Patterning During Embryo Development in *Pinus*

With Special Emphasis on Somatic Embryogenesis in Scots pine (*Pinus sylvestris* L.)

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Embryoutveckling hos släktet *Pinus* – med särskild tonvikt på somatisk embyoutveckling i tall (*Pinus sylvestris* L.)

Sammanfattning

Skogsindustrin har ett stort intresse av att kunna föröka ekonomiskt viktiga trädslag vegetativt, då det innebär stora fördelar i både förädlingsarbetet och för massförökning av det förädlade materialet. Somatisk embryogenes är en relativt ny metod för vegetativ förökning, där man från ett enda frö kan producera ett obegränsat antal genetiskt identiska plantor. Metoden innebär att somatiska celler stimuleras att utvecklas till embryogena celler som kan bilda embryon, så kallade somatiska embryon, som motsvarar fröembryon. Gran, men inte tall, kan förökas effektivt via somatiska embryon.

Till skillnad från gran, så multipliceras fröembryot i tall på ett tidigt stadium genom delning. Embryona börjar tävla för sin överlevnad, och så småningom blir ett embryo dominant medan de resterande underordnade embryona succesivt bryts ner. Embryogena cellkulturer av tall kan endast etableras från omogna fröembryon, under en tvåveckorsperiod varje sommar, när multipliceringen av fröembryot sker. Embryogena cellkulturer av tall växer mycket snabbt, men det är svårt att stimulera utvecklingen och mognad av somatiska embryon.

Genom att systematiskt följa utvecklingen av somatiska embryon från en normal och en abnorm cellinje, har skillnader i utvecklingen mellan dessa kunnat dokumenteras. Vi fann att förhållandet mellan den embryogena massan och suspensorn är i obalans i embryon från den abnorma cellinjen, vilket delvis kan förklaras av en störd polär transport av växthormonet auxin. Studien visade också att många tidiga embryon i båda cellinjerna inte utvecklas vidare. De blockerade embryona i den normala cellinjen bryts ner på ett liknande sätt som de underordnade embryona i ett tallfrö. De blockerade embryona i den abnorma cellinjen bildar istället nya embryon, vilket resulterar i en kontinuerlig slinga av avbruten utveckling och nybildning av nya embryon. Dessa resultat tyder på att somatiska embryon från abnorma cellinjer fastnar i ett skede av ihållande multiplicering, där övergången till ett dominant embryo inte går att kontrollera.

För att få kunskap om hur övergången från multipliceringen av ett embryo till vidare utveckling av ett dominant embryo regleras, har vi studerat hur genuttrycket förändras under den tidiga utvecklingen av fröembryon. Ett antal gener, vilka reglerar centrala utvecklingsprocesser, har identifieras. Funktionerna av dessa gener under embryoutvecklingen måste nu bekräftas. När vi har fått mer ingående kunskaper om hur embryoutvecklingen regleras i tall, kommer förhoppningsvis metoden att föröka tall via somatiska embryon kunna förbättras.

Patterning During Embryo Development in *Pinus*. With Special Emphasis on Somatic Embryogenesis in Scots Pine (*Pinus sylvestris* L.)

Abstract

Somatic embryogenesis is an attractive method to propagate conifers vegetatively. However, many species belonging to *Pinus* are recalcitrant to somatic embryo development. The overall aim of this thesis has been to gain a better understanding of the developmental pathway leading to cotyledonary somatic embryos of Scots pine (*Pinus sylvestris*).

By comparing the developmental pathway of somatic embryos in a normal and in an abnormal cell line, differences between the cell lines were identified. An important difference was the high ratio of early and late embryos carrying supernumerary suspensor cells in the abnormal cell line compared to embryos in the normal cell line. This unbalanced ratio between the embryonal mass and the suspensor is at least partly caused by a disturbed polar auxin transport. Furthermore, a high proportion of early and late embryos degenerated in both cell lines. The degenerating embryos in the normal cell line were eliminated in a similar way as subordinate embryos in the seed. Contrastingly, the degenerating embryos in the abnormal cell line were not eliminated; instead the degenerated embryos started to differentiate new embryos creating a loop of embryo degeneration and embryo differentiation. During initiation of embryogenic tissue, the protruding early zygotic embryo(s) started to degenerate before a proliferating embryogenic culture was established, indicating that the initiation of embryogenic cultures is not a direct continuation of cleavage polyembryony. The results further suggest that there is a high risk that cell lines initiated from early zygotic embryos at the stage of cleavage develop abnormally.

To be able to use more differentiated tissues as explants for initiation of embryogenic cultures, more knowledge is needed about totipotence and embryogenic potential. Treatment with the histone deacetylase inhibitor trichostatin A (TSA) during maturation of somatic embryos of Norway spruce inhibited the maturation progression of the embryos. Furthermore, although TSA-treatment maintained the embryogenic potential in germinating somatic embryos, it did not enable the embryos to regain embryogenic potential after it was lost.

By analysing global changes in gene expression during early zygotic embryo development in Scots pine, we identified genes and processes that might be important for regulating the cleavage process and for the development of a dominant embryo.

