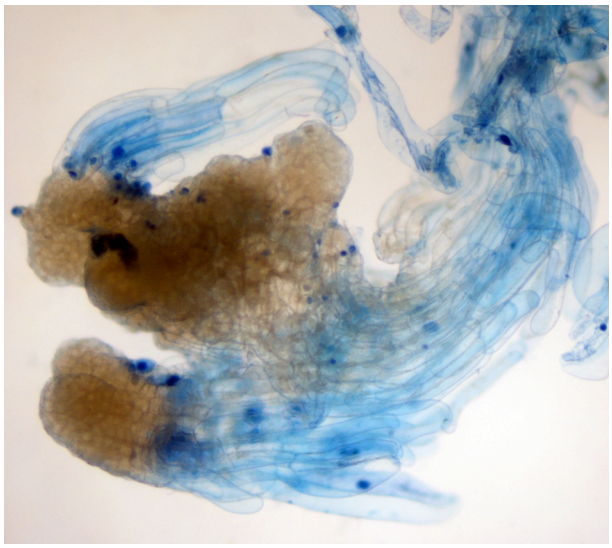
Programmed Cell Death and Genetic Stability in Conifer Embryogenesis

Andreas Helmersson
Faculty of Natural Resources and Agricultural Sciences
Department of Plant Biology and Forest Genetics
Uppsala



Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2007

Acta Universitatis Agriculturae Sueciae
2007: 127

Front cover illustration:
Light microscopy image showing early somatic embryos of Norway spruce (*Picea abies* L. Karst) after maturation treatment with abscisic acid for two weeks. Cells stained blue with Evans blue show dead or dying cells with disrupted plasma membranes.

ISSN 1652-6880
ISBN 978-91-85913-26-8

Tryck: SLU Service/Repro, Uppsala 2007

Abstract

Helmersson, A. 2007. *Programmed Cell Death and Genetic Stability in Conifer Embryogenesis*. Doctor's dissertation.
ISSN 1652-6880, ISBN 978-91-85913-26-8

Somatic embryogenesis, the generation of embryos from somatic cells, is a valuable tool for studying embryology. In addition, somatic embryos can be used for large-scale vegetative propagation, an application of great interest for forestry. A critical event during early embryo differentiation in conifers is the apical basal polarization, which proceeds through the establishment of two embryonic parts: the proliferating embryonal mass and the terminally differentiated suspensor. The development of both parts is strictly coordinated and imbalance causes embryonic defects. The suspensor cells are eliminated by programmed cell death (PCD). In animals, caspase family proteases are the main executioners of PCD. In this work we have used synthetic peptide substrates containing caspase recognition sites and corresponding specific inhibitors to analyse the role of caspase-like activity during early embryo differentiation in Norway spruce (*Picea abies* L. Karst.). We found that VEIDase is the principal caspase-like activity. This activity is localized specifically in suspensor cells, and its inhibition prevents normal embryo development by blocking the suspensor differentiation. The *in vitro* VEIDase activity was shown to be highly sensitive to pH, ionic strength, temperature and Zn^{2+} concentration. *In vivo* studies with Zinquin, a zinc-specific fluorescent probe, revealed a high accumulation of intracellular free zinc in the embryonal masses and an abrupt decrease in the suspensor. Increased zinc concentration in the culture medium suppresses terminal differentiation and PCD of the suspensor. In accordance, exposure of early embryos to TPEN, a zinc-specific chelator, induces ectopic cell death affecting embryonal masses. This establishes zinc as an important factor affecting cell fate specification during plant embryogenesis.

Before somatic embryos can be accepted for clonal propagation it is important to show that the regenerated plants have similar growth to that of seedlings and are genetically uniform. The genetic integrity during zygotic and somatic embryogenesis in Norway spruce and Scots pine (*Pinus sylvestris* L.) was investigated by comparing the stability of variable nuclear microsatellite loci. The stability varied significantly among families in both species during somatic embryogenesis. Scots pine families showing low genetic stability during establishment of embryogenic cultures had a higher embryogenic potential than those that were genetically more stable. In contrast, embryo development was suppressed in genetically unstable families. The stability of microsatellites was in general higher in zygotic embryos than in somatic embryos. No deviation in growth was observed in somatic embryo plants of Norway spruce carrying mutated microsatellites.

Key words: developmental PCD, gymnosperms, simple sequence repeat microsatellite marker, somatic embryogenesis, VEIDase, Zinc, Zinquin-etyl-ester

Author's address: Andreas Helmersson, Department of Plant Biology and Forest Genetics, SLU, Box 7080, SE-750 07 Uppsala, Sweden. *E-mail*: Andreas.Helmersson@vbsg.slu.se

*"*When you have to shoot, shoot don't talk*"*

The Good, The Bad and The Ugly, 1966

Contents

Appendix

The present thesis is based upon the following papers, which will be referred to by the corresponding Roman numerals.

I. Helmersson, A., von Arnold, S., Burg, K., Bozhkov, P.V. 2004. High stability of nuclear microsatellite loci during the early stages of somatic embryogenesis in Norway spruce. *Tree Physiology* 24(10), 1181-6.

II. Bozhkov PV, LH Filonova, MF Suarez, A Helmersson, AP Smertenko, B Zhivotovsky, von Arnold S. 2004. VEIDase is a principal caspase-like activity involved in plant-programmed cell death and essential for embryonic pattern formation. *Cell Death and Differentiation* 11(2), 175-82.

III. Helmersson, A., von Arnold, S., Bozhkov, P.V. The level of free intracellular zinc controls programmed cell death/cell survival decisions in plant embryos. (*Manuscript*).

IV. Burg, K., Helmersson, A., Bozhkov, P., von Arnold, S. 2007. Developmental and genetic variation in nuclear microsatellite stability during somatic embryogenesis in pine. *Journal of Experimental Botany* 58(3), 687-98.

V. Helmersson, A., Jansson, G., Bozhkov, P.V., von Arnold, S. Genetic variation in microsatellite stability of somatic embryo plants of *Picea abies*: a case study using six unrelated full sib families. (*Provisionally accepted for publication in Scandinavian Journal of Forestry*).

Introduction

Economic importance of forestry

The United Nations and Food and Agriculture Organization of the United Nations (FAO) have calculated that the world population will increase from 6.1 billion to about 7.6 billion from 2000 to 2020. The population growth will result in an increased requirement for forest products. As a result the global deforestation will continue. In the period 1990 – 2005 the deforestation was estimated to be 13 million hectares per year (an area about the size of Greece), with few signs of a significant decrease over time (FAO, 2005). Forests cover 30% of the total land area in the world. Thus the increasing worldwide demand for both wood products and other uses of forests (e.g. recreation and preservation of biodiversity) means that more productive, rather than extensive, commercial forests are needed. The forest plantations in the world are increasing but still account for less than five percent of the total forest area (FAO, 2007).

The Swedish land area is covered to 55% by productive forest. The two dominating species in Swedish forests are Norway spruce (*Picea abies*) (41%) and Scots pine (*Pinus sylvestris*) (39%) (Swedish Forest Industries Federation, 2006). Coniferous species have a wide range of commercial uses, e.g. timber for construction, raw material for pulp and paper production and as an energy source. Forestry and the forest industry play an important role in Sweden accounting for almost four percent of Sweden's GDP. The forest industry constitutes 10% of the employment in Sweden. In addition, it accounts for more that 12% of the exports (Swedish Forest Industries Federation, 2006). Sweden ranks third among the world's largest paper and sawn timber exporters and is the world's fourth largest exporter of pulp. Sweden supplies 13% of the paper demand in Europe and covers about 12% of the total consumption of sawn timber in the European Union.

Since 1990 the prerequisites for higher forest productivity in Sweden have changed dramatically. In 1994 a new forest law required that the use of forestland should be balanced between two equally important goals, production and nature conservation. The Swedish Forest Agency has calculated that between 1990 and 2004, the use of wood increased by 27%, the total harvesting by 30%, the net import more than 100% and at the same time the use of fuel wood also increased. As a result the net harvesting has today become larger than the net growth for Norway spruce, while it remains steady for Scots pine (Bräcke *et al*., 2007). To sustain the Swedish forest industry with high quality raw material the production has to increase from the existing Swedish forests. It is feasible to combine environmentally friendly silviculture with genetically improved plant material. Especially the production of large quantities of genetically improved and uniform seedlings will be a key component of sustainable forest management programs in the future.

Forest tree breeding and mass propagation

Breeding of forest trees is a slow process where valuable traits are successively improved. The improvement is obtained through well known quantitative genetic techniques involving recurrent testing, selection and crossing (Eriksson *et al*., 2007). Testing and selection are based on specific traits identified in the breeding programme. Selected plants, that best fulfil the requirements are used for further breeding and mass-propagation. The selection is done after about one fifth of the rotation period i.e. after about 15 years. However when the trees are selected they are too old to be propagated vegetatively. Therefore scions from the selected trees are grafted and seed orchards are established. The seed orchard begins to produce improved seeds after about 15-20 years. In general the genetic gain of first-round conifer seed orchards is 10% in volume growth per unit area, as compared with unimproved stock. The predicted gain from the second round of seed orchards ranges between 10% (totally untested plus trees) and 25% (intense selection from a large number of field-tested plus trees) (Rosvall *et al*., 2001). From the third round of seed orchards, which is currently being established, the expected gain can reach 25%. New seed orchards will thereafter be established every 20th year with an expected additional gain of 10% per round.

Pollen contamination is a serious problem in most conifer seed orchards. With the aid of biochemical markers it was estimated that the contamination amounts to as much as 50% on average and up to 70% not uncommonly (Eriksson *et al*., 2007). Furthermore, all propagation methods include some selection. For example, seed orchard clones vary significantly in flowering time as well as in pollen and seed production. In consequence seeds are often derived from a few clones in a seed orchard. CellFor (http://www.cellfor.com/; 30-august-2007) for example state that 80% of the seeds in an orchard can be produced by only 20% of the trees. Taken together, the true genetic gain from seed orchard seeds is difficult to predict owing to high pollen contamination, uneven flowering and pollen production, and recombination events during fertilization. The possibility to propagate the selected trees vegetatively would be an effective technique to produce large amounts of plant material and capture genetic gain more efficiently (Park & Bonga, 1992).

Plants can be propagated vegetatively by grafting, rooting of cuttings, micropropagation or somatic embryogenesis. Some methods are more feasible for reforestation purposes than others: grafting of scions to small plants is a simple technique but is labour-intensive and thus unrealistic for reforestation purposes. This method is mostly used to multiply trees selected for seed orchards and breeding archives. Rooting of cuttings in Norway spruce was first reported in the beginning of the 19th century (Pfifferling, 1830, cited in Kleinschmit *et al*., 1973). Several projects for cutting propagation of Norway spruce have been tried in Sweden (Högberg *et al*., 1995). Better growth and higher survival have been reported for cutting-propagated plants compared with seedlings of the same size and genetic origin, up to about 10 years of age (Gemmel *et al*., 1991). However there are substantial limitations with this method in clonal forestry. Only scions from young Norway spruce plants can be efficiently rooted at high rates in practice. Furthermore, scions from older trees have a tendency to plagiotropic growth (Sonesson, 2003). Micropropagation of conifers by axillary or adventitious shoots is hampered by high production costs, difficulties in rooting and low

multiplication rates even though adventitious shoots give high multiplication rates (Högberg, 2003). It has been shown that somatic embryogenesis is an effective method to propagate conifers vegetatively.

Vegetative propagation is of great importance for the Swedish long term breeding strategy. When the superior genotypes have been identified, based on clonal field trials, they are too old to be propagated by cuttings. Consequently, the identified elite genotypes are lost and they can only be used as parents for the next generation. However, if the genotypes, during testing, can be stored in such a way that they maintain their ability to be propagated vegetatively the specific genetic gain for each genotype could be preserved. By using somatic embryogenesis in combination with cryopreservation this goal can be achieved.

Somatic embryogenesis

In contrast to zygotic embryogenesis, somatic embryogenesis is an asexual propagation process where somatic cells differentiate into embryos. Somatic embryos pass through a similar sequence of developmental stages as their zygotic counterparts (Mordhorst *et al*., 1997). Since the first observation of somatic embryo formation in carrot (*Daucus carota*) cell suspensions by Steward *et al*., (1958) and Reinert (1958), the potential for somatic embryogenesis has been shown in a wide range of plant species. In gymnosperms, somatic embryos have been induced in a variety of cycads and conifers (Attree & Fowke, 1993).

In addition to somatic embryogenesis being used for clonal propagation it is also an important tool for studying embryology. In conifers where no embryo-specific mutants have been characterised, somatic embryogenesis has been very useful for these studies. An advantage is also that the developmental process of the somatic embryos can be controlled and synchronised, allowing collection of embryos at specific stages. Embryogenic cultures are also an attractive target for genetic transformation.

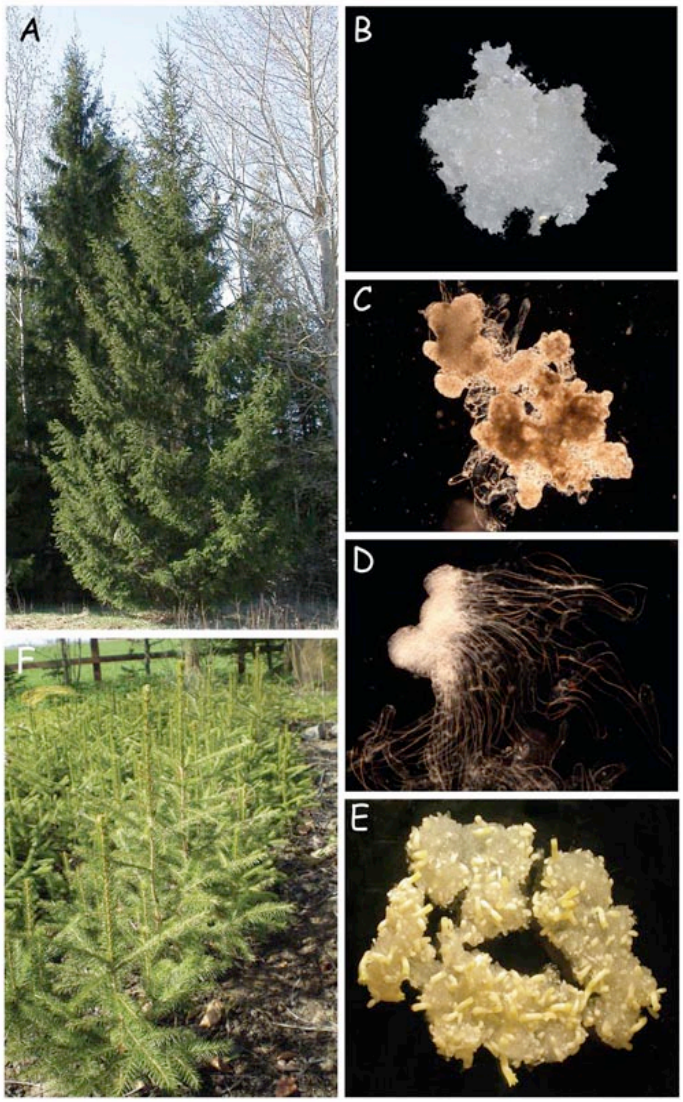


Figure 1. Propagation of Norway spruce via somatic embryogenesis. Seeds are collected from selected trees (A). Zygotic embryos are incubated on a medium containing plant growth regulators (PGRs), auxin and cytokinin, to form embryogenic cultures. Embryogenic culture established from one zygotic embryo is defined as an embryogenic cell line (B). In the presence of PGRs the cell lines proliferate as proembryogenic masses (PEMs) (C), after withdrawal of PGRs somatic embryos differentiate (D). Mature cotyledonary somatic embryos are formed when the cultures are exposed to ABA (E). The mature embryos are partially desiccated, germinated and acclimated to field conditions (F).

Norway spruce

Somatic embryogenesis in conifers was first reported for Norway spruce by Hakman *et al*., (1985) and Chalupa, (1985). Since then the characteristics of Norway spruce somatic embryogenesis have been thoroughly studied. Filonova *et al*., (2000a) used a time lapse tracking technique to analyse the developmental pathway, which resulted in a model that today is accepted for studies in plant embryology. The overall procedure of somatic embryogenesis in Norway spruce is shown in Figure 1 and the model of developmental pathway suggested by Filonova *et al*., (2000b) is outlined in Figure 2.

