.* 2002; Footitt *et al.* 2003; Ingouff *et al.* 2003). Overall, these studies indicate that alterations in gene expression affect the normal embryonic developmental pathway. As proper formation of SAM is a critical event for somatic embryogenesis, it is not surprising that several studies have focused on the identification of genes expressed within the apical pole of the embryos. Sundas-Larsson *et al.* (1998) isolated a novel spruce gene, *HBK1*, which shows sequence similarities with members of *KNOX* genes in flowering plants, including the maize *KN1* and the *Arabidopsis SHOOTMERISTEMLESS* (*STM*). Both *KN1* and *STM* are expressed in the central population of non-differentiated cells of the apical pole where they are involved in the formation and maintenance of the SAM (Barton and Poethig 1993; Smith *et al.* 1995). Genetic analyses also indicated that loss-of-function mutant plants of either of the two genes fail to develop functional SAM (Bart and Poethig 1993; Kerstetter *et al.* 1994). Besides sharing significant similarities throughout the coding region, *HBK1* includes the three conserved domains (*KNOX*, *ELK*, and *HOMEODOMAIN*) which are present in all members of *KNOX* genes, including *KN1* and *STM* (Sundas-Larsson *et al.* 1998). These similarities suggest that *KBK1*, like other *KNOX* members, is a transcription factor regulating the expression of target genes. In addition, the localization pattern of HBK1, which is also restricted in the central region of the apical pole indicates that this gene may regulate SAM development in spruce (Sundass-Larsson *et al.* 1998). In recent years our lab has used this gene as a marker to estimate SAM quality in developing spruce somatic embryos (Belmonte *et al.* 2006).

Besides *HBK1*, two other members of the *KNOX* gene family have identified in Norway spruce: *HBK2* and *HBK3* (Hjortwang *et al.* 2002). *HBK3* shares an overall 83% identity with *KBK1*, whereas *KBK2* has only 50% homology with *HBK1* and *HBK3*. Transformation experiments have shown that *HBK3* may also be involved in SAM formation, as embryos over-expressing this gene have enlarged SAMs (Belmonte *et al.* in press). In addition, lines down-regulating *HBK3* loose their ability to produce embryos (Belmonte *et al.* unpublished data).

In a further effort to identify genes involved in SAM development in conifers, we have isolated and characterized PgAGO, a spruce gene homologous to members of the *ARGONAUTE* family in Arabidopsis (Tahir *et al.* 2006). This gene contains the conserved domains PIWI and the PAZ, which are found in ARGONAUTE proteins where they are involved in gene silencing. The expression of PgAGO is restricted only in the meristematic cells of embryonic roots and shoots, suggesting a possible role of this gene in the control of apical meristems. Spruce lines down-regulating this gene produces embryos with abnormal SAMs and RAMs (Tahir *et al.* 2006). Further efforts are focused on the identification of other genes involved in the regulation of embryo growth and meristem formation, and on global changes of protein synthesis during embryogenesis (Lippert *et al.* 2005).

CONCLUSIONS

Since initial reports documenting the production of somatic embryos in conifers vast amount of work has been carried out on the optimization of media and culture conditions. These efforts have been rendered possible by our increasing knowledge on the developmental, physiological, and molecular events regulating embryogenesis *in vitro*. Early work has emphasized the importance of ABA and osmoticum during embryo development and maturation. Selection of proper osmoticum agents, especially PEG has been proven to be effective in stimulating water stress and increasing embryo yield and post-embryonic performance. Recent progresses in molecular biology techniques have broaden our understanding on the mechanisms regulating embryogenesis in conifers and on the genes involved in histodifferentiation and development. An exciting avenue to undertake is the identification of genes involved in the embryo development which would improve the embryo output of lines with otherwise low embryogenic potentials. Such studies would also represent a valuable tool for the optimization of culture media component.

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