Together these results contribute to the knowledge that in turn can lead to improved protocols for large scale propagation of *Pinus* species via somatic embryos.

Keywords: Conifer, embryogenic potential, polar auxin transport (PAT), programmed cell death (PCD), Scots pine, somatic embryos, somatic embryogenesis, transcriptome

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Dedication

To my beloved children Edvin and Nora.

You never fail until you stop trying.
Albert Einstein

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Abrahamsson, M., Valladares, S., Larsson, E., von Arnold, S. (2012). Patterning during somatic embryogenesis in Scots pine in relation to polar auxin transport and programmed cell death. *Plant Cell Tiss Organ Cult* 109:391-400.

II Abrahamsson, M., Valladares, S., Merino, I., Larsson, E., von Arnold, S. (2016). Degeneration pattern in somatic embryos of the gymnosperm *Pinus sylvestris* L. (submitted).

III Uddenberg, D., Valladares, S., Abrahamsson, M., Sundström, J.F., Sundås-Larsson, A., von Arnold, S. (2011). Embryogenic potential and expression of embryogenesis-related genes in conifers are affected by treatment with a histone deacetylase inhibitor. *Planta* 234:527-539.

IV Merino, I., Abrahamsson, M., Sterck, L., von Arnold, S. (2016) Functional switches in gene regulation during development of zygotic embryos in Scots pine (manuscript).

The contribution of Malin Abrahamsson to the papers included in this thesis was as follows:

I Malin Abrahamsson took part in planning the work and performed the experiments together with Silvia Valladares. Malin wrote the first version of the manuscript.

II Malin Abrahamsson was highly involved in planning the work, performed most of the laboratory work, analysed the data and had the main responsibility for writing the manuscript.

III Malin Abrahamsson sampled pine tissue and took part in the analyses of gene expression in pine.

IV Malin Abrahamsson took part in planning the work, in the laboratory work and analysing the data.

1 Introduction

Forests are essential for life on our planet and support a large portion of the world's biodiversity; and human societies and the global economy are highly dependent on forests. Approximately 30% of earth's land area is covered by forest and the demands on these forests are continuously increasing. It is a challenging and complex task to manage forests to ensure economic, social and environmental well-being of the world's rapidly growing population while at the same time conserving forests for future generations (North American Forest Commission, 1998). To be able to set aside large portions of forest for conservation, we must increase the use of intensively managed forests. To achieve this, the productivity (i.e. wood quality and trees' adaptation ability) must be maximized, which can be accomplished through the use of improved genetic material gained by forest tree breeding. In addition, intensively managed forests and increased forest growth provide an environmental benefit by increasing the carbon dioxide absorption. Conifers belong to the gymnosperm group, and grow naturally in almost all parts of the world. They are economically important in many countries as a source of timber, pulp, fuel and chemicals, and as ornamentals. They also include the tallest and oldest of trees (Raven et al. 1999).

Improving forest trees by breeding is a very slow process in which economically important traits are continuously improved (Fig. 1a). The improvement is obtained through well-known quantitative methods that involve recurrent crossings, testing and selection. In the case of conifers, every breeding cycle takes 20-25 years. When the superior genotypes have been selected, branches from the selected trees are grafted and grown into new trees in seed orchards. The seeds from these seed orchards are then collected to be used for reforestation (Högberg et al. 1998). However, parts of the genetic gain achieved in the breeding program are lost owing to sexual recombination and contamination of foreign pollen. The possibility to propagate selected trees

vegetatively on a large scale would thus make it possible to capture the genetic gain more efficiently. One way to achieve this is by using somatic embryos (Fig. 1b; somatic embryogenesis is further described in chapter 1.2.3). Many conifers belonging to the genus *Picea* and some species belonging to *Pinus* can be propagated on a large scale by somatic embryos (Park et al. 2006). However, for many economically important *Pinus* species there are still no protocols available for efficient regeneration of high quality cotyledonary embryos via somatic embryogenesis.