Initiation of embryogenic cell lines usually starts from zygotic embryos, which are incubated on a medium containing auxin and cytokinin (PGRs). The initiation of embryogenic culture from seedlings or older material is rare but has been reported (Ruaud *et al*., 1992, Harvengt *et al*., 2001). The best initiation frequency is attained when using seeds, which have not yet been desiccated. The cell lines are regarded as established when they proliferate as proembryogenic masses (PEMs). At this stage the embryogenic culture can be cryopreserved in liquid nitrogen.

Embryogenic cell lines proliferate as PEMs in the presence of PGRs. Three stages of PEMs are distinguished based on cell number and organization (Figure 2). Early somatic embryos differentiate from PEMs after withdrawal of PGRs. Their further development to mature embryos requires abscisic acid (ABA). Mature cotyledonary somatic embryos are partially dehydrated in a controlled manner to reduce water content to less than 55% (Cellfor. http://www.cellfor.com/; 30-august-2007), which is the prerequisite for synchronous germination. They are then germinated on medium lacking PGRs. The germinated embryos are transferred to vessels where the roots have access to liquid nutrient solution (Högberg, 2003). When the root system is well developed, having several lateral roots, the somatic embryo plants are acclimatised to *ex vitro* conditions following standard nursery schemes.

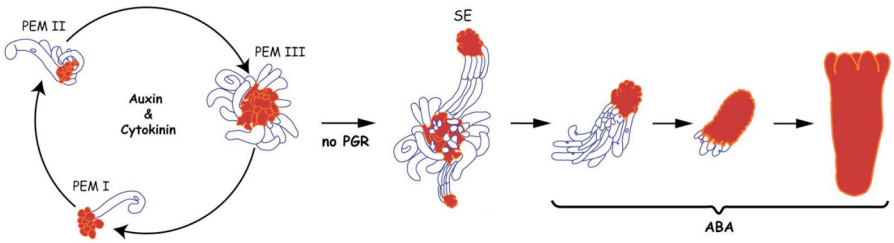


Figure 2. A schematic drawing of the developmental pathway of somatic embryogenesis in Norway spruce (adapted from Filonova *et al*., 2000b). PEMs are stimulated to proliferate in medium supplemented with PGRs. The individual PEM should pass through three different stages (I, II and III) before somatic embryos can differentiate. PEM I consists of a few meristematic cells and have one vacuolated cell joined to them. In PEM II several vacuolated cells have been formed and finally in stage III there are clusters of both vacuolated and densely packed cells as well as cells in the transition between the two forms. Somatic embryos are stimulated to differentiate from PEM III after withdrawal of PGRs.

Further development and maturation of somatic embryos requires ABA. Living cells stain red with acetocarmine. Dead or dying cells stain blue with Evans blue.

Scots pine

The earliest reports of achieving somatic embryo cultures followed by plantlet regeneration with pine species (*Pinus sp.*) were with cultures derived from mature zygotic embryos of sugar pine (*Pinus lambertiana*) (Gupta & Durzan, 1986) and loblolly pine (*Pinus taeda*) (Gupta & Durzan, 1987). The first report of all steps for somatic embryogenesis in Scots pine was described by Keinonen-Mettälä *et al*., (1996). The different steps to achieve somatic embryogenesis in Scots pine are similar to those in Norway spruce including: initiation and proliferation of embryogenic cultures, early embryo differentiation, maturation and desiccation of the embryos followed by germination and plant development.

Polyembryony is a characteristic feature of several conifer species. It may result from the fertilization of several archegonia (simple polyembryony) or from the cleavage of the embryonal masses of one or more zygotic embryos (cleavage polyembryony). Cleavage polyembryony, which is common in the genera *Pinus*, *Cedrus*, *Tsuga* and *Abies* (Buchholz, 1931; Chowdhury, 1962), has been most studied in *Pinus*. At an early developmental stage the primary embryo starts to go through several rounds of cleavage resulting in four to twelve embryos of equal size. The embryos start to compete resulting in one embryo becoming dominant, while the subordinate embryos are aborted (Nagmani *et al*., 1995; Filonova *et al*., 2002).

In most pine species, initiation of somatic embryogenesis is limited to the first few weeks of zygotic embryo development (Keinonen-Mettälä *et al*., 1996) or prior to the emergence of cotyledonary primordia (Finer *et al*., 1989; Becwar *et al*., 1990; Lelu *et al*., 1999; Park *et al*., 1999; Salajova *et al*., 1999; Percy *et al*. 2000). Bozhkov *et al*. (1997) indicated that embryogenic cultures of Korean pine (*Pinus koraiensis*) arise from the continuation of the zygotic embryo cleavage process. Park *et al*., (2006) further showed that somatic embryogenesis initiation in jack pine (*Pinus banksiana*) primarily resulted from auxin-induced cleavage of the four-celled proembryo and that cleavage polyembryony becomes continuous. Hence identification of the stage of cleavage polyembryony is important for somatic embryogenesis initiation in pine species. In Scots pine the initiation frequency was highest using immature zygotic embryos (1-3 weeks after fertilization, waf) (Park *et al*., 2006).

The continuous proliferation of early embryos in Scots pine brings further problems for regulating the embryogenic process. In Norway spruce, when embryogenic cultures are proliferating as PEMs, a pre-treatment in PGR-free medium is sufficient to initiate embryo development. In contrast, Scots pine embryos proliferate even after several weeks in PGR-free medium. It has also been shown that initiation and proliferation of embryogenic cultures in Scots pine do not require exogenous PGRs (Lelu *et al*., 1999). In many cases the quality of cotyledonary somatic embryos is low as indicated by the low germination frequency. Results from Rodriguez *et al.,* (2006) indicate that chloroplast development had been initiated in maritime pine (*Pinus pinaster*) cotyledonary

somatic embryos before they were artificially brought to a dormant state, suggesting that the germination programme might be activated during somatic embryo maturation.

Integration of somatic embryogenesis into breeding programmes

A prerequisite for integrating somatic embryogenesis into breeding programmes for reforestation are that most of the selected families can be propagated via somatic embryos and that the somatic embryo plants grow normally and are genetically stable.

Selection

Before somatic embryos can be used for practical purposes the technique must be applicable to a broad range of genotypes. This means that no selection favouring a limited number of genotypes should take place between the initiation of embryogenic tissue and the production of plants. Initiation frequency of embryogenic cultures varies among species, families and genotypes (Cheliak & Klimaszewska, 1991; Park *et al*., 1993; MacKay *et al*., 2006). An important risk to consider is that a strong genetic selection on embryogenic capacity could be correlated with unfavourable responses in other traits. However, in Norway spruce no correlations were found between embryogenic capacity and phenological traits in two populations of Norway spruce (Ekberg *et al*., 1993). In addition, Högberg *et al*. (1998) found no indication that propagation capacity at different stages of somatic embryogenesis is correlated with important traits in breeding.

A skewed distribution of propagated clones can reduce the genetic diversity of the produced plant population and reduce the genetic gain (Högberg, 2003). Therefore the method for propagating Scots pine via somatic embryogenesis has to be improved. It has been shown that optimal medium composition varies among cell-lines (genotypes) (Pullman *et al*., 2005; Park *et al*., 2006; Pullman & Skryabina, 2007). Therefore, by including several standard culture media in propagation programs the number of genotypes that can be propagated can be increased.

Growth

The growth of somatic embryo plants is affected by the treatments given during the *in vitro* phase and during the *ex vitro* establishment phase. An inappropriate treatment often leads to negative after-effects on plant growth. Bozhkov & von Arnold (1998) showed that the osmotic potential during maturation affects the quality of the somatic embryos. The time of contact with ABA during embryo maturation and the length of continuous light treatment during the first growth period strongly affect the height growth during the first two growth periods (Högberg *et al*., 2001). Högberg *et al*. (2003) found that critical selection among young somatic embryo plants following *ex vitro* transfer could improve performance of clonal stock propagated by somatic embryogenesis. Grossnickle *et al*., (1994) found that somatic embryo plants of interior spruce (*Picea glauca* × *Picea engelmannii*) were significantly shorter than seedlings at the time of *ex vitro* transfer. These differences remained after one season in the nursery and after two years in the field, although height growth was similar for both plant types

(Grossnickle & Major 1994a, 1994b). It is usually difficult to compare growth of somatic embryo plants and seedlings because of the problem with producing plants of similar size. In many cases the seedlings have become much larger when the plants are transferred to *ex vitro* conditions.

The first season growth, and several other properties, were compared between somatic embryo plants and seedlings in white spruce (*Picea glauca*). Clonal effects were present in most of the characteristics studied, but the growth was not significantly different among families (Lamhamedi *et al*., 2000). Grossnickle & Folk (2005) compared the quality of somatic embryo plants in standard operational nursery production practices and concluded that container-grown somatic embryo plants of interior spruce can meet the standards used in reforestation programs.

Genetic stability

Micropropagation allows large-scale propagation of selected genotypes, however, problems with somaclonal variation have been encountered. Somaclonal variation refers to any phenotypic or genotypic modifications that arise from *in vitro* culture (Larkin & Scowcroft, 1981; Jain, 2001). Somaclonal variation is associated with several types of genomic disorder, including changes in ploidy (Karp *et al.*, 1989), DNA methylation modifications (Peschke & Phillips, 1991; Smulders *et al*., 1995), point mutations (Brettell *et al*., 1986), insertion of mobile DNA elements or retroelements (Peschke *et al*., 1987; Brettell & Dennis, 1991; Pouteau *et al*., 1991; McKenzie *et al*., 2002) and chromosomal rearrangements (Phillips *et al*., 1994, Tremblay *et al*., 1999). Although a few somaclonal variation changes are stable and genetically inherited, others are not inherited or reversible or inherited in a non-Mendelian fashion.

The frequency of somaclonal variation in tissue culture-derived plants depends on the type of explant (Karp *et al*., 1984), the genotype (Galiba *et al*., 1985, Linacero & Vazquez, 1993; Saieed *et al*., 1994; Tremblay *et al*., 1999; Etienne & Bertrand, 2003) and species (Wilhelm *et al*., 2005). In addition, the type of regeneration process (e.g., somatic embryogenesis or organogenesis) may also be a contributory factor determining the frequency of somaclonal variation (Armstrong & Phillips, 1988). *In vitro* induced stress during cellular reprogramming by PGRs such as 2,4-dichlorophenoxyacetic (2,4-D) can cause somaclonal variation (Saieed *et al*., 1994). Some authors have reported that prolonged time in *in vitro* culture can promote somaclonal variation (Orton 1985; Lee & Phillips, 1987; Hartmann *et al*. 1989; Tremblay *et al*., 1999; Etienne & Bertrand, 2003).

A number of PCR-based techniques for analysing somaclonal variation have been tested including AFLP (Amplified Fragment Length Polymorphism), RAPD (Random Amplification of Polymorphic DNA) and SSR (Simple Sequence Repeat). However, when evaluating somaclonal variation, it is usually more advantageous to use more than one DNA amplification technique (Palombi & Damiano, 2002).

The AFLP technology originally proposed by Vos *et al*. (1995) detects polymorphisms in different genomic regions, between 50 and 100 fragments at the same time. It is a highly sensitive and reproducible technique and no prior

sequence information for amplification in needed. As a result, AFLP has become widely used for studying genetic variation in strains or closely related species of bacteria, fungi, plants and animals (Savelkoul *et al*., 1999).

RAPD markers are DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence. Even though RAPD markers mostly are dominant, they may be used to differentiate between genetically distinct individuals.
RAPD markers were used for showing:

- Genetic stability in micropropagated tetraploid black locust (*Robinia pseudoacacia*) (Shu *et al*., 2003) and in somatic embryos of Norway spruce (Heinze & Schmidt, 1995; Passerieux *et al*., 1999).
- Genetic instability in plants of white spruce regenerated from phenotypically abnormal somatic embryos (De Verno *et al*., 1999).
- Genetic instability in four plants out of 2270 regenerated from somatic embryos of white spruce (Isabel *et al*., 1996).

Microsatellites or SSRs (Simple Sequence Repeats), are polymorphic loci present in nuclear and organellar DNA that consist of repeated units of 1-4 base pairs in length (Turnpenny & Ellard, 2005). The size of the repeat unit, the number of repeats, the presence of complex repeats and the frequency of transcription in the area of the DNA repeat are factors determining how suitable the SSR is for marker application. SSRs owe their variability to an increased rate of mutation compared to other neutral regions of DNA. These high mutation rates are most frequently caused by slipped strand mispairing during DNA replication on a single strand of DNA double helix (Levinson & Gutman, 1987a). Recombination events during meiosis may also induce mutations (Blouin *et al*., 1996). Mutation frequencies for microsatellites are reported in the range of 10^3 in humans (Weber & Wong, 1993), 10^4–10^5 in yeast (Strand *et al*., 1993) and 10^2 in bacteria (Levinson & Gutman, 1987b) per generation. The specificity of SSR amplification prevents any interference from contaminating DNA, which could be a strong concern with analysis of field-grown plant material (Camacho *et al*., 1997). This renders microsatellite regions appropriate targets for monitoring identity of clonally propagated plants as well as for population genetics studies.

SSR markers were used for showing:

- Genetic stability in somatic embryos initiated from primary (derived from seedlings) and secondary (derived from somatic embryo plants) embryogenic cells lines of Norway spruce (Harvengt *et al.*, 2001).
- Genetic instability in micropropagated trembling aspen *(Populus tremuloides*) (Rahman & Rajora, 2001).
- Genetic instability in somatic embryos of Norway spruce (Burg *et al*., 1999).
- Genetic instability in one of eight markers in cork oak (*Quercus suber*) somatic embryos (Lopes *et al*., 2006).

Somaclonal variation can sometimes favour the breeding objective by inducing higher genetic variation (Larkin & Scowcroft, 1981). However, the occurrences of abnormal somatic embryo plants are unlikely to constitute the main stock of productive forest owing to genetic and natural thinnings over a rotation period. In

addition, we have to keep in mind that molecular markers never permit complete genetic conformity to be demonstrated. The best method would be to check the expression of a large number of important genes at several developmental stages using, for example, microarray techniques.

Embryology

Most morphogenic events in plants occur after seed germination, however, the embryonic phase is crucial as it is here the plant meristems and the shoot-root plant body pattern are specified, and the primary tissue types differentiate. The genetic regulation of embryo pattern formation in the model plant *Arabidopsis* has been reviewed (Laux *et al*., 2004). *Arabidopsis* is a dicotyledonous plant and is a good model for embryology, having the advantage that mutants can be isolated and genes cloned (Berleth, 1998). Historically, the embryo specific mutants were the subject of the earliest large-scale mutant screens in *Arabidopsis* (Castle *et al*., 1993).

In contrast to *Arabidopsis* hardly anything is known about the regulation of embryo pattern formation in gymnosperms. Gymnosperms have several disadvantages as experimental organisms. They have large genomes, about 200 to 400 times bigger than *Arabidopsis*. Furthermore, they have a large size and a long generation time. According to molecular data, the last common ancestor of extant seed plants existed about 300 million years ago (Savard *et al*., 1994; Goremykin *et al*., 1997), and earliest fossil evidence of gymnosperms dates back about 350–365 million years ago (Beck, 1988; Taylor & Taylor, 1993). From an evolutionary point of view it is important that we learn more about the regulation of embryogenesis in gymnosperms.

Angiosperms

Several stages have been identified during embryo development in *Arabidopsis* including the octant, globular, triangular, heart, torpedo, bent-cotyledon and mature embryo stages (Jürgens & Mayer, 1992). The regulation of embryo development has been reviewed (Berleth & Chatfield, 2002; Laux *et al*., 2004; Weijers & Jürgens, 2005). Initially the zygote goes through an asymmetric division resulting in two daughter cells, which have different sizes and developmental fates. The small apical cell will form the embryo proper and the vacuolated basal cell will form the suspensor.

The *Arabidopsis* basal cell divides horizontally and produces a filamentous suspensor. The suspensor is considered to play an important role in transporting nutrients to the growing embryo proper early in its development (Johansson & Wales, 1993). Radioactively labelled ^{14}C-sucrose was transported from the endosperm through the suspensor to the embryo proper in broad bean (*Vicia faba* L.) indicating that nutrients are transported via the suspensor to the embryo proper (Yeung, 1980). In *Arabidopsis* when the embryo proper has reached the late heart stage, the suspensor degenerates and is usually eliminated before the embryo is mature. Developmental programs of the embryo proper and the suspensor are closely coordinated and an imbalance causes embryonic defects or lethality (Lukowitz *et al*., 2004). Experimental abortion of the embryo can induce the

formation of a secondary embryo from the suspensor cells (Gerlach-Cruse, 1969). Moreover, mutations in several genes give similar effects (Schwartz *et al*., 1994; Vernon & Meinke, 1994; Zhang & Somerville, 1997).