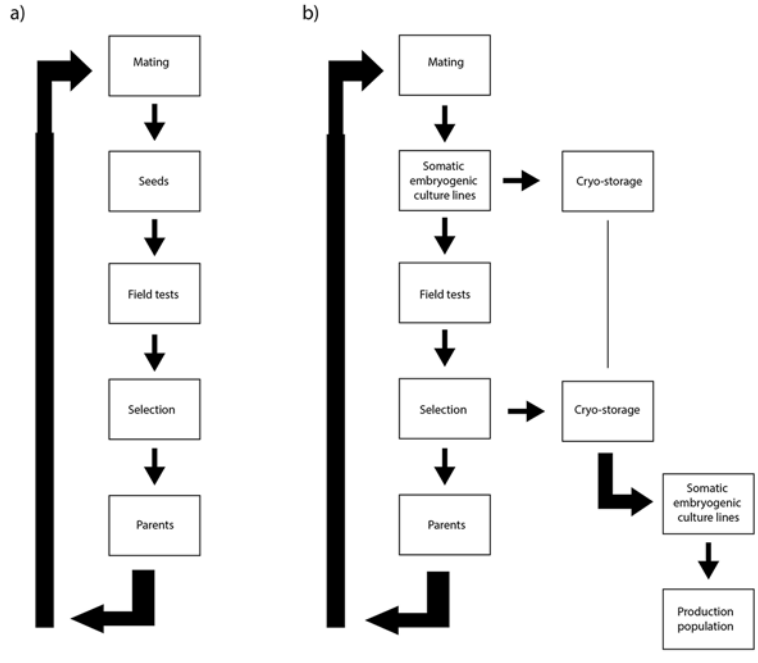


Figure 1. The breeding process (After Högberg 2003). **a** An illustration of the strategy of conventional breeding. Conventional breeding begins with the selection of parents followed by pollination i.e. mating. The resulting full-sib seeds are then used to produce seedlings that are planted in field tests. Once the field tests have identified the best performing plants, these plants will become the parents for the next generation of breeding. **b** An illustration of the strategy of clonal forestry with somatic embryogenesis and cryopreservation. Clonal forestry with somatic embryogenesis begins with the selection of parents followed by pollination i.e. mating. The resulting full-sib seeds are then used for establishing embryogenic cultures (cell lines). Subsequently, one part of each embryogenic cell line is cryopreserved simultaneously as one part is used for plant production via somatic embryogenesis. All plants regenerated from the same cell line are genetically identical. The regenerated plants are planted into clonal field-tests. Once the best clones have been identified, the corresponding embryogenic cell lines are retrieved from the cryopreservation, and further used to establish a production population. Simultaneously, the best performing plants in the field tests will become the parents for the next generation of breeding.

One major public concern is that clonal-based forestry will bring genetic variation to an end, with a huge failure of intensively managed forests as a result. However, coniferous trees are dependent on environmental factors, such as the interplay between day length and temperature. Therefore, a specific clone can grow well only in a limited geographical area, and the use of many different clones is necessary in clonal-based forestry (Eriksson et al. 2013). By selecting appropriate clone mixtures, the risk of losing a clone or affecting the biological diversity is greatly reduced. Furthermore, *Cryptomeria japonica* has in Japan been propagated vegetatively for centuries for the use in extensive reforestation.

About 55% of the Swedish land area is covered by productive forest, of which 81% consists of the coniferous species Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) (Swedish Forest Agency, 2014). Today Scots pine and Norway spruce are used for building and construction, furniture, floors, paper, poles, joinery and moldings, sleepers, plywood, lumber, boat boards, keels and masts, and for artificial fibers (Skogssverige, 2016). From the pine wood, it is also possible to extract chemicals such as tar, pine oil, turpentine, phenol and resin.

This thesis describes the patterning during early embryo development in Scots pine, with a special focus on the process of somatic embryogenesis. The developmental pathway of somatic embryogenesis, from initiation to the maturation of cotyledonary embryos, has been analysed in normal and abnormal cell lines in order to pinpoint deviations from the normal path. Furthermore, a transcriptome analysis of early zygotic embryos was performed in order to reveal genes and putative processes involved in cleavage polyembryony, and in the development of a dominant embryo. Taken together, these results will provide means to improve the possibilities to propagate Scots pine via somatic embryos.

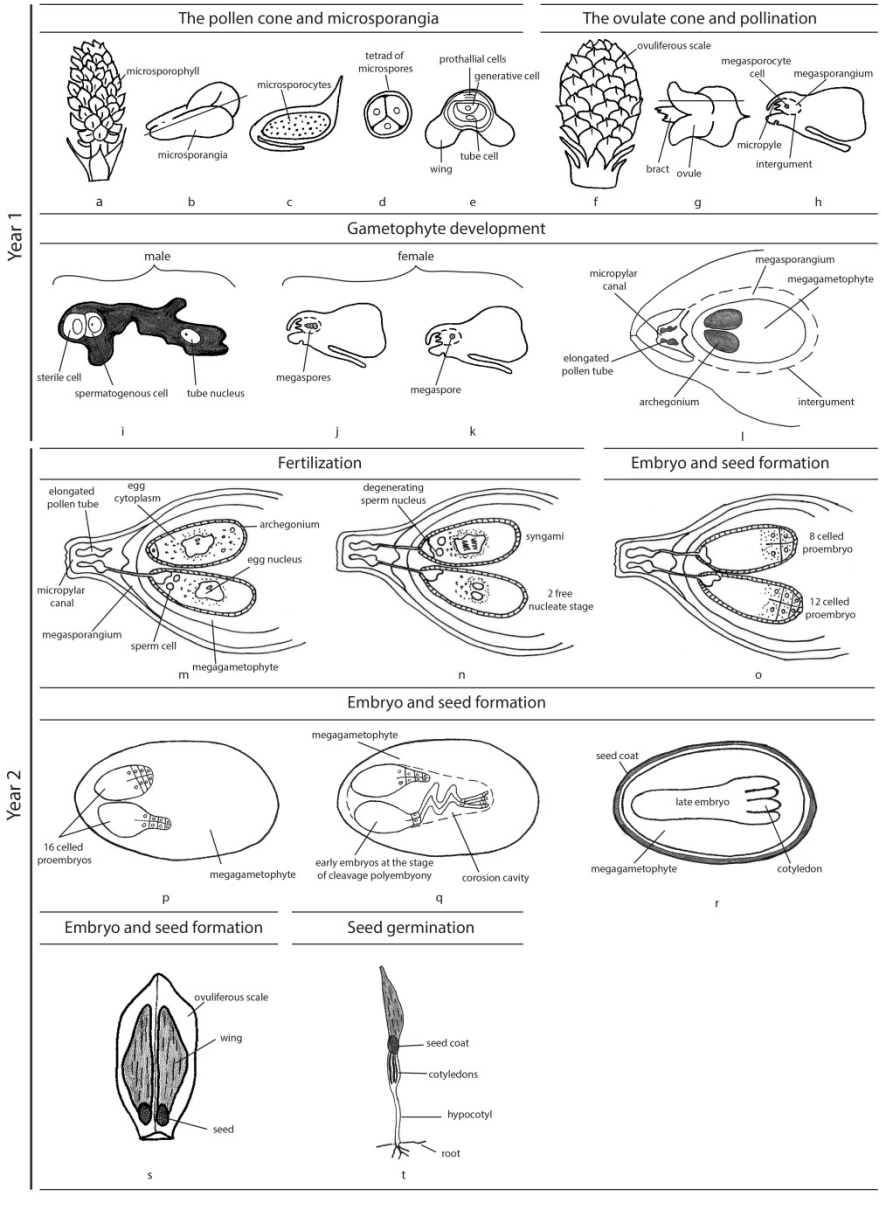
1.1 Reproductive cycle in *Pinus* species

The reproductive cycle of *Pinus* species (Fig. 2) is similar to that of most conifers. Exceptions include species beloniging to *Picea*, which complete their reproductive cycle in one year, whereas *Pinus* species complete their reproductive cycle in two years. Furthermore, cleavage polyembryony, characteristic for *Pinus* species, does not occur in all conifers.

Pinus species are monoecious, producing separate pollen cones and ovulate cones on the same tree. The pollen cone (Fig. 2a-e) consists of many small spirally arranged microsporophylls, while the ovulate cone (Fig. 2f-h) consists

of numerous spirally arranged megasporophylls (Raven et al. 1999). After pollination, the male and female gametophytes continue their development (Fig. 2i-l). Inside the ovule, the pollen grain develops a pollen tube that starts to slowly digest its way through the megasporangium towards the developing megagametophyte (female gametophyte) (Raven et al. 1999; Williams 2009 with ref). Early in the second year, fertilization takes place (Fig. 2m-n). One of the sperm cells unites with the egg nucleus to create a diploid zygote. Adjacent archegonium may also be fertilized if more than one pollen grain is present. A megagametophyte may therefore contain more than one zygote. Protected inside the ovule the fertilized eggs develop into embryos (Fig. 2o-r). A detailed description of the embryo development in *Pinus* species (Fig. 2p-r) will follow under zygotic embryogenesis in *Pinus* species (1.2). At seed maturation (Fig. 2s), the embryo will desiccate and enter the dormancy stage (Raven et al. 1999).

The zygotic embryo of gymnosperms develops within the megagametophyte. The megagametophyte is considered as the functional homologue of the endosperm in angiosperms, owing to its main function in providing nutrients for the developing and germinating embryo (Costa et al. 2004). Unlike angiosperms, see 1.3 below, gymnosperms display single fertilization creating a diploid embryo that develops within a haploid megagametophyte (Singh 1978). After pollination but before fertilization, the megagametophyte in *Pinus* species starts to develop slowly. The development of the megagametophyte proceeds through three major phases (Williams 2009 with ref): (1) *A free nuclear phase*, where the megaspore nucleus undergoes several rounds of division to form the free nuclear gametophyte. The megagametophyte often contains about 2000 free nuclei, although the number varies between species (Konar and Moitra 1980 with ref). (2) *A cellularization phase*, where cell wall formation starts about 13 months after pollination (Raven et al. 1999; Williams 2009 with ref). (3) *The cellular growth phase*, which is a phase of additional growth and development, during which usually two or three archegonia differentiate (each with a single egg cell) about 15 months after pollination (Friedman and Carmichael 1998; Raven et al. 1999). When the ovules have been fertilized the cells in the megagametophyte will start to accumulate storage material (Singh 1978; Friedman and Carmichael 1998).


Year 1
The pollen cone and microsporangia
The ovulate cone and pollination
microsporophyll
microsporangia
microsporocytes
tetrad of microspores
prothallial cells
generative cell
tube cell
wing
ovuliferous scale
megasporocyte cell
megasporangium
micropyle
intergument
bract
ovule
a
b
c
d
e
f
g
h
Gametophyte development
male
female
sterile cell
spermatogenous cell
tube nucleus
megaspores
megaspore
micropylar canal
megasporangium
megagametophyte
elongated pollen tube
archegonium
intergument
i
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l
Fertilization
Embryo and seed formation
elongated pollen tube
egg cytoplasm
archegonium
egg nucleus
micropylar canal
megasporangium
sperm cell
megagametophyte
degenerating sperm nucleus
syngami
2 free nucleate stage
8 celled proembryo
12 celled proembryo
m
n
o
Year 2
Embryo and seed formation
16 celled proembryos
megagametophyte
megagametophyte
early embryos at the stage of cleavage polyembyony
corosion cavity
seed coat
late embryo
megagametophyte
cotyledon
p
q
r
Embryo and seed formation
Seed germination
ovuliferous scale
wing
seed
seed coat
cotyledons
hypocotyl
root
s
t