Polar transport of auxin within the embryo plays a central role for apical-basal-polarization and embryo patterning. Polar auxin transport requires auxin-efflux transporters belonging to the PINFORMED (PIN) family (Friml *et al*., 2003; Weijers *et al*., 2005). Four members of the PIN family of auxin efflux facilitators are expressed in the embryo – *PIN1*, *PIN3*, *PIN4* and *PIN7* (Friml *et al*., 2002; Friml *et al*., 2003). After the first zygotic division, PIN7 is located in the basal cell, facing the smaller apical cell, resulting in polar auxin transport and auxin accumulation in the proembryo (Friml *et al*., 2003). During transition to the globular stage, PIN7 localization shifts from the upper to the lower side of the suspensor cells. Weijers & Jürgens, (2005) suggest that GNOM (GN) re-localizes PIN7 in a similar way as PIN1 (Steinmann *et al*., 1999). Within the globular embryo auxin is transported towards the uppermost suspensor cell, creating an auxin maximum which specifies the hypophysis and triggers root pole specification.

During the octant stage all proembryo cells express *MONOPTEROS* (*MP*) and *BODENLOS* (*BDL*) (Weijers & Jürgens, 2005). *MP* encodes the AUXIN RESPONSE FACTOR5 (ARF5) protein (Hardtke & Berleth, 1998), a transcription factor that activates auxin-response genes (Ulmasov *et al*., 1999). MP is thought to be kept inactive by binding to the indoleacetic acid (IAA) protein BDL, which is degraded upon *PIN7*-directed auxin flow into the embryo proper, allowing MP to activate its target genes (Hamann *et al*., 2002). Loss of MP (Berleth & Jürgens, 1993) or gain of BDL function (Hamann *et al*., 1999, 2002) interfere with the specification of the apical cell, and prevent the formation of an embryonic root. Immediate targets of MP and BDL are not known, but homeodomain-containing transcription factors are assumed to be one of their targets (Weijers & Jürgens, 2005). At the globular stage *MP* and *BDL* are expressed in the inner cell of the embryo (Friml *et al*., 2003). The MP- and BDL-mediated auxin response in embryo proper cells appears to be required for signalling from the embryo proper to the subjacent hypophysis to allow for normal root development.

At the globular stage the hypophysis undergoes asymmetric cell division, and becomes part of the embryo itself. Its descendants make up the quiescent centre (QC) of the root and the columella stem cell initials (stem cells are a small population of relatively undifferentiated cells whose daughters can either remain stem cells or differentiate). The asymmetric division of the hypophysis is marked by the expression of *SCARECROW* (*SCR*) and *WOX5*, first in the hypophyseal cell and subsequently only in the lens-shaped cell and the QC (Wysocka-Diller *et al*., 2000; Haecker *et al*., 2004). The QC maintains the undifferentiated state of the surrounding stem cells by local signalling (van den Berg *et al*., 1997; Sabatini *et al*., 2003). Early defects in QC development are often followed by abnormal differentiation of the neighbouring stem cell precursors (Willemsen *et al*., 1998). The position of the QC depends on the presence of an auxin maximum in the columella, which is maintained by basipetal auxin transport (Sabatini *et al*., 1999; Friml *et al*., 2002, 2003).

The shoot apical meristem is subdivided into several zones. The central zone contains an organizing centre (OC) and a stem cell niche. Cells surrounding the central zone start to differentiate organ primordia. The first indication of shoot meristem initiation is expression of *WUSCHEL* (*WUS*) in the four sub-primordial cells of the 16-cell stage (Mayer *et al*., 1998). A *WUS*-expression domain is established but not effective until the heart stage. At the heart stage *WUS* activity in the OC results in the specification of stem cell identity in the three outermost cell layers, by inducing expression of *CLAVATA3* (*CLV3)*. *CLV3* limits the size of the OC by restricting *WUS* expression. This regulatory feedback loop between stem cells and the OC explains how the plant can assess and adjust the size of the stem cell pool (Laux *et al*., 2004 and references herein).

The first histological sign of differentiation of cotyledonary primordia can be seen from the flanks of the late globular embryo. In the centre of the apical domain, outgrowth is repressed by *CUP-SHAPED COTYLEDON1* (*CUC1*), *CUC2*, and *CUC3*. *CUC1* and *CUC2* are first expressed in isolated patches of apical cells before expression spreads into a stripe across the embryo apex that divides it into a central and two peripheral zones (Aida *et al*., 1999; Takada *et al*., 2001). The *CUC* genes encode putative transcription factors homologous with the petunia (*Petunia hybrida*) NO APICAL MERISTEM (NAM) protein, which also affects cotyledon separation and shoot meristem formation (Souer *et al*., 1996). Auxin is involved in signalling to the apical domain, because the spatial expression pattern of *CUC1* and *CUC2* require *MP* and *PIN1* activities (Liu *et al*., 1993; Hadfi *et al*., 1998; Aida *et al*., 2002).

Gymnosperms

The development of gymnosperm embryos has previously been summarized (von Arnold *et al*., 2002). The sequence of embryo development in gymnosperms can be divided into three phases (Singh, 1978). 1) Proembryogeny – stages before elongation of the suspensor; 2) Early embryogeny – the stages after elongation of suspensor, and before the establishment of root meristem and 3) late embryogeny – establishment of the root and shoot meristems and further development of the embryo until maturity. Embryo development occurs entirely within the female gametophytic tissue, which serves as the nutrient supply for the developing embryo (Owens, 1985). In most gymnosperms, the nucleus in the zygote divides without cytokinesis so that four free nuclei are formed, which become arranged in a tier (Singh, 1978). After several divisions, the proembryo becomes cellularised. In Norway spruce the 16-cell stage is organized in four distinct tiers, of which two constitute embryonal tiers which give rise to the embryonal mass (analogous to the embryo proper in angiosperms) and to the secondary suspensor, one elongates and forms the primary suspensor, and the upper tier degenerates. In the outer layer of the embryonal mass, cells divide both periclinally and anticlinally, thereby not permitting the differentiation of the classical protoderm (Singh, 1978). The patterning during early embryogeny proceeds through the establishment of three major cell types: the meristematic cells of the embryonal mass, the embryonal tube cells and terminally differentiated suspensor cells. The embryonal mass cells are small and spherical with dense cytoplasm and high mitotic activity. The asymmetric divisions of the most basally situated cells within the embryonal mass give rise to a layer of elongated embryonal tube cells that differentiate to form one

layer of the suspensor cells. Mediated by this asymmetric division suspensor cells are continuously added and form several layers of highly vacuolated cells. The transition between early and late embryogeny is considered to take place when the embryonal mass is pushed through the archegonial wall into the megagametophytic cavity (Gifford & Foster, 1989). During the early phase of late embryogeny the root and shoot apical meristems are delineated. The cotyledonary primordia arise in a ring around the distal end of the embryo.

Embryo development is difficult to study in conifers owing to the inaccessibility of the embryo inside the megagamethophyte. The possibilities to study the mechanisms underlying conifer embryogenesis have been enhanced considerably during the last years by using somatic embryos of Norway spruce.

Global changes in gene expression during successive stages of embryo development can be followed by microarray analysis of synchronized embryogenic cultures. RNA extracted from embryogenic cultures of Norway spruce representing successive developmental stages was analysed on DNA microarrays (Van Zyl *et al*., 2003; Stasolla *et al*., 2004). The variation in expression of specific genes suggests that a high level of DNA methylation is important for the differentiation of somatic embryos from PEMs. Furthermore, downregulation of genes involved in auxin metabolism is important for the apical-basal polarization during early embryogeny and downregulation of genes involved in cell wall formation is important for differentiation of primary meristems.

The key morphogenic event in plant embryogenesis is formation of the apical-basal pattern via establishment of the proliferating embryo proper (apical) and the terminally differentiated suspensor (basal). Expression and activation of metacaspase *mcII-pa* is essential for maintaining the proper balance between cell proliferation and PCD (Suarez *et al*., 2004; Bozhkov *et al*., 2005b). Suspensor cells do not divide but instead become committed to PCD as soon as they are formed. While cells in the upper layer of the suspensor (i.e. adjacent to the embryonal mass) are in the commitment phase of PCD, the cells in the lower layers exhibit a gradient of stages of autophagy-mediated cell dismantling towards the basal end of the suspensor where cell corpses are located. Recently the auxin transport inhibitor NPA was used for studying polar auxin transport in somatic embryogenesis of Norway spruce (Larsson *et al*., 2007). It has been found that polar auxin transport is essential for correct patterning of both apical and basal parts of the embryos throughout the whole developmental process. Furthermore, mature embryos showed aberrant phenotypes comparable with several auxin response and transport mutants in *Arabidopsis,* indicating that the role of polar auxin transport is conserved between angiosperms and gymnosperms. Differentiation of the outer cell layer in the embryonal mass during early embryogeny is regulated by *PaHB1* (*Picea abies* Homeobox1), whose encoded protein is highly similar to *ATML1* (for *Arabidopsis thaliana* meristem – L1 layer) (Lu *et al*., 1996). As in angiosperms, proper functioning of the outer cell layer in Norway spruce requires the specific expression pattern of a gene *Pa18,* encoding a lipid transfer-like protein (Sabala *et al*., 2000). The expression of *PaHB1* and *Pa18* switches from ubiquitous expression in PEMs to an outer cell layer-specific localization in early embryos.

During late embryogeny *PaHB2* participates in the maintenance of the radial patterning by specifying cell identity in the cortical cell layers (Ingouff *et al*., 2003). Three members of the *KNOX* (*KNOTTED1*-like homeobox) family (*HBK1*, *HBK2* and *HBK3*) are expressed at all stages during somatic embryo development in Norway spruce (Hjortswang *et al*., 2002). Based on its localization *HBK1* is suggested to be involved in regulation of the shoot apical meristem (Sundas-Larsson *et al*., 1998; Belmonte *et al*., 2005). *HBK2* is not expressed in embryo defective cell lines suggesting that expression of *HBK2* is necessary for correct embryonic patterning (Hjortswang *et al*., 2002). Furthermore, *HBK3* has recently been shown to be involved in transdifferentiation of PEMs into somatic embryos and in the formation of the embryonic shoot apical meristem (Belmonte *et al*., 2007). Over-expression of *HBK3* results in embryos having enlarged shoot apical meristems and up-regulated expression of *PgAGO* (*Picea glauca* Argonaute family of proteins). Transcripts of *PgAGO* are preferentially localized in cells in the shoot and root apical meristems from the early phases of embryo development in white spruce (Tahir *et al*., 2006). Suppression of *PgAGO* results in poorly organized shoot and root meristems leading to severe abnormalities during embryo development such as meristem abortion and growth cessation.

The maize *Vivipaurous 1* gene homolog (*Pavp1*) of Norway spruce is expressed during maturation of somatic embryos (Footitt *et al*., 2003). When the early cotyledonary stage is reached, the expression of *Pavp1* declines which is similar to what has been shown in angiosperms. It has recently been shown that loblolly pine and *Arabidopsis* express similar genes during embryogenesis. Comparative analyses using expressed sequence tags (ESTs) between *Arabidopsis* and loblolly pine reveal that a substantial number of genes are conserved (Cairney *et al*., 2006). Out of 108 *Arabidopsis* embryogenesis-related expressed genes, loblolly pine homologs were present for 83 of these genes. In addition several sequences identified from the loblolly pine embryogenic transcriptome were not found to have any known function in *Arabidopsis* and may contribute to the knowledge of gene expression during embryo development.

Programmed cell death

There are many ways for the cell to commit suicide. The genetically regulated PCDs are distinct from necrosis, which is a process of rapid pathological cell death induced by gross injury to the cell. PCD is a fundamental biological process, crucial for normal development, immunity and stress response in all eukaryotic organisms. Apoptosis and autophagic cell death are the two main types of PCD in animals.

The apoptotic route is hallmarked by the condensation of both chromatin and cytoplasm, followed by the breakdown of the cell into apoptotic bodies that are still surrounded by an intact membrane. The apoptotic cell finally disappears through caspase-mediated dismantling of cellular constituents and engulfment of cell remnants by macrophages (Clarke, 1990). In contrast to apoptosis, autophagic PCD does not involve cellular breakdown and macrophage-mediated phagocytosis of cell remnants, but relies on the cell's own degradation machinery that is based upon the formation of autophagosomes and their fusion with lysosomes and lytic vacuoles (Baehrecke, 2002). The core of the basic, conserved, machinery, driving

the apoptotic form of PCD is comprised of three proteins, Ced-9, Ced-4 and Ced-3, originally discovered in the worm, *Caenorhabditis elegans*, and then shown to have direct homologs – the Bcl-2 family proteins, Apaf-1, and the caspase family proteases, respectively, in *Drosophila* and mammals (Horvitz, 2003). Diverse forms of regulated cell-death processes have been identified in the plant and fungal kingdoms. Unlike animals, fungi and plants have rigid cell walls, lack macrophages, and do not possess structural homologues of the apoptotic core cell death machinery. These features explain why fungal and plant cells cannot die by apoptosis and instead use the autophagic cell death pathway (Bozhkov *et al*., 2005a; Bozhkov & Jansson, 2007). The mechanisms of PCD in fungi and plants, as well as those operating during autophagic PCD in animals are still poorly understood. In plants, PCD occurs for example during embryogenesis, seed development, aerenchyma formation, xylogenesis and leaf senescence (Pennell & Lamb, 1997). Furthermore, PCD contributes to plant defence against environmental stresses and pathogens (Greenberg & Yao, 2004; Lam, 2004).

Cell death proteases

Caspases are a family of cysteine-dependent aspartate-specific proteases involved in the initiation and execution of the apoptotic process in animals. Fourteen caspases have been identified in mammals (Wolf & Green, 1999). Caspases are constitutively expressed in cells as inactive pro-enzymes and can be processed to form active enzymes (Grütter, 2000). Activated effector caspases cleave key structural components of the cytoskeleton and the nucleus, as well as numerous proteins involved in signal transduction, gene expression, the cell-cycle and cell-death control (Earnshaw *et al*., 1999). Collectively, these proteolytic events disrupt survival pathways and induce those morphological changes that are characteristic for dying cells.

Metazoan caspases represent the phylogenetically youngest branch of a highly divergent superfamily of proteins that all share a catalytic diad of histidine and cysteine as well as a caspase-like secondary structure (Uren *et al*., 2000; Aravind & Koonin, 2002). Members of this superfamily that are not canonical caspases have been classified as metacaspases (typical for plants, fungi and protozoa) and paracaspases (found in *Dictyostelium* and metazoans). Paracaspases are unrelated to cell death control (Ruefli-Brasse *et al*., 2003; Roisin-Bouffay *et al*., 2004), while metacaspases are involved in PCD (Bozhkov *et al*., 2005b). Metacaspases have different substrate specificity than animal caspases, as they can only cleave peptides after Arg or, to a lesser extent, after Lys residues. Metacaspase *mcII-Pa* from Norway spruce is up-regulated in embryo-suspensor cells, where it translocates from the cytoplasm to the nucleus and binds to the nuclear pore complex and chromatin causing nuclear envelope disassembly and DNA fragmentation. Silencing of *mcII-Pa* results in suppression of PCD and early developmental arrest (Suarez *et al*., 2004). The cell-death function of mcII-Pa depends on its proteolytic activity, which in turn requires proteolytic processing of the mcII-Pa pro-enzyme. Accordingly, mutations in the catalytic cysteine of mcII-Pa abrogate its activation and block nuclear degradation (Bozhkov *et al*., 2005b).

Plants possess at least three types of aspartate-specific proteases, which are responsible for the increased caspase-like activities that are systematically observed during PCD (Bozhkov *et al.*, 2005a):

(i) Subtilisin-like serine proteases (called saspases after serine aspartate-specific proteases) which are triggered by the fungal toxin victorin in oat plants (Coffeen & Wolpert, 2004).