Figure 2. (Figure shown on previous page) Reproductive cycle of *Pinus* sp. (After Schmeil 1932; Owens et al. 1988; Raven et al. 1999). **a** A pollen cone consisting of many small spirally arranged scales. Each scale is called microsporophyll. **b**,**c** Two microsporangia, located on the underside of each microsporophyll, contain massive amounts of spore-producing cells called microsporocytes. **d** In early spring, the nucleus in each microsporocyte divides by meiosis to produce four microspores. **e** Each microspore develops two gas-filled wings. Successively three mitotic divisions take place, each creating two prothallial cells, one generative cell and one tube cell. **f** The ovulate cone consists of numerous spirally arranged ovuliferous scales. **g** Each ovuleferous scale has a small bract fused to the lower surface, and two ovules located on the upper surface. **h** Each ovule consists of a megasporangium surrounded by an outer protective layer called the integument. An opening in the integument, the micropyle, consists of a canal covered by a sticky secretion. Furthermore, each megasporangium contains a megasporocyte cell. **i** The generative cell of the pollen grain undergoes division, giving rise to a sterile cell and a spermatogenous cell. **j** Inside the megasporangium, the single sporocyte cell undergoes meiosis giving rise to four megaspores. **k** Only one of these megaspores will develop into the megagametophyte, and the rest will soon degenerate . **l** At the micropylar end of the megagametophyte, several pores start to form of which each leads to an archegonium, containing a single egg cell. **m** Early during the second year, the pollen tube grows through the megasporangium to the megagametophyte. As a pollen tube approaches an archegonium, the spermatogenous cell divides producing two sperm cells. The tube nucleus and the two sperm cells and the sterile cell are discharged into the egg cytoplasm. One sperm nucleus unites with the egg nucleus (syngamy) to create a diploid zygote, and the other degenerates. **n** Protected inside the ovule the fertilized eggs are ready to form embryos. **o, p** Proembryogeny – before suspensor elongation. **q** Early embryogeny – all stages during and after the elongation of the suspensor and before the establishment of the root meristem. **r** Late embryogeny – establishment of the root and shoot meristem and further development of the embryo. **s** Seed maturation is also accompanied by the transformation of the integuments into a tough protective seed coat. The seed and surface tissue are separated from the cone scale and form a winged seed. The seeds of *Pinus* species are usually dispersed in the autumn of the following year after the initial appearance of the cones and the pollination. **t** The seed can germinate in the spring the year after the seed is released from the cone.

1.2 Zygotic embryogenesis in *Pinus* species

In plants, zygotic embryogenesis covers the development from the time of fertilization until seed germination. Embryo development is generally divided into an early morphogenic phase, and a late maturation phase (Goldberg et al. 1994). Most cell divisions and differentiation processes occur during the morphogenic phase, while the embryo increases in size by cell expansion during the maturation phase.

The development of the embryo in *Pinus* can be divided into three phases (Singh 1978); (1) proembryogeny – before suspensor elongation. (2) Early embryogeny – all stages during and after the elongation of the suspensor but before the establishment of the root meristem. (3) Late embryogeny – establishment of the root and shoot meristem and further development of the embryo.

Proembryogeny

Proembryogeny starts with a free nuclear stage (Fig. 3a-i). After cell wall formation, four tiers are formed (Fig. 3j-m) of which the lowest tier (E) will form the embryonal mass and the second lowest (E1) will form the embryonal suspensor (Fig. 3m). The phase of proembryogeny ends just as the E1 tier starts to elongate (Fig. 3n).

Early embryogeny

During early embryogeny, the E1 tier continues to elongate (Fig. 3o) (Singh 1978). The E2 tier splits or cleaves longitudinally into four disjoined tiers, giving rise to so-called cleavage polyembryony (Grifford and Foster 1989). Each of the four disjoined E tiers gives rise to an embryo that begins the development by apical cell growth (Buchholz 1920). The apical cell disappears as the embryonal mass start to form (Fig. 3p). At this stage, the embryos will start to compete for nutrients and space to grow and survive. However, it is not only a competition among the four embryos within the same archegonium, but also with those of the other archegonia within the same seed. The embryo that gets (by chance) the best position within the megagametophyte, will most likely possess the greatest growth vigour, get a competitive advantage, and win the competition (Williams 2009 with ref.; Buchholz 1926). Usually, only one embryo, *the dominant embryo*, will win the competition and reach maturity while the others, *the subordinate embryos*, are suppressed, arrested and subsequently eliminated by programmed cell death (PCD) at various stages of development (Roy Chowdhury 1962; Filonova et al 2002). The dominant embryo suppresses the growth and development of the subordinate embryos by exerting mechanical suppression with its suspensor system, and thereby causes starvation. It has also been suggested that the dominant embryo secretes growth inhibiting factors that suppress the subordinate embryos (Dogra 1967).