(ii) Vacuolar processing enzymes (VPEs) are phylogenetically-conserved proteases from the legumain family, with a high preference for the caspase-1 substrate YVAD (Tyr-Val-Ala-Asp). Active VPEs reside in the lytic vacuoles (Hatsugai *et al*., 2004; Hara-Nishimura *et al.*, 2005)

(iii) Caspase-like proteases involved in PCD in plants and fungi having strong preference for the mammalian caspase-6 substrate VEID (Val-Glu-Ile-Asp) (Paper II; Madeo *et al*., 2002). Recently, Boren *et al*. (2006) have established an autophagosomal localization of the VEIDase activity at the early stages of PCD in barley (*Hordeum vulgare*) endosperm.

Based on present knowledge it is believed that VEIDase enzyme(s) stand downstream to metacaspases in the proteolytic cell degradation pathway (Bozhkov & Jansson, 2007).

Research objectives

This project has focused on (i) how PCD regulates somatic embryo development in Norway spruce and (ii) genetic stability during somatic embryogenesis in Norway spruce and Scots pine.

Specific aims were to investigate:

- whether PCD correlates with nuclear microsatellite (SSR) instability during early stages of somatic embryogenesis, especially during PEM to somatic embryo transition;
- if caspase-like activity is involved in PCD during embryo pattern formation;
- how intracellular free zinc affects PCD and embryo development;
- stability of nuclear microsatellites during somatic embryogenesis in Scots pine;
- genetic stability in somatic embryo plants of Norway spruce.

Results and Discussion

Programmed cell death during somatic embryogenesis in Norway spruce

Genetic stability during early stages of somatic embryogenesis (Paper I)

During abortion of mammalian embryos, abnormal embryos are selectively eliminated by PCD (Hardy, 1999; Qumsiyeh *et al*., 2000; Hardy *et al*., 2001). Although the roles of embryo abortion in survival and evolution strategies of plant species are well recognized (e.g., Moller, 1996), little is known about the mechanisms of plant embryo abortion. By using Scots pine natural polyembryony as a model system, it was demonstrated that autophagic PCD eliminates all but one monozygotic embryo in a seed (Filonova *et al*., 2002). Any genetic defects in the aborted Scots pine embryos were not analysed. However Martínéz-Gomez & Gradziel (2003) have reported chromosomal aberrations in abnormal monozygotic embryos of almond (*Prunus dulcis*). In Norway spruce differentiation of somatic embryos from PEMs involves elimination of PEMs by PCD (Bozhkov *et al*., 2002, Filonova *et al*., 2000b). We hypothesized that activation of PCD in PEMs following withdrawal of PGRs might be caused by genetic aberrations in PEMs.

In mammalian studies, microsatellite instability has been suggested as a reliable marker for embryo abortion (Kiaris *et al*., 1996; Spandidos *et al*., 1998). Although the exact mechanism responsible for microsatellite instability remains unknown, it has been established that it reflects an elevated mutational rate and is the result of an inaccuracy in DNA replication and repair caused by strand misalignment (Schlotterer & Tautz, 1992). In this work we used three nuclear SSR microsatellite markers, SpAGC1, SpAGC2 and SpAGG3 developed by Pfeiffer *et al*., (1997) to determine the genetic integrity of embryogenic cultures. These microsatellites had previously been used for analysing genetic stability during somatic embryogenesis

(Harvengt *et al*., 2001; Burg *et al*., 1999) as well as in population genetics studies (Geburek *et al*., 1998; Yazdani *et al*., 2003).

Two embryogenic cell lines from the same full-sib family were analysed. The cell lines were treated according to two protocols, one of which included a pre-treatment in medium lacking PGRs, which stimulates embryo differentiation and PCD. In the second protocol the PCD stimulating treatment was excluded (Figure 3). Samples were collected at four stages during somatic embryogenesis for the pre-treatment containing protocol and at three stages for the protocol lacking pre-treatment. The chosen successive stages during embryogenesis were: PEM proliferation, early embryo differentiation, late embryogeny (2 weeks with ABA) and cotyledonary embryos. Needles from the parental trees were used as reference.

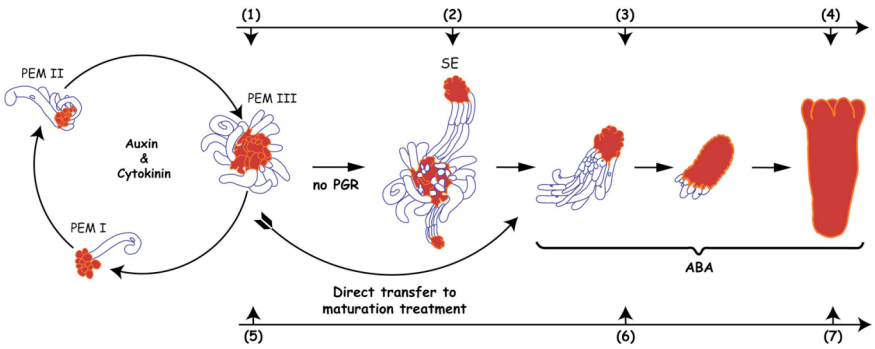


Figure 3. Experimental design when analysing genetic stability during somatic embryogenesis. The upper part shows the standard protocol, where samples were collected during PEM proliferation in PGR containing medium (1); early embryo differentiation one week after withdrawal of PGRs (2); late embryogeny after two weeks on ABA containing medium (3) and cotyledonary embryos after seven weeks of ABA treatment (4). The lower part shows the modified protocol, where the step in PGR-free medium was omitted. Samples were collected as for the standard protocol (5), (6) and (7). In total 104 samples were collected at seven sampling occasions.

The one week pre-treatment in PGR-free medium increased the number of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) positive nuclei and gave significantly higher yield of both early somatic embryos and cotyledonary embryos than the cultures transferred directly from PGR-containing proliferation medium to maturation medium with ABA (Table 1). Surprisingly neither of the two cell lines demonstrated mutations in any of the three SSR regions at any of the successive stages of somatic embryogenesis.

Table 1. *Effects of pre-treatment on PCD and embryogenesis. Embryogenic cell suspension cultures (cell line 95:88:22) were given a one-week pre-treatment with (+PGRs) or without (-PGRs) before being plated on maturation medium containing ABA. The data presented show the frequency of TUNEL positive nuclei as well as the frequency of early somatic embryos after one week pre-treatment in media containing or lacking PGRs. After seven weeks of maturation treatment the number of cotyledonary somatic embryos (SE) formed per gram fresh weight was calculated. The data show mean (*± SEM)

	TUNEL %	Early SE %	Number of cotyledonary SE per gram fresh weight
+PGR	5 (± 0.9)	9 (± 4.3)	2 (± 0.5)
-PGR	28 (± 4.3)	38 (± 5.3)	8 (± 1.8)

This paper confirms previous results (Bozhkov *et al*., 2002) that PCD stimulates embryo formation and supports the reports of high genetic stability of clonal Norway spruce material (Harvengt *et al*., 2001). However we cannot exclude that mutations occur at a low rate (lower than 1 out of 1346 SSRs). Neither can we exclude that a small fraction of mutated PEM cells could have escaped detection owing to the high number of cells in each sample used for DNA isolation. Therefore complementary studies using single-cell PCR techniques have to be used to test our hypothesis that genetically aberrant PEMs exist and are eliminated by PCD.

Caspase-like activity during embryogenesis (Paper II)

Activation of the caspase family of proteases is paramount for control of PCD in animals. Caspases have a unique strong preference for cleavage of the target proteins next to the asparagine residue. In this work we have used synthetic peptide substrates containing caspase recognition sites and corresponding specific inhibitors to analyse the role of caspase-like activity in the regulation of PCD during plant embryogenesis.

First, we measured the proteolytic activity of a large number of cell extracts (>50) prepared at different stages of Norway spruce somatic embryogenesis against six peptide substrates specific for different members of mammalian caspases. We observed constant relative rates for proteolytic activities against all six peptide substrates in all tested extracts. Although the absolute cleavage rates for individual substrates varied significantly depending both on *in vitro* assay conditions and on the stage of embryogenesis, VEID-AMC was found to be the most efficiently cleaved substrate. Next in order of cleavage rate to VEID-AMC was IETD-AMC (≈ 44% of the VEID-AMC cleavage rate) followed by four other peptides whose relative cleavage rates were 7 to 20 times lower than that of VEID-AMC. The similar pattern of relative cleavage rates of synthetic peptides, with the strongest preference for Val in P4 position (as in VEID) and tolerance for Ile in the same position (as in IETD), is a distinguishing characteristic of mammalian caspase-6 (Talanian *et al*., 1997).

In general, caspase activities cannot be inhibited by protease inhibitors other than caspase-specific ones. Accordingly, we observed no inhibitory effect of a range of serine, cysteine, aspartic acid and proteosomal protease inhibitors on the cleavage of VEID-AMC by cell extracts of Norway spruce. In contrast, caspase-6 substrate-mimetic inhibitor Ac-VEID-CHO almost completely inhibited this activity at the

concentration of 10 μM. These results suggest that embryogenic Norway spruce cells contain a single protease, or a group of related proteases, with the substrate preference and inhibitor specificity similar to those of mammalian caspase-6. Considering the large number of cell extracts analysed, it is also evident that there are no caspase-like proteases other than VEIDase activated during embryogenesis in Norway spruce.

In the comparative study of biochemical characteristics of caspases-3, -6, -7, and -8, Stennicke & Salvesen (1997) observed high sensitivity of catalytic activity of purified mammalian caspase-6 towards changes in pH, ionic strength and Zn^{2+} concentration. We therefore examined whether Norway spruce VEIDase has a similarly high sensitivity to the same factors. The biochemical assays showed that plant VEIDase activity resembles that of mammalian caspase-6, as it exhibited high sensitivity to pH (optimum at pH 7.0) and can be readily inhibited by salt and micromolar concentrations of ionic zinc.

To examine the role of VEIDase activity during embryo development and associated PCD we first analysed the kinetics of this activity over the whole developmental pathway. Samples were taken at three important stages of embryogenesis: PEM proliferation (3 and 7d, +PGR), early embryo differentiation (1, 4 and 7d, -PGR) and embryo maturation (7 and 35d, ABA) and analysed for VEID-AMC cleavage (for stages see Figure 2). The activity increased about three-fold under depletion (PEM proliferation) and subsequent withdrawal of PGRs (early embryo differentiation), with the maximal activity observed during early embryogeny at the time of active elongation of the embryo-suspensor (four days after withdrawal of PGRs). Thereafter the activity began to decline and was barely detectable in embryos matured on ABA for 35 days, which coincide with the developmental stage where embryo shaping is no longer required. Secondly, to verify that the VEIDase activity was located specifically in those embryonic cells that undergo PCD, we stained living embryos by the cell permeable fluorogenic substrate containing the –VEIDN– sequence for fluorescent microscopy analysis. This substrate is composed of two fluorophores covalently linked to a caspase-6-specific peptide. In the uncleaved substrate, fluorescence is quenched owing to the formation of intramolecular excitonic dimers. Upon cleavage of the peptide, the fluorophore–fluorophore interaction is abolished, leading to an increase in fluorescence. The strongest florescence signal was detected in the embryonal tube-cells and in the first layer of suspensor cells, whereas the embryonal mass and the distal end of the suspensor revealed a lack of VEIDase activity. This demonstrates that VEIDase activity is localized specifically in the cells during both the commitment (embryonal tube cells) and in the beginning of the execution phase of PCD (proximal suspensor cells).

The localization of VEIDase in cells destined for PCD suggested that the activity might be critical for correct embryonic pattern formation. Therefore, we tested if inhibition of this activity could affect embryonic pattern formation and associated PCD *in vivo*. In these experiments, two different cell permeable caspase inhibitors (pan-caspase inhibitor, zVAD-fluoromethyl ketone (fmk), and caspase-6-specific inhibitor, zVEID-fmk) were compared with both a control (DMSO) and zLEHD-fmk (caspase-9-specific inhibitor) for the effect on embryogenesis and associated PCD. The number of TUNEL positive nuclei in the whole culture was significantly

($P < 0.01$) reduced by zVAD-fmk and zVEID-fmk at concentrations of 2 μM and 200 nM, respectively, whereas the treatment with zLEHD-fmk at higher concentrations (20 μM) had no effect. There was a clear change in pattern formation of spruce embryos when using either zVAD-fmk or zVEID-fmk. The effect correlated well with the concentration of added inhibitor: (i) at 2 μM the embryonal masses were unable to separate from each other and proliferated in clumps having short suspensors and showed no signs of cell death revealed by *in vivo* VEIDase staining and TUNEL, (ii) when using 20 μM the differentiation of embryo suspensors was completely blocked.

These results demonstrate that the VEIDase activity is an essential part of the developmental cell death program, and that this activity is necessary for correct embryo pattern formation.

Availability of free ionic zinc during embryogenesis (Paper III)

In this paper we have studied how intracellular free zinc affects and controls the balance between cell survival and PCD during plant embryo development using Norway spruce somatic embryogenesis as a model system. We have shown in the previous paper (Paper II) that Norway spruce cultures have proteolytic activity similar to mammalian caspase-6. In animal cells zinc inhibits apoptotic cell death by inactivation of several members of the caspase family proteases (mainly caspases-3, -6 and -9) (Stennickle & Salvesen, 1997; Truong-Tran *et al*., 2000). Accordingly, cells supplemented with exogenous zinc experience lower susceptibility to apoptotic stimuli, establishing zinc as an important physiological suppressor of cell death in animals. The metabolic networks for proper zinc transport and homeostasis in plants are beginning to be understood (Grotz & Guerinot, 2006). However, to our knowledge, no reports to date have shown the involvement of zinc in the control of plant PCD.

First, we determined the localization of labile pools of intracellular free zinc in early zygotic and somatic embryos of Norway spruce by fluorescent microscopy using two membrane-permeant zinc-specific fluorophores, Zinquin (Zinquin-etyl-ester) (Zalewski *et al*., 1993) and Dansylaminoethyl-cyclen (Koike *et al*., 1996). Both fluorescent probes gave uniform fluorescence from the living cells of the embryonal mass and showed an abrupt decrease in the suspensor cells. This decrease of fluorescence was seen already in the embryonal tube cells that are in the commitment phase of PCD and have large part of the cell contents preserved (Smertenko *et al*., 2003; Bozhkov *et al*., 2005a), suggesting that zinc deprivation occurs very early in the PCD pathway. These data show that distribution of free intracellular zinc correlates with cell pattern development in plant embryos. Free zinc is high in the living cells of the embryonal mass and is low in the suspensor that is composed of cells committed to or executing PCD.

In animal cells, addition of the cell permeable zinc chelator TPEN was shown to have a strong pro-apoptotic effect on diverse cell types (e.g. McCabe *et al*., 1993; Zalewski *et al*., 1993; Ahn *et al*., 1998; Meerarani *et al*., 2000; Chimienti *et al*., 2001). To assess the effect of zinc depletion on cell death in our model system we compared the frequency of cells with nuclear DNA fragmentation using TUNEL-assay. Zinc-deprived embryos had increased frequency of TUNEL-positive cells

compared to control embryos. In the control embryos TUNEL-positive cells were mainly located in the suspensors, while after TPEN treatment TUNEL-positive cells were also detected in the embryonal masses. To quantify the effect of TPEN we estimated the frequency of embryos containing two or more TUNEL-positive cells in the embryonal masses. The frequency increased from 12% in the control embryos to 42% and 60% in the embryos treated with 1 μM and 5 μM TPEN, respectively. These results demonstrate that zinc deprivation induces ectopic cell death, which affects the apical domains of the embryos. Activation of ectopic cell death was confirmed by analysis of the plasma membrane integrity using Evans blue, the vital dye excluded by living cells but penetrating through disrupted plasma membranes into dead and dying cells (Bozhkov *et al*., 2002). TPEN-treated embryos showed strong Evans blue staining in the embryonal masses and the suspensor cells, in contrast to the control embryos where only the suspensors were stained. The zinc depletion-induced cell death in the embryonal masses had a lethal effect, as no cotyledonary embryos were regenerated from the zinc chelated cultures. Altogether, these data suggest that free intracellular zinc protects the embryonal mass, enabling embryo survival and correct pattern formation.

Terminal differentiation and PCD are two interrelated processes underlying suspensor cell identity during plant embryogenesis (Bozhkov *et al*., 2005a). Genetic or pharmacological dysregulation of this identity causes developmental aberrations often leading to embryo lethality (Vernon & Meinke, 1994; Rojo *et al*., 2001; Smertenko *et al*., 2003; Lukowitz *et al*., 2004; Suarez *et al*., 2004). Abrupt decline of zinc content detected by specific fluorophores in suspensor cells might indicate that zinc deprivation in the basal domain is important for establishing suspensor cell identity and embryonic patterning. In order to investigate this possibility, we attempted to counteract the naturally occurring decline of zinc in the basal domain by growing embryos with increased concentrations of zinc sulphate. Zinc sulphate was added continuously starting at early embryo differentiation until embryo maturation (Figure 2). The early embryos developing in PGR-free medium supplemented with 100 μM or 300 μM zinc had a significantly lower proportion of TUNEL-positive cells than the control embryos growing with 5 μM zinc. However we could not detect any obvious morphological alterations caused by zinc supplementation over the 7-day period of early embryo development.

We next investigated whether the zinc-mediated suppression of embryonic PCD had a developmental effect on the transition from early to late embryogeny. For this, we analysed the frequency distribution of all embryos in the control and extra zinc-supplemented cultures nine days after addition of ABA into four typical phenotypes based on their developmental stage and apical-basal patterning: (i) few-celled embryos with polarized morphology composed of fewer than 16 cells (ii) morphologically normal early somatic embryos with well defined rounded embryonal masses and massive suspensors (iii) embryos showing no exact border between embryonal mass and suspensor region exhibiting elongated embryonal masses and (iv) late embryos without suspensors or possessing remnants of suspensors. The aberrant phenotype (iii) was caused by equal instead of asymmetric cell divisions in the basal part of the embryonal masses, so that the suspensor cell specification was suppressed. The aberrant and the few-celled embryos were rarely observed in control cultures (less than 3 and 1%,

respectively) which were mainly represented by morphologically normal early embryos (47%) and late embryos without suspensors (49%). Addition of 100 and 300 μM zinc-sulphate increased the frequency of aberrant embryos to 34% and 40% respectively; concomitantly the fraction of late embryos without suspensors decreased to 13 % and 6%, respectively. As a result, the number of cotyledonary somatic embryos formed after 10 weeks was 2.3- and 5.4-fold lower in 100 and 300 μM zinc-supplemented cultures, respectively, indicating that abnormal early embryos are arrested at the transition to late embryogeny. Localization of zinc in the aberrant embryos, using Zinquin, showed that free zinc accumulated both in the embryonal masses and suspensors of the embryos. These data suggest that zinc supplementation greatly enhances cell survival, which can impair embryonic pattern formation.

Zinc was previously shown to inhibit mcII-Pa (Bozhkov *et al*., 2005b) and VEIDase activity *in vitro* (Paper II). Both protease activities were shown to be essential for establishing correct apical-basal pattern in Norway spruce and were implicated in the regulation of PCD. In the present study, we asked whether zinc-mediated regulation of cell death in the embryos correlates with the level of metacaspase activity. For measuring metacaspase activity in cell lysates we used the fluorogenic substrate Boc-EGR-AMC, which has previously been shown to be one of the preferred substrates for mcII-Pa both *in vitro* and *in vivo* (Bozhkov *et al*., 2005b). Zinc supplementation or chelation treatments started simultaneously with withdrawal of PGRs. We measured the activity of cell lysates prepared from control cultures and cultures supplemented with either TPEN or zinc sulphate and sampled at 0, 24, 48, 96 and 168 hours after withdrawal of PGRs. The control cultures exhibited normal developmental dynamics and consequently showed an increase in the metacaspase-like activity between 96 and 168 hours, coinciding with the period of apical-basal pattern formation of the early embryo (Filonova *et al*., 2000b). Both the 100 μM and the 300 μM zinc-sulphate supplemented cultures showed a reduction in mcII-Pa activity after 24 hours. In consistence with this observation, chelation of free intracellular zinc by TPEN led to a pronounced increase in the metacaspase-like activity over the initial period of 48 hours followed by a decrease to below control level. Evans blue staining confirmed that all cells were already dead at 168 hours, so the loss of metacaspase activity at the end of TPEN treatment was not unexpected. Taken together our results indicate that metacaspases can be one of the targets of zinc-inhibited PCD.

Inactivation of several members of caspase family proteases (primarily caspases 3, 6 and 9) by zinc is thought to be the main process underlying the anti-apoptotic effect of zinc in mammalian cells (for review see Truong-Tran *et al*., 2001). Although the exact biochemical mechanisms of caspase inhibition by zinc are unknown, it has been demonstrated that zinc blocks the process of pro-caspase-3 activation rather than the already activated enzyme (Truong-Tran *et al*., 2000). It is proposed that zinc binds reversibly to the thiol group of catalytic Cys^{163}, inhibiting activation of the proenzyme and protecting the essential residue from oxidation (Maret *et al*., 1999; Truong-Tran *et al*., 2001). Despite metacaspases and caspases display low sequence similarity and have distinct substrate specificities, they have a conserved catalytic diad of histidine and cysteine and both require cysteine-dependent processing of proenzyme for enzyme activation (Vercammen *et al*., 2004; Bozhkov *et al*., 2005b). Here we show that, like activity of mammalian

caspases, plant metacaspase activity is suppressed by zinc *in vivo*, suggesting that metacaspases could be one of the targets for zinc during zinc-mediated regulation of plant PCD. However, it remains to be investigated whether zinc binds to a critical cysteine residue of metacaspases to keep the enzyme in an unprocessed inactive form and/or to inactivate already active enzyme.

Variation in nuclear microsatellite stability

Developmental and genetic variation in nuclear microsatellite stability during somatic embryogenesis in Scots pine (Paper IV)

In vitro culture conditions appear to affect the stability of the plant genomes with various plant species and genotypes responding differently under various conditions (Larkin & Scowcroft, 1981; Galiba *et al*., 1985; Armstrong & Phillips, 1988; Linacero & Vazquez, 1993; Saieed *et al*., 1994; Tremblay *et al*., 1999; Jain, 2001; Etienne & Bertrand, 2003). In this work, the following questions have been raised. (i) Does the genetic instability induced by *in vitro* stress vary among families? (ii) Does the genetic instability influence the adaptation to *in vitro* conditions? (iii) Does the genetic instability affect the competence for plant regeneration? To address these questions, somatic embryogenesis of Scots pine was used as a biological system.

Embryogenic cultures of Scots pine were established from immature zygotic embryos collected from half-sib families. The time span for isolation of ovules was designated one, two and three weeks after fertilization owing to the narrow period of *in vitro* embryonic competence in Scots pine (Keinonen-Mettälä *et al*., 1996). The whole ovules were cultured on proliferation medium containing 9.0 μM 2,4-D and 4.4 μM benzyladenine (BA). The initiation frequency was on average low and varied from 0.2 to 11 percent depending on seed family and harvest time. An embryogenic cell line was regarded as established when the multiplication rate was at least two-fold during the three-week subculture interval, which took about six months. After establishment the cell lines were proliferated for four months before they were cryopreserved. After thawing, the cell lines were proliferated for another three months prior to maturation treatment on medium containing ABA (60 μM). After 6 months individual cotyledonary somatic embryos were formed.

The genetic stability of four hypervariable nuclear microsatellite regions SPAC11.4, SPAC11.6, SPAG11.8, and SPAC12.5 (Soranzo *et al*., 1998) was analysed in 268 newly established embryogenic cell lines representing 10 families, in 48 proliferating cell lines representing 4 families three months after thawing and in 85 cotyledonary somatic embryos from two families. As control, the zygotic counterparts from the selected 10 families were isolated from at least 45 seeds for each family. Since we used embryos from half-sib families we were only able to determine the genetic stability of the maternal alleles during establishment of embryogenic cultures and in zygotic embryos.

The instability of the analysed alleles in zygotic embryos was highest in the SPAC11.6 locus (9%), while the SPAG11.8 and SPAC12.5 yielded fewer mutations, 3.5% and 1% respectively, and no mutations were found in the SPAC11.4 locus. The frequency of perfect genotypes (FPG %) was on average

84% for the zygotic embryos. However, the FPG value varied significantly, from 66% to 98%, among families in the zygotic embryos resulting in lower stability than expected.

A high mutation rate in microsatellites was observed during establishment of embryogenic cultures. On average 40% of the newly established cell lines carried a mutated allele. The four loci showed various mutation frequencies. Loci SPAC11.4 and SPAC12.5 yielded 2% and 6% mutated maternal alleles respectively, while the loci SPAC11.6 and SPAG11.8 yielded more than 12% mutated maternal alleles. However, there was a significant difference in mutation frequency between families. The FPG varied from 40% to 100% in different families.

Based on the FPG value for newly established cell lines, the 10 families were divided into two groups. Families with FPG values higher than 70% were classified as stable while families with lower FGP were classified as unstable. One genetically stable and one unstable family were selected from each category and from each family both stable and unstable cell lines were selected (in total 48 cell lines). Of the 48 cell lines analysed, 13 showed altered genotypes during proliferation compared with after establishment. Twenty-six percent of the cell lines with perfect genotype after establishment showed new alleles during proliferation while 30% of the cell lines with altered genotypes after establishment showed further mutations during proliferation. In order to confirm that new mutations occurred in the two groups during proliferation we analysed different sectors in proliferating embryogenic cultures. The analysis showed that embryogenic cultures of Scots pine contain both mutated and non-mutated cells. Phenotypic or genotypic alterations after prolonged proliferation *in vitro* have also been shown in several other species including conifers (De Verno *et al*., 1994, 1999; Isabel *et al*., 1996; Fourré *et al*., 1997).

One family classified as stable and one as unstable after establishment from each of the two groups was selected for appearance of mutated nuclear SSR loci in mature cotyledonary somatic embryos. In the unstable family, 38% of the analysed embryos had perfect genotypes, which corresponds well with the 45% FPG observed in the established cell lines. Of the cotyledonary embryos analysed for the stable family, 83% had a perfect genotype, which coincides with the FPG value (82%) found during establishment of embryogenic cultures.

Interestingly, we found that the embryogenic potential of the families significantly correlated with the genetic instability, less stable families showing higher embryogenic potential. In contrast, maturation of somatic embryos was higher in genetically stable families. Our results suggest that the possibility to establish embryogenic cultures is higher in genetically unstable families, but also that the possibility to regenerate somatic embryos is higher for genetically stable families. This suggests that severely mutated cell lines lose the capacity to differentiate cotyledonary somatic embryos. A high genetic instability was also found in zygotic embryos. It is important to stress that no correlation was found between the genetic stability of the families observed in zygotic embryos versus those in established cell lines. Consequently, it is not possible to predict the genetic stability of embryogenic cell lines established from different families by

analysing the inherent stability of zygotic embryos. The genetic instability induced by in *vitro stress* varies among families. An interesting question is therefore if the genetic instability of a family reflects the plasticity of the family?

Genotypic variation of somatic embryo plants in Norway spruce (Paper V)

Plant regeneration through somatic embryogenesis is currently possible for several coniferous species. One very important aspect is to determine the genetic fidelity of somatic embryo plants relative to comparable seedlings. Several field tests have been established for plants derived from somatic embryos of several coniferous species (Sutton, 2002; Högberg *et al*., 2003). These trials hold great promise for clonal propagation and integration of somatic embryogenesis into breeding programs. However, before this method can be accepted it is important to show that the plants are "true to type". In previous reports of genetic stability in plants derived from somatic embryos, relatively few genotypes have been analysed. In Paper IV we found that the genetic stability varies significantly among families of Scots pine. If this is true, a larger number of genotypes in Norway spruce has to be analysed.

Somatic embryo plants were regenerated from embryogenic cultures, derived from full-sib families, using the protocol previously described (Högberg *et al*., 1998). We analysed the stability of 314 Norway spruce somatic embryo plants originating from six families representing 38 clones, with 2 to 11 plants per clone. The somatic embryo plants were between 2 and 7 years old. As reference material, needles from the parental trees and 208 open pollinated seedlings from 4 of the 6 families were analysed. The material was analysed for genetic stability in four hypervariable nuclear microsatellite regions SpAGC1, SpAGG3, SpAC1H8 and SpAC1F7 (Pfeiffer *et al*., 1997).

In order to establish if the procedure of somatic embryogenesis favours accumulation of specific alleles, we compared the frequency of different alleles within each locus in all clones, excluding mutated alleles, with the allele frequency in the parental trees. No significant deviations from the parental distribution could be found. This indicates that allelic diversity is not affected (when propagating Norway spruce using somatic embryos) by somatic embryogenesis in Norway spruce.

Altered alleles were observed in both seedlings and somatic embryo plants. Somatic embryo plants derived from six of the 38 clones tested carried mutated microsatellites. In four of the six clones all plants within the clone had identical mutations, while the two remaining clones only showed altered microsatellite pattern in a few somatic embryo plants. The six clones carrying mutated microsatellites were derived from four of the six families included in this study. The number of plants with altered alleles was high enough to establish statistical significance for the effect of family in the full-sib families (somatic embryo plants) but was too low for estimating any family effect in the corresponding half-sib families (seedlings).

In order to elucidate if somatic embryo plants derived from clones carrying mutated microsatellite alleles showed any change in growth capacity we compared

growth of somatic embryo plants from mutated and non-mutated clones in two of the six analysed families. The growth characteristics for the plants in the mutated clones did not differ from the other plants in that clone except for one clone that showed significantly higher relative growth. Neither aberrant nor reduced growth was therefore observed in clones carrying mutations in microsatellite regions.

A much higher mutation rate was detected in somatic embryo plants than in seedlings. Since all somatic embryo plants in some clones carried the same mutation we cannot rule out that some or all of the mutations had occurred already in the zygotic embryo. However, in Scots pine we showed that families showing a low genetic stability during establishment of embryogenic cultures had a higher embryogenic potential than those that were genetically stable (Paper IV). As a consequence there is a risk that genetically unstable families are overrepresented in embryogenic cell lines. Although maturation was suppressed in genetically unstable families of Scots pine it was still possible to regenerate plants. We assume that the higher mutation frequency in clones than in seedlings of Norway spruce might at least partially be explained by the fact that selection for unstable cell lines was made already during establishment of embryogenic cell lines. However, in two clones we observed both mutated and non-mutated plants, indicating that a few mutations were also induced during the proliferation phase and/or during the maturation phase. In order to avoid these types of mutations it is important to use optimized protocols.

Conclusions

This thesis describes the progress towards better understanding of plant embryogenesis. Norway spruce somatic embryogenesis was used as a model to generate information about how PCD regulates embryo development. The genetic stability during embryogenesis in Norway spruce and Scots pine was also studied. The main conclusions from the results presented in this thesis are as follows:

Programmed cell death (Paper I, II and III)

- We confirm that PCD is required for embryo differentiation and eliminates suspensor cells during embryo development. However, our hypothesis that genetically aberrant PEMs or embryos are eliminated by PCD could not be proved using our experimental design. Techniques that allow higher resolution than bulk-sampled cells like singe cell PCR are required.
- We identified VEIDase as the main caspase-like activity during somatic embryogenesis in Norway spruce. Inhibition of VEIDase activity *in vivo* disrupted embryonic pattern formation. Biochemical characteristics of VEIDase activity resemble those of mammalian caspase-6, including high sensitivity to zinc *in vitro*.
- We showed that an increased level of intracellular free zinc inhibits PCD and disrupts the apical-basal patterning during early embryogenesis. Decreased level of zinc induces ectopic cell death in the embryonal masses.

Genetic stability (Paper IV and V)

- The mutability of microsatellites varies among families of Scots pine both in zygotic and somatic embryos. Embryogenic potential is higher in more unstable families. In contrast, regeneration of mature cotyledonary embryos is higher in stable families.
- Plants regenerated from somatic embryos of Norway spruce grow normally regardless of the presence of mutated microsatellites.

Future perspectives

Vegetative propagation by somatic embryogenesis of a few economically important conifer species has today reached a stage of commercial application. Somatic embryo plants are mainly used for clonal testing and selection of superior genotypes but also for large-scale re-forestation purposes. Several million somatic embryo plants being adapted to field conditions each year. The key to commercial applications has been the common effort of many scientists to optimize the protocols. However, many processes during embryogenesis in plants are still not fully understood and need further investigation. I suggest some possible areas of research in conifer embryology below:

- In-depth understanding of how PCD regulates plant embryogenesis will be useful both for improving protocols and for insights into evolutionary aspects of PCD.
    - VEIDase caspase-like activity was not caused by the metacaspase mcII-Pa (Bozhkov *et al.*, 2005b). Further work is needed to purify VEIDase enzyme and to investigate its interactions with mcII-Pa.
    - How does intracellular free zinc inhibit cell death proteases *in vivo?* Do auxin and zinc have any co-regulation on embryo patterning? Intracellular transport and storage mechanisms for zinc are also areas requiring further study.
- Can microsatellite instability be linked to phenotype and plant quality?
- Improved methods to propagate pine using somatic embryogenesis:
    - Is there an evolutionary benefit of polyembryo? Can this lead to increased fitness of the species?
    - Filonova *et al*., 2002 showed that subordinate embryos are aborted by PCD but the molecular identity of death trigger remains to be identified?