During early embryogeny (Fig. 3o-p), the most basally situated cells in the embryonal mass undergo asymmetric division to form embryonal tube cells (Et) (Fig. 3p). These are subsequently added to the suspensor system as new tube cells are formed. Thereby, several files of secondary suspensor cells are continuously formed (E2, E3…etc.), leading to a rapid growth of the suspensor system (Roy Chowdhury 1962; Filonova et al. 2002). The suspensor cells do not divide but they elongate and become highly vacuolated (Singh 1978; Smertenko et al. 2003). Furthermore, the suspensor cells are committed to PCD as soon as they are formed, and the oldest part of the suspensor system will continuously degenerate (Roy Chowdhury 1962; Filonova et al. 2000;

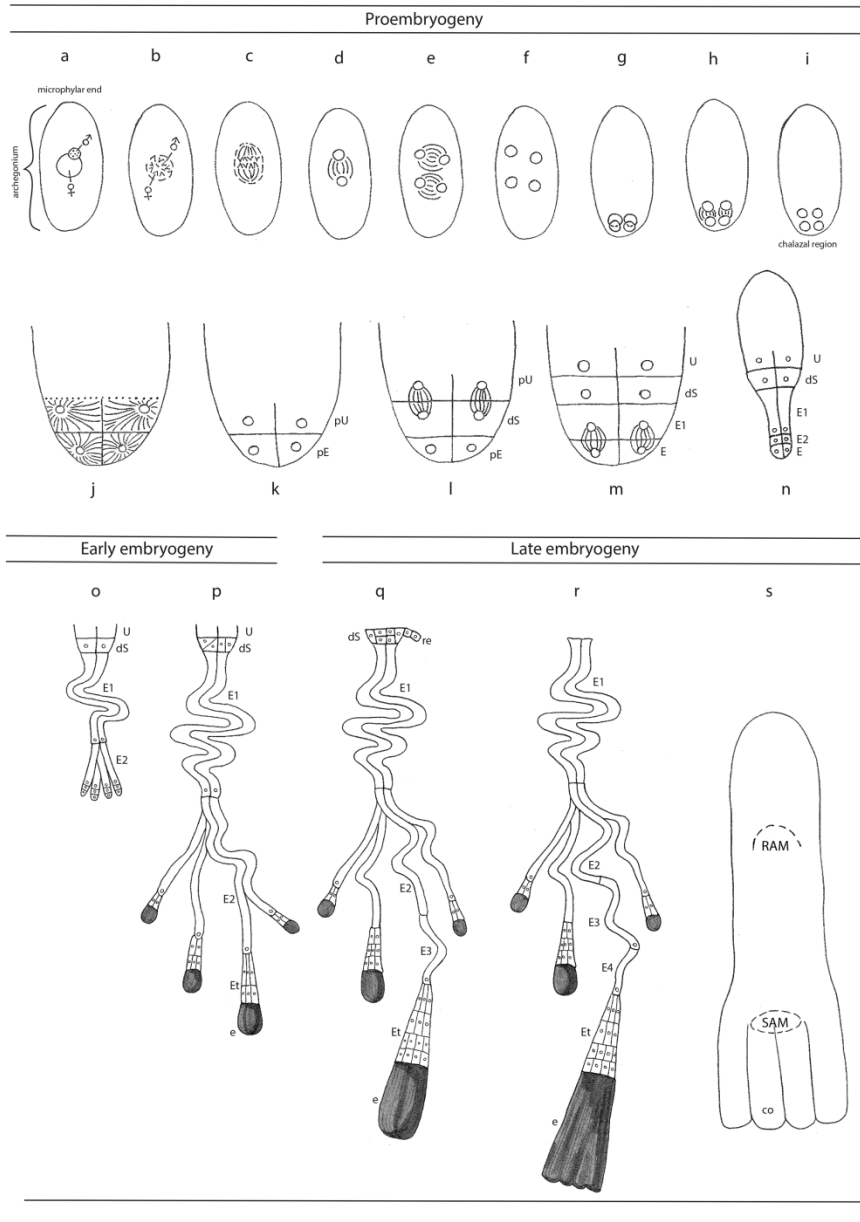
Bozhkov et al. 2005). The rapid growth of the suspensor system will force the apical tier out of the archegonium and into the megagametophyte (Roy Chowdhury 1962; Skinner 1992).

Higher plants have proximal stem cells and distal stem cells (Scheres 2007). Both types of stem cells continuously produce daughter cells through cell divisions. However, the daughter cells of proximal stem cells continue to divide, while the daughters of distal stem cells do not continue to divide. It has been suggested that the basal cells of the embryonal mass of early embryos in conifers are distal stem cells that by asymmetric divisions give rise to both apical meristematic daughter cells in the embryonal mass and basal vacuolated suspensor cells (or embryonal tube cells) (Zhu et al. 2016). However, this has so far been difficult to confirm since there are no clear differences between these cells and the rest of the cells in the embryonal mass.

Late embryogeny

Late embryogeny is the phase of histogenesis and organogenesis (Fig. 3q-s). During this stage, the root organizing centre (ROC) i.e. the precursor of the root apical meristem (RAM) forms near the centre of the embryo, and the shoot apical meristem (SAM) forms at the apical part of the embryonal mass. The cotyledon primordia arise in a ring around the apical end of the embryo (Fig. 3r) (Singh 1978). Finally by seed maturity, the SAM is surrounded by a whorl of cotyledons and the RAM is covered by a root cap (Fig. 3s) (Raven et al. 1999).

Pinus species can show meristematic cells in the dS tier (Fig. 3q). The cells in the dS tier do not elongate as the secondary suspensor cells (Dogra 1967; Sing 1978), however, they can start to divide to form lobes, resulting in the formation of so-called “rosette embryos” (Roy Chowdhury 1962). Since these cell masses never form embryos, but are instead eventually aborted, the term “rosette embryo” has been questioned (Roy Chowdhury 1962; Doyle 1963, Dogra 1967).


Proembryogeny
a
b
c
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e
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i
microphylar end
archegonium
chalazal region
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l
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pU
pE
dS
U
E1
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Early embryogeny
Late embryogeny
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E3
E4
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Figure 3. (Figure shown on previous page) Schematic drawing of zygotic embryo development in *Pinus* sp. (After Dogra 1967; Singh 1978; Gifford and Foster 1989). **a** The male and female nuclei fuse. The fusing nuclei are large and full of dense nucleoplasm. **b** After the nuclei (including the nucleoplasms) have merged, separate chromatin networks are formed. **c** The first mitosis is intranuclear and the resulting nuclei are formed within the nucleoplasm of the zygote. The nuclear membrane of the zygote disappears at the end of the mitosis. **d-f** Two free nuclear divisions take place resulting in four free nuclei. **g** The four free nuclei move towards the chalazal portion of the zygote, i.e. the base of the archegonium, where they become arranged in one layer. **h, i** The four nuclei divide with vertical spindles to give rise to eight nuclei arranged in two layers of four each. **j, k** Secondary spindles develop and wall formation results in two tiers: the primary upper (or open) tier (pU) and the primary embryonal tier (pE), each comprising four cells. The pU tier is open towards the micropylar end, i.e. the neck of the archegonium, and is continuous with the general cytoplasm of the proembryo. **l, m** The pU tier and the pE tier go through internal divisions to form four tiers. **l** First the pU tier divides into an upper (or open) tier (U) and into a dysfunctional suspensor tier (dS; sometimes called rosette tier). However, it should be noted that the dS tier, in some literature, is also called the rosette tier. The U tier remains open towards the archegonium. **m** Then the pE tier divides into an embryonal tier (E) and into an embryonal tier 1 (E1). The E1 tier is often also referred to as the primary suspensor. **n** The upper four cells of the E tier (E1) elongate to function as a suspensor, and the lower four cells of the E tier will form the embryonal mass. **o** An embryo that has cleaved (at E2) into four disjoined E tiers. The U tier has started to degenerate. **p** One of the embryos has started to become dominant and the growth of the remaining embryos is progressively reduced. The basal cells of the embryonal mass, the embryonal tubes (Et), divide predominantly in a transverse plane and elongate. **q** The dominant embryo has developed further to a stage just before cotyledon differentiation. Note the developing rosette embryo (re) from the dS tier. **r** The dominant embryo is starting to differentiate a ring of cotyledons. **s** A mature cotyledonary embryo with two polar meristems (root and shoot meristem) and a whorl of cotyledons.
E - Embryonal tier, E1 – Embryonal tier 1, co – cotyledon, pU - primary upper (open) tier, pE - primary embryonal tier, RAM – root apical meristem, re - rosette embryo, SAM – shoot apical meristem, U - upper (open) tier

1.3 Embryogenesis in the angiosperm model species *Arabidopsis thaliana*

Arabidopsis (*Arabidopsis thaliana*) embryogenesis has been well studied, and compared to conifer embryogenesis, there is a lot of knowledge available (Goldberg et al. 1994; Jenik et al. 2007; Wendrich and Weijers 2013; ten Hove et al. 2015). However, even though this knowledge cannot be directly applied to conifer species, it can be used as a foundation in the process of gaining more knowledge about conifer embryogenesis.

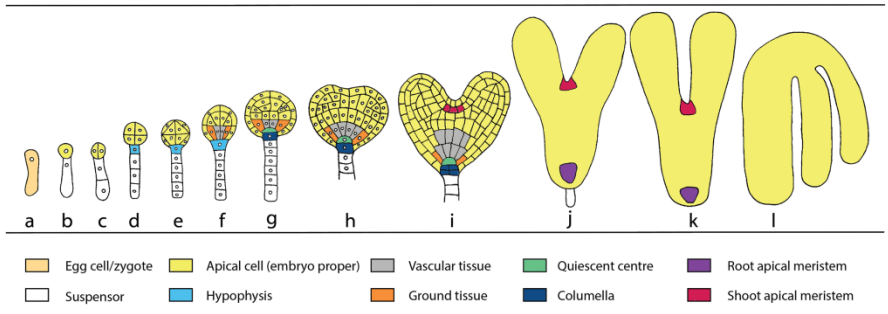

Figure 4. A simplified schematic drawing of zygotic embryo development in Arabidopsis (After ten Hove et al. 2015; Wendrich and Weijers 2013). **a** Zygote. **b** One-celled embryo. **c** Two- celled embryo. **d** Octant stage. **e** Dermatogen stage. **f** Early globular stage. **g** Late globular stage. **h** Transition stage. **i** Early heart stage. **j** Late heart stage. **k** Torpedo stage. **l** Bent cotyledonary stage.