References

Ahn, Y.H., Kim, Y.H., Hong, S.H. & Koh, J.Y. 1998. Depletion of intracellular zinc induces protein synthesis-dependent neuronal apoptosis in mouse cortical culture. *Experimental Neurology* 154, 47-56.

Aida, M., Ishida, T. & Tasaka, M. 1999. Shoot apical meristem and cotyledon formation during *Arabidopsis* embryogenesis: interaction among the *CUP-SHAPED COTYLEDON* and *SHOOT MERISTEMLESS* genes. *Development* 126, 1563-1570.

Aida, M., Vernoux, T., Furutani, M., Traas, J., & Tasaka, M. 2002. Roles of PIN-*FORMED1* and *MONOPTEROS* in pattern formation of the apical region of the *Arabidopsis* embryo. *Development* 129, 3965-3974.

Aravind, L. & Koonin, E.V. 2002. Classification of the caspase-hemoglobinase fold: detection of new families and implications for the origin of the eukaryotic separins. *Proteins* 1;46(4), 355-67.

Armstrong, C.L. & Phillips, R.L. 1988. Genetic and cytogenetic variation in plants regenerated from organogenic and friable embryogenic tissue cultures of maize. *Crop Science* 28, 363 – 369.

Attree, S. & Fowke, L.C. 1993. Embryogeny of gymnosperms; advances in synthetic seed technology of conifers. *Plant Cell Tissue and Culture* 35, 1-35.

Baehrecke, E. H. 2002. How death shapes life during development. *Nature Reviews: Molecular cell Biology* 3, 779-787.

Beck, C.B. 1988. Origin and Evolution of Gymnosperms. *Columbia University Press, New York*.

Becwar, M.R., Nagmani, R. & Wann, S.R. 1990. Initiation of embryogenic cultures and somatic embryo development in loblolly pine (*Pinus taeda*). *Canadian Journal of Forest Research* 20, 810-817.

Belmonte, M.F., Donald, G., Reid, D.M., Yeung, E.C. & Stasolla, C. 2005. Alterations of the glutathione redox state improve apical meristem structure and somatic embryo quality in white spruce (*Picea glauca*). *Journal of Experimental Botany* 56, 2355-2364.

Belmonte, M.F., Tahir, M., Schroeder, D. & Stasolla, C. 2007. Overexpression of *HBK3*, a class I KNOX homeobox gene, improves the development of Norway spruce (*Picea abies*) somatic embryos. *Journal of Experimental Botany* 58 (11), 2851-2861.

Berleth, T. & Jürgens, G. 1993. The role of the monopteros gene in organising the basal body region of the *Arabidopsis* embryo. *Development* 118, 575-587.

Berleth, T. 1998. Experimental approaches to *Arabidopsis* embryogenesis. *Plant Physiology and Biochemistry* 36, 69-82.

Berleth, T. & Chatfield, B. 2002. Embryogenesis: Pattern formation from a single cell. In: Somerville CR, Meyerowitz EM (eds) *The Arabidopsis Book*. American Society of Plant Biologists, Rockville, MD.

Blouin, M.S., Parsons, M., Lacaille, V. & Lotz, S. 1996. Use of microsatellite loci to classify individuals by relatedness. *Molecular Ecology* 5, 393 - 401.

Boren, M., Höglund, A.S., Bozhkov, P. & Jansson, C. 2006. Developmental regulation of a VEIDase caspase-like proteolytic activity in barley caryopsis. *Journal of Experimental Botany* 57(14), 3747-53.

Bozhkov, P.V., Ahn, I.S. & Park, Y.G. 1997. Two alternative pathways of somatic embryo origin from polyembryonic mature stored seeds of *Pinus koraiensis* Sieb et Zucc. *Canadian Journal Botany* 75, 509–512.

Bozhkov, P.V. & von Arnold, S. 1998. Polyethylene glycol promotes maturation but inhibits further development of *Picea abies* somatic embryos. *Physiologia Plantarum* 104, 211-224.

Bozhkov, P.V., Filonova, L.H. & von Arnold, S. 2002. A key developmental switch during Norway spruce somatic embryogenesis is induced by withdrawal of growth regulators and associated with cell death and extracellular acidification. *Biotechnology and Bioengineering* Vol. 77. 6, 658-667.

Bozhkov, P.V., Filonova, L.H. & Suarez, M.F. 2005a. Programmed cell death in plant embryogenesis. *Current Topics in Developmental Biology*. 2005;67:135-79.

Bozhkov, P.V., Suarez, M.F., Filonova, L.H., Daniel, G., Zamyatnin, A.A.Jr., Rodriguez-Nieto, S., Zhivotovsky, B. & Smertenko, A. 2005b. Cysteine protease mcII-Pa executes programmed cell death during plant embryogenesis. *Proceedings of the National Academy of Sciences of the United States of America*. Vol 102, 40, 14463-14468.

Bozhkov, P. & Jansson, C. 2007. Autophagy and cell-death proteases in plants: Two wheels of a funeral cart. *Autophagy* 3, 136-138.

Brettell, R.I.S., Dennis, E.S., Scrowcroft W.R. &. Peacock, W.J. 1986. Molecular analysis of a somaclonal mutant of maize alcohol dehydrogenase. *Molecular and General Genetics* 202, 235-239.

Brettell, R.I.S. & Dennis, E.S. 1991. Reactivation of a silent Ac following tissue culture is associated with heritable alterations in its methylation pattern. *Molecular and General Genetics* 229, 365-372.

Bräcke, J-O., Joshi, S. & Svensson, S.A. 2007. *Virkesbalanser för år 2004*. Rapport nr 4. Skogsstyrelsen pp 1-60.

Buchholz, J.T. 1931. The pine embryo and the embryos of related genera. III. *Trans Illinois State Academia Science* 23, 117-125.

Burg, K., Hristoforoglu, K., Fluch, S., Hohl, K., Burg, A. & Schmidt, J. 1999. Analysis of the fidelity of DNA replication in embryogenic cultures of Norway spruce. In: Espinel S, Ritter E, eds. *Proceedings of Application of Biotechnology to Forest Genetics*. Biofor 99 Vitoria-Gasteiz, Spain, 231–236.

Cairney, J., Zheng, L., Cowels, A., Hsiao, J., Zismann, V., Liu, J., Ouyang, S., Thibaud-Nissen, F., Hamilton, J., Childs, K., Pullman, G. S., Zhang, Y., Oh, T. & Buell, C.R. 2006. Expressed Sequence Tags from loblolly pine embryos reveal similarities with angiosperm embryogenesis. Plant Molecular Biology 62, 485–501.

Camacho, F. J.,Gernandt, D. S., Liston, A., Stone, J. K. & Klein, A. S. 1997. Endophytic fungal DNA, the source of contamination in spruce needle DNA. *Molecular Ecology* 6, 983-987.

Castle, L.A., Errampalli, D., Atherton, T.L., Franzmann, L.H., Yoon, E.S. & Meinke, D.W. 1993. Genetic and molecular characterization of embryonic mutants identified following seed transformation in *Arabidopsis*. *Molecular and General Genetics* 241, 504–514.

CELLFOR, interactive webpages 2007. http://www.cellfor.com/; accessed 30-march-2007

Cheliak, W.M. & Klimaszewska, K. 1991. Genetic variation in somatic embryogenic response in open-pollinated families of black spruce. *Theoretical and Applied Genetics* 82, 185–190.

Chimienti, F., Seve, M., Richard, S., Mathieu, J. & Favier, A. 2001. Role of cellular Zinc in programmed cell death: temporal relationships between zinc depletion, activation of caspases, and cleavage of Sp family transcription factors. *Biochemical Pharmacology* 62, 51-62.

Chalupa, V. 1985. Somatic embryogenesis in and plantlet regeneration from cultured immature and mature embryos of *Picea abies* (L.) Karst. *Commonwealth Forestry Institute* 14, 57-63.

Chowdhury, C.R. 1962. The embryogeny of conifers: a review. *Phytomorphology* 12, 313-338.

Clark, P.G.H. 1990. Developmental cell death: morphological diversity and multiple mechanisms. *Anatomy and Embryology* 181, 195-213.

Coffeen, W. C. & Wolpert, T. J. 2004. Purification and characterization of serine proteases that exhibit caspase-like activity and are associated with programmed cell death in Avena sativa. *Plant Cell* 16, 857-873.

De Verno, L.L., Charest, P.J. & Bonen, L. 1994. Mitochondrial DNA variation in somatic embryogenic cultures of Larix. *Theoretical and Applied Genetics* 88, 727–732.

De Verno, L.L., Y.S. Park, J.M. Bonga & Barrett, J.D. 1999. Somaclonal variation in cryopreserved embryogenic clones of white spruce (*Picea glauca* (Moench) Voss.). Plant Cell Reports 18, 948–953.

Earnshaw, W.C., Martins, L.M. & Kaufmann, S.H. 1999. MAMMALIAN CASPASES: structure, activation, substrates, and functions during apoptosis. Annual Review of Biochemistry 68, 383–424.

Ekberg, I., Norell, L. & von Arnold, S. 1993. Are there any associations between embryogenic capacity and phenological traits in two populations of Norway spruce? *Canadian journal of forest research* 23, 731-737.

Eriksson, G., Ekberg, I. & Clapham, D. 2007. *An Introduction to Forest Genetics, Second edition*. 187 pp.

Etienne, H. & Bertrand, B. 2003. Somaclonal variation in *Coffea arabica*: effects of genotype and embryogenic cell suspension age on frequency and phenotype of variants. *Tree Physiology* 23, 419-426.

FAO, 2005. Global Forest assessment, Progress towards sustainable forest development

FAO, 2007. State of the World's Forests (SOFO), Corporate document repository, 144 pg

Filonova, L.H., Bozhkov, P.V. & von Arnold, S. 2000a. Developmental pathway of somatic embryogenesis in *Picea abies* as revealed by time laps tracking. *Journal of Experimental Botany* 51, 249-264.

Filonova, L.H., Bozhkov, P.V., Brukhin, V.B., Daniel, G., Zhivotovsky, B. & von Arnold, S. 2000b. Two waves of programmed cell death occur during formation and development of somatic embryos in the gymnosperm, Norway spruce. *Journal of Cell Science* 113, 4399-4411.

Filonova, L.H., von Arnold, S., Daniel, G., & Bozhkov, P.V. 2002. Programmed cell death eliminates all but one embryo in a polyembryonic plant seed. *Cell Death and Differentiation* 9(10), 1057-62.

Finer, J.J., Kriebel, H.B. & Becwar, M.R. 1989. Initiation of embryogenic callus and suspension cultures of eastern white pine (*Pinus strobus* L.). *Plant Cell Reports* 8, 203-206.

Footitt, S., Ingouff, M., Clapham, D. & von Arnold, S. 2003. Expression of the viviparous 1 (*Pavp1*) and $p34^{cdc2}$ protein kinase (*cdc2Pa*) genes during somatic embryogenesis in Norway spruce (*Picea abies* [L.] Karst). *Journal of Experimental Botany* 54(388), 1711-1719.

Fourre, J.L., Berger, P., Niquet, L. & Andre, ´ P. 1997. Somatic embryogenesis and somaclonal variation in Norway spruce: morphogenetic, cytogenetic and molecular approaches. *Theoretical and Applied Genetics* 94, 159–169.

Friml, J., Benkova, E., Blilou, I., Wisniewska, J., Hamann, T., Ljung, K., Woody, S., Sandberg, G., Scheres, B., Jürgens, G. & Palme K. 2002. AtPIN4 mediates sink-driven auxin gradients and root patterning in *Arabidopsis*. *Cell* 108, 661-673.

Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwartz, H., Hamann, T., Offringa, R. & Jürgens, G. 2003. Efflux-dependent auxin gradients establishes the apical-basal axis of *Arabidopsis*. *Nature* 426, 147-153.

Galiba, G., Kertesz, Z., Utka J. & Sagi, L. 1985. Differences in somaclonal variation in three winter wheat (*Triticum aestivum*) varieties. *Cereal Research Communications* 13, 343-350.

Geburek, T., Mottinger-Kroup, S. & Burg, K. 1998. Genetic variation of Norway spruce (*Picea abies* (L.) Karst.) populations in Austria. II. Microspatial patterns derived from nuclear sequence tagged microsatellite sites. *Forest Genetics* 5, 231-237.

Gemmel, P., Örlander, G. & Högberg, K.-A. 1991. Norway spruce cuttings perform better than seedlings of the same genetic origin. *Silvae genetica* 40, 198-202.

Gerlach-Cruse, D. 1969. Embryo- und Endospermentwicklung nach einer Röntgen-bestrahlung der Fruchtknoten von *Arabidopsis thaliana*. *Radiation Botany* 9, 433–442.

Gifford, E. M. & Foster, A. S. 1989. Morphology and evolution of vascular plants. Third Edition. eds. D. Kennedy and R. B, Park. W. H. Freeman and company, New York. ISBN 0-7167-1946-0.

Goremykin, V.V., Hansmann, S. & Martin, W.F. 1997. Evolutionary analysis of 58 proteins encoded in six completely sequenced chloroplast genomes: revised molecular estimates of two seed plant divergence times. Plant Systematics and Evolution 206, 337–351.

Greenberg, J.T. & Yao, N. 2004. The role and regulation of programmed cell death in plant-pathogen interactions. *Cell Microbiology* 6, 201–211.
Grossnickle, S. C. & Major, J. E. 1994a. Interior spruce seedlings compared with emblings produced from somatic embryogenesis. II. Stock quality assessment prior to planting. *Canadian journal of forest research* 24, 1385-1396.
Grossnickle, S. C. & Major, J. E. 1994b. Interior spruce seedlings compared with emblings produced from somatic embryogenesis. III. Physiological response and morphological development on a reforestation site. *Canadian journal of forest research* 24, 1397-1407.
Grossnickle, S. C., Major, J. E. & Folk, R. S. 1994. Interior spruce seedlings with emblings produced from somatic embryogenesis. I. Nursery development, fall acclimation, and over-winter storage. *Canadian journal of forest research* 24, 1376-1384.
Grossnickle, S.C. & Folk, R. 2005. Stock quality assessment of a somatic interior spruce seedlot. *Northern Journal of Applied Forestry* 22(3), 197-202(6).
Grotz, N. & Guerinot, M.L. 2006. Molecular aspects of Cu, Fe and Zn homeostasis in plants. *Biochimica et Biophysica Acta* 1763, 595-608.
Grütter M. G. 2000. Caspases: key players in programmed cell death. *Current Opinion in Structural Biology* 10, 649-655.
Gupta, P.K. & Durzan, D.J. 1986. Somatic polyembryogenesis from callus of mature sugar pine embryos. *Bio/Technology* 4, 643–645.
Gupta, P. K. & Durzan, D. J. 1987. Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblolly pine. *Bio/Technology* 5. 147–151.
Hadfi, K., Speth, V., & Neuhaus, G. 1998. Auxin-induced developmental patterns in *Brassica juncea* embryos. *Development* 125, 879-887.
Haecker A, Gross-Hardt R, Geiges B, Sarkar A, Breuninger H, Herrmann M, Laux T. 2004. Expression dynamics of WOX genes mark cell fate decisions during early embryonic patterning in *Arabidopsis thaliana*. *Development* 131, 657-668.
Hakman, I. & von Arnold, S. 1985. Plantlet regeneration through somatic embryogenesis in *Picea abies* (Norway spruce). *Journal of Plant Physiology*. 121, 149-158.
Hamann, T., Mayer, U., & Jürgens, G. 1999. The auxin- insensitive bodenlosmutation affects primary root formation and apical-basal patterning in the *Arabidopsis* embryo. *Development* 126, 1387-1395.
Hamann, T., Benkova, E., Bäurle, I., Kientz, M., & Jürgens, G. 2002. The *Arabidopsis BODENLOS* gene encodes an auxin response protein inhibiting MONOPTEROS-mediated embryo patterning. *Genes and Development* 16, 1610–1615.
Hara-Nishimura, I., Hatsugai, N., Nakaune1, S., Kuroyanagi, M. & Nishimura, M. 2005. Vacuolar processing enzyme: an executor of plant cell death *Current Opinion in Plant Biology* 8, 404–408.
Hardtke, C. S. & Berleth, T. 1998, The *Arabidopsis* gene *MONOPTEROS* encodes a transcription factor mediating embryo axis formation and vascular development. *The EMBO Journal* 17, 1405-1411.
Hardy, K. 1999. Apoptosis in the human embryo. *Reviews of Reproduction* 4, 125-134.
Hardy, K., Spanos, S., Becker, D., Iannelli, P., Winston, R. M. & Stark, J. 2001. From cell death to embryo arrest: mathematical models of human preimplantation embryo development. *Proceedings of the National Academy of Sciences of the United States of America* 98, 1655–1660.
Hartmann, C., Henry, Y., De Buyser, J., Aubry, C. & Rode, A. 1989. Identification of new mitochondrial genome organizations in wheat plants regenerated from somatic tissue cultures. *Theoretical and Applied Genetics* 77, 169–175.
Harvengt, L., Trontin, J.F., Reymond, I. Canlet, F. & Paques, M. 2001. Molecular evidence of true-to-type propagation of a 3-year-old Norway spruce through somatic embryogenesis. *Planta* 213, 828–832.
Hatsugai, N., Kuroyanagi, M., Yamada, K., Meshi, T., Tsuda, S., Kondo, M., Nishimura, M. & Hara-Nishimura, I. 2004. A plant vacuolar protease, VPE, mediates virus-induced hypersensitive cell death. *Science* 305, 855-858.

Heinze, B. & Schmidt, J. 1995. Monitoring genetic fidelity and somaclonal variation in Norway spruce (*Picea abies*) somatic embryogenesis by RAPD analysis. *Euphytica* 85, 341-345.

Hjortswang, H., Sundås, A., Bharathan, G., Bozhkov, P., von Arnold, S. & Vahala, T. 2002. *KNOTTED 1*-like homebox genes of a gymnosperm, Norway spruce, expressed during somatic embryogenesis. *Plant Physiology and Biochemistry* 40, 837-843.

Horvitz, H. R. 2003. Worms, Life and Death (Nobel Lecture). *ChemBioChem* 4, 697-711.

Högberg, K.-A., Eriksson, U. & Werner, M. 1995. Vegetativ förökning och klonskogsbruk - med tonvikt på gran. *Redogörelse 1*, 1995. Skogforsk, Uppsala.

Högberg, K.-A. & Karlsson, B. 1998. Nursery Selection of *Picea abies* Clones and Effects in Field Trials. *Scandinavian Journal of Forest Research* 13. 12-20.

Högberg, K.-A., Bozhkov, P.V., Grönroos, R. & von Arnold, S. 2001. Critical factors affecting ex vitro performance of somatic embryo plants of *Picea abies*. *Scandinavian Journal of Forest Research* 16, 295-304.

Högberg, K.-A., Bozhkov, P.V. & von Arnold, S. 2003. Early selection improves clonal performance and reduces intraclonal variation of Norway spruce plants propagated by somatic embryogenesis. *Tree Physiology* 23, 211-216.

Högberg, K.-A., 2003. Possibilities and Limitations of Vegetative Propagation of Norway spruce. Doctoral thesis. Swedish University of Agricultural Sciences. *Silvestria* 294.

Ingouff, M., Farbos, M., Wiweger, M. & von Arnold S. 2003. The molecular characterization of *PaHB2*, a homeobox gene of the HD-GL2 family expressed during embryo development in Norway spruce. Journal of Experimental Botany 54, 1343-1350.

Isabel, N., Boivin, R., Levasseur, C., Charest, P.M., Bousquet, J. & Tremblay, F.M. 1996. Occurrence of somaclonal variation among somatic embryo-derived white spruces (*Picea glauca*. Pinaceae). *American Journal of Botany* 83, 1121–1130.

Jain, S.M. 2001. Tissue culture-derived variation in crop improvement. *Euphytica* 118, 153-166.

Johansson, M. & Walles, B. 1993. Functional anatomy of the ovule in broad bean, *Vicia faba* L. II. Ultrastructural development up to early embryogenesis. *International Journal of Plant Sciences* 154(4), 535-549.

Jürgens, G. & Mayer, U. 1992. *Arabidopsis. In A colour atlas of developing embryos*. J. Bard London, ed., Wolfe Publ.

Karp, A., Rissiott, R., Jones, M.G.K. & Bright, S.W.J. 1984. Chromosome doubling in monohaploid and dihaploid potatoes by re-generation from cultured leaf explants. *Plant Cell Tissue Organ Culture* 3, 363-373.

Karp, A., Jones, M.G.K., Foulger, D., Fish N. & Bright, S.W.J. 1989. Variability in potato tissue culture. *American Potato Journal* 66, 669-684.

Keinonen-Mettälä, K., Jalonen, P., Eurola, P., von Arnold, S. & von Weissenberg, K. 1996. Somatic embryogenesis of *Pinus sylvestris*. *Scandinavian Journal of Forest Research* 11, 242–250.

Kiaris, H., Koumantakis, E., Ergazaki, M. & Spandidos, D.A. 1996. Microsatellite instability in aborted embryos. *Molecular Human Reproduction* 2, 72-74.

Kleinschmit, J., Müller, W., Schmidt, J. & Racz, J. 1973. Entwicklung der Stecklingsvermehrung von Fichte (*Picea abies* (L.) Karst.) zur Praxisreife. *Silvae genetica* 26, 197-203.

Koike, T., Watanabe, T., Aoki, S., Kimura, E. & Shiro, M. 1996. A novel biomemetic zinc(II)-fluorophore, dansylaminoethyl-pendant macrocyclic tetraamine 1,4,7,10-tetrazacyclododecane (Cyclen). *Journal of the American Chemical Society* 118, 12696-12703.

Lam, E. 2004. Controlled cell death, plant survival and development. *Nature Reviews: Molecular Cell Biology* 5, 305–315.

Lamhamedi, M.S., Chamberland, H., Bernier, P.Y. & Tremblay, F.M. 2000. Clonal variation in morphology, growth, physiology, anatomy and ultrastructure of container-grown white spruce somatic plants. *Tree Physiology* 20, 869 – 880.

Larkin, P.J. & Scowcroft, W.R. 1981. Somaclonal variation: a new source of variability from cell cultures for plant improvement. *Theoretical and Applied Genetics* 60, 197-214.
Larsson, E., Sitbom, F., Ljung, K & von Arnold, S. 2007. Inhibited polar auxin transport results in aberrant embryo development in Norway spruce. *In press.*
Laux, T., Würschum, T. & Breuninger, H. 2004. Genetic regulation of embryogenic pattern formation. *The Plant Cell* 16, 190-202.
Lee, M. & Phillips, R.L. 1987. Genetic variants in progeny of regenerated maize plants. Genome 29, 834 – 838.
Lelu, M.A., Bastien, C., Drugeault, A., Gouez, M.L. & Klimaszewska, K. 1999. Somatic embryogenesis and plantlet development in *Pinus sylvestris* and *Pinus pinaster* on medium with and without plant growth regulators. *Physiologia Plantarum* 105, 719–728.
Levinson, G. & Gutman, G. A. 1987a. Slipped-Strand Mispairing: A Major Mechanism for DNA Sequence Evolution. *Molecular Biology and Evolution* 4(3), 203-221.
Levinson, G. & Gutman, G.A. 1987b. High frequencies of short frame shifts in poly-CA/TG tandem repeats born by bacteriophage M13 in *Escherichia coli* K-12. *Nucleic Acids Research* 15, 5323-5338.
Linacero, R. & Vazquez, A.M. 1993. Somaclonal variation in rye. *Mutation Research* 302, 201-205.
Liu, C., Xu, Z., & Chua, N.H. 1993. Auxin polar transport is essential for the establishment of bilateral symmetry during early plant embryogenesis. *The Plant Cell* 5, 621–630.
Lopes, T., Pinto, G., Loureiro, J., Costa A. & Santos, C. 2006. Determination of genetic stability in long-term somatic embryogenic cultures and derived plantlets of cork oak using microsatellite markers. *Tree Physiology* 26, 1145 –1152.
Lu, P., Porat, R., Nadeau, J. A. & O'Neil, S. D. 1996. Identification of a Meristem LI Layer-Specific Gene in *Arabidopsis* That is Expressed during Embryonic Pattern Formation and Defines a New Class of Homeobox Genes. *The Plant Cell* 8, 2155-2168.
Lukowitz, W., Roeder, A., Parmenter, D. & Somerville, C. 2004. A MAPKK Kinase Gene Regulates Extra-Embryonic Cell Fate in *Arabidopsis*. *Cell* 116, 109 –119.
MacKay, J.J., Becwar, M.R., Park, Y.S., Corderro, J.P. & Pullman, G.S. 2006. Genetic control of somatic embryogenesis initiation in loblolly pine and implications for breeding. *Tree Genetics and Genomes* 2, 1–9.
Madeo, F., Herker, E., Maldener, C., Wissing, S., Lächelt, S., Herlan, M., Fehr, M., Lauber, K., Sigrist, S. J., Wesselborg, S. & Fröhlich, K-U. 2002. A Caspase-Related Protease Regulates Apoptosis in Yeast. *Molecular Cell* 9, 911–917. 184, 765–770.
Maret, W., Jacob, C., Vallee, B.L. & Fischer, E.H. 1999. Inhibitory sites in enzymes: zinc removal and reactivation by thionein. *Proceedings of the National Academy of Sciences of the United States of America* 96, 1936–1940.
Martínez-Gomez, P. & Gradziel, T. M. 2003. Sexual polyembryony in almond. *Sexual Plant Reproduction* 16, 135–139.
Mayer, K. F. X., Schoof, H., Haecker, A., Lenhard, M., Jürgens, G. & Laux, T. 1998. Role of *WUSCHEL* in Regulating Stem Cell Fate in the *Arabidopsis* Shoot Meristem. *Cell* 95, 805-815.
McCabe, M.J., Jiang, S.A. & Orrenius, S. 1993. Chelation of intracellular zinc triggers apoptosis in mature thymocytes. *Laboratory Investigation* 69, 101-110.
McKenzie, N., Wen, L.Y. & Dale, J. 2002. Tissue-culture enhanced transposition of the maize transposable element Dissociation in *Brassica oleracea* var. 'Italica'. *Theoretical and Applied Genetics* 105, 23-33.
Meerarani, P., Ramadass, P., Toborek, M., Bauer, H.C., Bauer, H. & Hennig, B. 2000. Zinc protects against apoptosis of endothelial cells induced by linoleic acid and tumor necrosis factor alpha. *American Journal of Clinical Nutrition* 71, 81-87.
Moller, A.P. 1996. Developmental stability of flowers, embryo abortion, and developmental selection in plants. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 263, 53-56.
Mordhorst, A.P., Toonen, M.A.J. & De Vries, S.C. 1997. Plant embryogenesis. *Critical Reviews in Plant Sciences* 16, 535–576.

Nagmani, R., Diner, A.M., Garton, S. & Zipf, A.E. 1995. Anatomical comparison of somatic and zygotic embryos in conifers. *In: Jain S, Gupta P, Newton R (eds.) Somatic embryogenesis in woody plants: 23-48. Kluwer Academic Publishers, Dordrecht*

Orton, T.J. 1985. Genetic instability during embryogenic cloning of celery. *Plant Cell Tissue and Organ Culture* 4, 159–169

Owens, J. N. and Blake, M. D. 1985. Forest tree seed production. *Petawawa National Forestry Institute Information Report*. PI-X-53.

Palombi, M.A. & Damiano, C. 2002. Comparison between RAPD and SSR molecular markers in detecting genetic variation in kiwifruit (*Actinidia deliciosa* A. Chev). *Plant Cell Reports* 20, 1061–1066

Park, Y.-S. & Bonga, J.M. 1992. Conifer micropropagation: its function in tree improvement programs. *In Micropropagation of Woody Plants. Ed. M.R. Ahuja. Kluwer Academic, Dordrecht*, pp 457–470.

Park, Y.S., Pond, S.E. & Bonga, J.M. 1993. Initiation of somatic embryogenesis in white spruce (*Picea glauca*): genetic control, culture treatment effects, and implications for tree breeding. *Theoretical and Applied Genetics* 86, 427–436.

Park, Y.S., Bonga, J.M., Cameron, S.I., Barrett, J.D., Forbes, K., De Verno, L. & Klimaszewska, K. 1999. Somatic embryogenesis in jack pine (*Pinus banksiana* Lamb). *In: Jain, M.S., Gupta, P.K., Newton, R.J., eds. Somatic embryogenesis in woody plants, vol 4. Dordrecht: Kluwer Academic Publishers* 491-504.

Park, Y.S., Lelu-Walter, M.A., Harvengt, L., Trontin, J.F. Mac Eacheron, I., Klimaszewska, K. & Bonga, J.M. 2006. Initiation of somatic embryogenesis in *Pinus banksiana*, *P. strobus*, *P. pinaster*, and *P. sylvestris* at three laboratories in Canada and France. *Plant cell organ tissue culture* 86, 87-101.

Passerieux, E., Baud, S., Dullieu, H. & Pâques, M. 1999. Brief communication. RAPD variation in a Norway spruce seedlot: consequences of somatic embryogenesis. *The Journal of Heredity* 90, 662-666.

Pennell, R.I. & Lamb, C. 1997. Programmed cell death in plants. *Plant Cell* 9, 1157–1168.

Percy, R., Klimaszewska, K. & Cyr, D.R. 2000. Evaluation somatic embryogenesis for clonal propagation of western white pine. *Canadian Journal of Forest Research* 30, 1867-1876.

Peschke, V.M., Phillips, R.L. & Gegenbach, B.G. 1987. Discovery of transposable element activity among progeny of tissue culture-derived maize plants. *Science* 238, 804-807.

Peschke, V.M. & Phillips, R.L. 1991. Activation of the maize transposable element Suppressor-Mutator (Spm) in tissue culture. *Theoretical and Applied Genetics* 81, 90 – 97.

Pfeiffer, A., Olivieri, A.M. & Morgante, M. 1997. Identification and characterization of microsatellites in Norway spruce (*Picea abies* K.). *Genome* 40(4), 411-419.

Phillips, R.L., Kaeppler S.M. & Peschke, V.M. 1994. Genetic instability of plant tissue cultures: breakdown of normal controls. *Proceedings of the National Acadademy of Sciences of the Unites States of America* 91, 5222-5226.

Pouteau, S., Huttner, E., Grandbastien, M.A. & Caboche, M. 1991. Specific expression of the tobacco Tnt1 retrotransposon in protoplasts. *EMBO Journal* 10, 1911-1918.

Pullman, G. S., Johnson, S., Tassel, van. S. & Zhang, Y. 2005. Somatic embryogenesis in loblolly pine (*Pinus taeda*) and Douglas fir (*Pseudotsuga menziesii*): improving culture initiation and growth with MES pH buffer, biotin, and folic acid. *Plant Cell, Tissue and Organ Culture* 80, 91–103.

Pullman, G. S. & Skryabina, A. 2007. Liquid medium and liquid overlays improve embryogenic tissue initiation in conifers. *Plant Cell Reports* 26(7), 873-887.

Qumsiyeh, M.B., Kim, K.R., Ahmed, M.N. & Bradford, W. 2000. Cytogenetics and mechanisms of spontaneous abortions: increased apoptosis and decreased cell proliferation in chromosomally abnormal villi. *Cytogenetetics and Cell Genetics* 88, 230-235.

Rahman, M.H. & Rajora, O.P. 2001. Microsatellite DNA somaclonal variation in micropropagated trembling aspen (*Populus tremuloides*). *Plant Cell Reports* 20, 531–536.

Reinert, J. 1958. Untersuchungen über die Morphogenese an Gewebenkulturen. *Bericht der Deutschen botanischen Gesellschaft* 71:15.

Rodríguez, M. J., Suárez, M. F., Heredia, R., Avila, C., Breton, D., Trontin. J. F., Filonova, L., Bozhkov, P., von Arnold, S., Harvengt, L. & Cánovas, F. M. 2006. Expression patterns of two glutamine synthetase genes in zygotic and somatic pine embryos support specific roles in nitrogen metabolism during embryogenesis. *New Phytologist* 169, 35-44.

Roisin-Bouffay, C., Luciani, M-F., Klein, G., Levraud, J-P., Adam, M. & Golstein. P. 2004. Developmental cell death in *Dictyostelium* does not require paracaspases. *Journal of Biological Chemistry* 279, 11489-11494.

Rojo, E., Gillmor, C.S., Kovaleva, V., Soerville, C.R. & Raikhel, N.V. 2001. VACUOLELESS1 is an essential gene required for vacuole formation and morphogenesis in *Arabidopsis*. *Developmental Cell* 1, 303-310.

Rosvall, O., Jansson, G., Andersson, B., Ericsson, T., Karlsson, B., Sonesson, J. & Stener, L-G. 2001. *Genetic gain from present and future seed orchards and clone mixes*. Redogörelse No. 1, 2001.

Ruaud, J. N., Bercetche, J. & Paques, M. 1992. First evidence of somatic embryos deriving from needles of 1-year-old *Picea abies* plants. *Plant cell reports* 11, 563-566.

Ruefli-Brasse, A. A., French, D. M. & Dixit, V. M. 2003. Regulation of NF-kB-dependent lymphocyte activation and development by paracaspases. *Science* 302, 1581-1584.

Sabala, I., Clapham, D., Elfstrand, M., & von Arnold, S. 2000. Tissue-specific expression of *Pa18*, a putative lipid transfer protein gene, during embryo development in Norway spruce (*Picea abies*). *Plant Molecular Biology* 42, 461-478.

Sabatini, S., Beis, D., Wolkenfelt, H., Murfett, J., Guilfoyle, T., Malamy, J., Benfey, P., Leyser, O., Bechtold, N., Weisbeek, P., & Scheres, B. 1999. An auxin-dependent distal organizer of pattern and polarity in the *Arabidopsis* root. *Cell* 99, 463-472.

Sabatini, S., Heidstra, R., Wildwater, M., & Scheres, B. 2003. SCARECROW is involved in positioning the stem cell niche in the *Arabidopsis* root meristem. *Genes and Development* 17, 354–358.

Saieed, N.T., Douglas G.C. & Fry, D.J. 1994. Induction and stability of somaclonal variation in growth, leaf phenotype and gasexchange characteristics of poplar regenerated from callus culture. *Tree Physiology*, 14, 1-16.

Salajova, T., Salaj, J. & Kormutak, A. 1999. Initiation of embryogenic tissues and plantlet regeneration from somatic embryos of *Pinus nigra* Arn. *Plant Science* 145, 33–40.

Savard, L., Li, P., Strauss, S.H., Chase, M.W., Michaud, M. & Bousquet, J. 1994. Chloroplast and nuclear gene sequences indicate Late Pennsylvanian time for the last common ancestor of extant seed plants. *Proceedings of the National Academy Sciences of the United States of America* 91, 5163–5167.

Savelkoul, P.H.M., Aarts, H.J.M., De Haas, J., Dijkshoorn, L., Duim, B., Otsen, M., Rademaker, J.L.W., Schouls, L. & Lenstra, J.A, 1999. Amplified-fragment length polymorphism analysis: the state of an art. *Journal of Clinical Microbiology* 37, 3083-3091.

Schlotterer, C. & Tautz, D. 1992. Slippage synthesis of simple sequence DNA. *Nucleic Acids Research* 20, 211-215.

Schwartz, B.W., Yeung, E.C., & Meinke, D.W. 1994. Disruption of morphogenesis and transformation of the suspensor in abnormal suspensor mutants of *Arabidopsis*. *Development* 120, 3235-3245.

Shu, Q.Y., Liu, G.S., Qi, D.M., Chu, C.C., Liu, J. & Li, H.J. 2003. An effective method for axillary bud culture and RAPD analysis of cloned plants in tetraploid black locust. *Plant Cell Reports* 22(3), 175-80.

Sing, H. 1978. *Embryology of gymnosperms*. Berlin: Borntrager.

Smertenko, A.P., Bozhkov, P.V., Filonova, L.H., von Arnold, S. & Hussey, P.J. 2003. Re-organisation of the cytoskeleton during developmental programmed cell death in *Picea abies* embryos. *Plant Journal* 33(5), 813-24.

Smulders, M.J.M., Rus-Kortekaasand, W. & Vosman, B. 1995. Tissue culture-induced DNA methylation polymorphisms in repetitive DNA of tomato calli and regenerated plants. *Theoretical and Applied Genetics* 91, 1257-1264.

Sonesson, J. 2003. Results and experiences from the central Swedish clonal forestry program. In *Abstracts from a conference of the Nordic Goup for the Management of Genetic Resources of Trees in Barony Castle, Scotland, 4-7 September, 2002. (ed. T. Eysteinsson). Rit mógilsár rannsóknastöðvar skógræktar* 17: 38

Soranzo, N., Provan, J., Powell, W. 1998. Characterization of microsatellite loci in *Pinus sylvestris* L. *Molecular Ecology* 7, 1260–1261.

Souer, E., van Houwelingen, A., Kloos, D., Mol, J., & Koes, R. 1996. The *NO APICAL MERISTEM* gene of Petunia is required for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. *Cell* 85, 159–170.

Spandidos, D., Koumantakis, E., Sifakis, S. & Sourvinos, G. 1998. Microsatellite mutations in spontaneously aborted embryos. *Fertility and Sterility* 70, 892-895.

Stasolla, C., Bozhkov, P., ChuT-M., van Zyl, L., Egertsdotter, U., Suarez, M., Craigh, D., Wolfinger, R., von Arnold, S. & Sederoff, R. 2004. Variation in transcript abundance during somatic embryogenesis in gymnosperms. *Tree Physiology* 24, 1073-1085.

Steinmann, T., Geldner, N., Grebe, M., Mangold, S., Jackson, C. L., Paris, S., Gälweiler, L., Palme, K. & Jürgens, G. 1999. Coordinated Polar Localization of Auxin Efflux Carrier PIN1 by GNOM ARF GEF. *Science* 286, 316-318.

Stennicke, H.R. & Salvesen, G.S. 1997. Biochemical characteristics of caspases-3, -6, -7, and -8. *Journal of Biological Chemistry* 272, 25719-25723.

Steward, F.C., Mapes, M.O. & Hears, K. 1958. Growth and organized development of cultured cells. II. Growth and division of freely suspended cells. *American Journal of Botany* 45, 705-708.

Strand, M., Prolla, T.A., Liskay, R.M. & Petes, T.D. 1993. Destabilization of tract of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair. *Nature* 365, 274-276.

Suarez, M.F., Filonova, L.H., Smertenko, A., Savenkov, E.I., Clapham, D.H., von Arnold, S., Zhivotovsky, B. & Bozhkov, P.V. 2004. Metacaspase-dependent programmed cell death is essential for plant embryogenesis. *Current Biology* 14, R339-R340.

Sundås-Larsson, A., Svenson, M., Liao, H. & Engström, P. 1998. A homeobox gene with potential developmental control function in the meristem of the conifer *Picea abies*. *Proceedings of the National Academy of Sciences of the United States of America* 95, 15118–15122.

Sutton, B. 2002. Commercial delivery of genetic improvement to conifer plantations using somatic embryogenesis, *Annals of Forest Science* 59, 657–661.

Swedish Forest Industries Federation, 2006. www.skogsindustrierna.org.

Tahir, M., Law, D.A. & Stasolla, C. 2006. Molecular characterization of *PgAGO*, a novel conifer gene of the ARGONAUTE family expressed in apical cells and required for somatic embryo development in spruce. *Tree Physiology* 26, 1257-1270.

Takada, S., Hibara, K., Ishida, T. & Tasaka, M. 2001. The *CUP- SHAPED COTYLEDON1* gene of *Arabidopsis* regulates shoot apical meristem formation. *Development* 128, 1127-1135.

Talanian, R.V., Quinlan, C., Trautz, S., Hackett, M.C., Mankovich, J.A., Banach, D., Ghayur, T., Brady, K.D. & Wong, W.W. 1997. Substrate specificities of caspase family proteases. *Journal of Biological Chemistry* 272, 9677-9682.

Taylor, T.N., Taylor, E.L. 1993. The Biology and Evolution of Fossil Plants. Prentice Hall, Englewood Cliffs, NJ.

Tremblay, L., Levasseur, C., Tremblay, F.M. 1999. Frequency of somaclonal variation in plants of black spruce (*Picea mariana*, *Pinaceae*) and white spruce (*P. glauca, Pinaceae*) derived from somatic embryogenesis and identification of some factors involved in genetic instability. *American Journal of Botany* 86 (10), 1373-1381.

Truong-Tran, A.Q., LH, Ho., Chai, F. & Zalewski, P.D. 2000. Cellular zinc fluxes and the regulation of apoptosis/gene-directed cell death. *Journal of Nutrition* 130,1459-1466.

Truong-Tran, A.Q., Carter, J. Ruffin, R.E. & Zalewski P.D. 2001. The role of zinc in caspase activation and apoptotic cell death. *BioMetals* 14, 315-330.

Turnpenny, P. & Ellard, S. 2005. *Emery's Elements of Medical Genetics*, 12th. ed. Elsevier, London.

Ulmasov, T., Hagen, G. & Guilfoyle, T. J. 1999. Activation and repression of transcription by auxin-response factors. *Proceedings of the National Academy of Sciences of the United States of America* 96, 5844-5849.

Uren, A.G., O'Rourke, K., Aravind, L., Pisabarro, M.T., Seshagiri, S., Koonin, E.V. & Dixit, V.M. 2000. Identification of Paracaspases and Metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. *Molecular Cell* 6, 961-967.

van den Berg, C., Willemsen, V., Hendriks, G., Weisbeek, P., and Scheres, B. 1997. Short-range control of cell differentiation in the *Arabidopsis* root meristem. *Nature* 390, 287-289.

Van Zyl, L,. Bozhkov, P., Clapham, D., Sederoff, R. & von Arnold, S. 2003. Up, down and up again is a signature global gene expression pattern at the beginning of gymnosperm embryogenesis. *Mechanisms of Development, Gene Expression Patterns* 3, 83-91.

Vercammen, D., van de Cotte, B., De Jaeger, G., Eeckhout, D., Casteels, P. & Vandepoele, K. 2004. Type II Metacaspases Atmc4 and Atmc9 of *Arabidopsis thaliana* Cleave Substrates after Arginine and Lysine. *The Journal of Biological Chemistry* 279(44), 45329 –45336.

Vernon, D.M. & Meinke, D.W. 1994. Embryogenic transformation of the suspensor in twin, a polyembryonic mutant of *Arabidopsis*. *Developmental Biology* 165, 566-573.

von Arnold, S., Sabala, I,. Bozhkov, P., Dyachok, J. & Filonova, L. 2002. Developmental pathways of somatic embryogenesis. *Plant Cell, Tissue and Organ Culture* 69, 233–249

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Lee, van de. T., Hornes, M., Frijters, A., Pot, J., Peleman, J. & Kuiper, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* 23(21), 4407-4414.

Wadenbäck, J. 2006. *Lignin Studies of Transgenic Norway Spruce*. Doctoral diss., Dept. of Plant Biology and Forest Genetics, SLU. Acta Universitatis Agriculturae Sueciae. No. 64.

Weber, L.J. & Wong, C. 1993. Mutation of human short tandem repeats. *Human Molecular Genetics* 2, 1123-1128.

Weijers, D., Sauer, M., Meurette, O., Friml, J., Ljung, K., Sandberg, G., Hooykaas, P. & Offringa, R. 2005. Maintenance of Embryonic Auxin Distribution for Apical-Basal Patterning by PIN-.FORMED-Dependent Auxin Transport in *Arabidopsis*. *The Plant Cell* 17, 2517-2526.

Weijers, D. & Jürgens, G. 2005. Auxin and embryo axis formation: the ends in sight? *Current Opinion in Plant Biology* 8, 32-37.

Wilhelm, E., Hristoforoglu, K., Fluch, S. & Burg, K. 2005. Detection of microsatellite instability during somatic embryogenesis of oak (*Quercus robur* L.). *Plant Cell Reports* 23, 790–795.

Willemsen, V., Wolkenfelt, H., de Vrieze, G., Weisbeek, P., & Scheres, B. 1998. The HOBBIT gene is required for formation of the root meristem in the *Arabidopsis* embryo. *Development* 125, 521-531.

Wolf, B. B. & Green, D. R. 1999. Suicidal Tendencies: Apoptotic Cell Death by Caspase Family Proteinases. *Journal of Biological Chemistry* 274, 20049-20052.

Wysocka-Diller, J.W., Helariutta, Y., Fukaki, H., Malamy, J.E., Benfey, P.N. 2000. Molecular analysis of SCARECROW function reveals a radial patterning mechanism common to root and shoot. *Development*. 127, 595–603.

Yazdani, R., Scotti, I., Jansson, G., Plomion, C. & Mathur, G. 2003. Inheritance and diversity of simple sequence repeat (SSR) microsatellite 16 markers in various families of *Picea abies*. *Hereditas* 138, 219-227.

Yeung, E.C. 1980. Embryology of *Phaseolus*: the role of the suspensor. *Z. Pflanzenphysiologie* 96, 17-28.

Zalewski, P.D., Forbes, I.J. & Betts, W.H. 1993. Correlation of apoptosis with change in intracellular labile Zn(II) using Zinquin [(2-methyl-8-p-toluenesulphonamido-6-quinolyloxy)acetic acid], a new specific fluorescent-probe for Zn(II). *Biochemical Journal* 296, 403-408.

Zhang, J.Z., & Somerville, C.R. 1997. Suspensor-derived polyembryony caused by altered expression of valyl-tRNA synthetase in the twn2 mutant of *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America* 94, 7349-7355.

Acknowledgements

My journey started 30 years ago in the little but not unknown place called "Bialitt", by many known from the TV-show in the early 60-ties. My first recall of trees was the resin smell from freshly chain sawed Norway spruce trees, from the storm 1981. Since then my character have evolved through practical forestry school in Osby to advanced forestry lectures in Umeå and finally resulted in this doctoral thesis in Uppsala. As stated by Wadenbäck (2006) a receipt for successful completion of a doctoral thesis is to collect a good set of supervisors and a good subject. In my case, I certainly agree that both premises have been fulfilled.

First and foremost, I am grateful to my supervisors for their support and enthusiasm, Sara von Arnold for her sparkling positivism, Peter Bozhkov for his practical and theoretical "know how" in the field of programmed cell death and David not being my formal supervisor but sill a vital injection to the scientific discussions. The people at Skogforsk in Ekebo and Uppsala for their invaluable help and support, especially Karl-Anders Högberg for sending me plant material without notice, excavating freezers and sampling our babies and Gunnar Jansson for the discussions about tree breeding. The people at the Institute of Biotechnology, ARCSeibersdorf in Vienna, Austria who made my visit an adventure in DNA-extraction, PCR-amplification and SSR-sequencing and in Austrian social events like "Mahlzeit". Most of all to my co-author Kornel Burg for his support and invaluable discussions about the methods mentioned. Thanks Silvia Fluch for your help with shopping smoked Salomon at IKEA and the bike you borrowed me, allowing me to work much to late in the weekdays and weekends.

Secondly, I sincerely would like to thank all my co-workers at our department of Plant Biology and Forest Genetics for all kinds of social and scientific events such as the WESE, GC-barbecues, Tuesday-floor hockey and nation-beer-nights. Special thanks to fellow doctoral students of our group, Gosia, Johan, Emma, Malin x II and Daniel x II for valuable non-scientific discussions. Social activities beside office hours also have to be acknowledged like parties at the "Ultuna kåren", Norrland's Nation for their invaluable big band jazz evenings, Swingkatten for dance lessons in Lindy Hop, Jazz and Boogie-Woogie and Philochoros for their passionate folk dance.

Finally, I want to thank my family and friends for the support and all the fun times we have together. Many thanks to all my old friends from Skåne that keeps me updated about things outside the lab, SKOGIS people from Jönköping to Umeå for keeping old memories in place, all relatives that asked me for updates and PowerPoint presentations about my research, and foremost my parents Ulla and Sune who let me plant as many trees as I wanted in their garden and took care of them while I was studying and my sister Emma just because she's my sister, and last but not least, a big hug for my Princess Johanna – Thank you all so much!

This work, carried out at the Department of Plant Biology and Forest Genetics at SLU Uppsala, Sweden and was funded by The Forest Tree Breeding Association, The Forestry Research Institute of Sweden (SkogForsk), The Swedish Research

Council (VR), and The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS), and the Institute of Biotechnology, ARCSeibersdorf Research GmbH, Austria and the commission of the European Communities, Community research programme 'Quality of life and Management of Living Resources' (Project SEP QLRT1-CT99-0679).