Embryogenesis in Arabidopsis begins with double fertilization, where one sperm cell fuses with the egg cell to form the diploid zygote, and another sperm cell fuses with the central cell to form the triploid endosperm mother cell. After the fertilization has taken place, the zygote starts to elongate (Fig. 4a) and divide asymmetrically to generate a small apical cell and a large vacuolated basal cell (Fig. 4b). The fate of these two cells is already determined at this stage. The apical cell will now start to divide in both a transversal and a longitudinal plane in order to develop the embryo proper (Fig. 4c). The basal cell will divide only in the longitudinal plane and give rise to one single cell file of which the apical-most cell is called the hypophysis, while the rest of the file forms the suspensor. At the octant stage, the embryo proper consists of eight cells which are divided into an upper and a lower tier (Fig. 4d). During the following step to the dermatogen stage, the cells of the embryo proper start to divide diagonally, which separates the protoderm from the inner tissues (Fig. 4e). The protoderm is the precursor of the epidermis and the inner tissues are the precursors of the ground and vascular tissues. During the transition to the early globular stage, the protodermal cells divide anticlinally to extend the outer layer, and the inner cells divide longitudinally. During the following step to the late-globular stage (Fig. 4f,g), the hypophysis divides asymmetrically to give rise to a small lens-shaped cell which is the precursor of the quiescent centre, and a larger basal cell which will give rise to the columella stem cells. Also during this period, the stem cells for the vasculature and ground tissues are specified. During the transition from the late-globular stage to the heart stage, the cotyledons grow out and the embryo proper shifts morphological symmetry from radial to bilateral (Fig. 4-j). Also during this

period the specification of the shoot and root apical meristems takes place. The cotyledons, RAM and SAM are in place by the torpedo stage (Fig. 4k), and by the bent cotyledonary stage, the embryo is fully mature and ready for germination (Fig. 4l).

1.4 Somatic embryogenesis in conifers

Somatic embryogenesis was first reported in carrot (*Daucus carota*) (Steward et al. 1958; Reinert 1959). The first report on somatic embryogenesis in a woody plant was in 1965 for the angiosperm Sandalwood (*Santalum album*) (Rao et al. 1965), and the first reports on somatic embryogenesis in gymnosperms (i.e. conifer) were in 1985 for Norway spruce (*Picea abies*) (Chalupa 1985; Hakman et al. 1985) and European larch (*Larix decicua*) (Nagmani et al. 1985). Since then, somatic embryogenesis has been achieved for several other conifers, and several companies are producing commercial quantities of somatic embryo plants of conifers (Lelu-Walter et al. 2013); for example, of loblolly pine (*Pinus taeda*) in USA (Grossnickle et al. 2008), Nordman fir (*Abies nordmanniana*) in Denmark (Find and Krogstrup 2009) and Norway spruce in Sweden (Egertsdotter, personal communication).

Somatic embryogenesis is an asexual process that leads to the formation of an embryo from a somatic cell(s). Somatic embryos in conifers follow a similar pattern of development as their zygotic counterparts, except during the first steps of development in which proliferating embryogenic cultures consist of a mixture of small meristematic cells and elongated vacuolated cells. Another difference is that the somatic embryo does not develop enclosed in the megagametophyte like the zygotic embryo.

The procedure to regenerate conifers via somatic embryogenesis comprises several phases such as initiation and proliferation of embryogenic tissue, differentiation of early embryos and development of late and cotyledonary embryos (von Arnold et al. 2008). Embryogenic cultures of most conifers are initiated by incubating the primary explant, i.e. immature or mature zygotic embryos, on culture medium containing plant growth regulators (PGRs), usually auxin and cytokinin. The newly initiated embryogenic tissue is proliferated in culture medium supplemented with the same PGRs as during initiation. To stimulate differentiation of early embryos and the development of late and cotyledonary embryos, the embryogenic cultures are first transferred to PGR-free culture medium (the period of pre-maturation) and then to culture medium containing abscisic acid (ABA) (the period of maturation).

Table 1. *Explants used for initiation of embryogenic cultures of Pinus sp.* ***MgClePol*** *- Whole megagagametophytes containing embryos at the stage of cleavage polyembryony or prior cleavage polyembryony (four celled proembryo);* ***MgPreCot*** *- Whole megagametophytes containing immature zygotic embryos at precotyledonary stage;* ***PreCot*** *- Immature zygotic embryos at the precotyledonary stage excised from the megagametophyte;* ***ME*** *- Mature embryos*

Explant	Species
MgPreCot	*P. armandii* (Maruyama et al. 2007)
MgClePol	*P. banksiana* (Park et al. 2006)
MgClePol	*P. caribaea* (Laine et al. 1990)
PreCot	*P. elliotti* (Jain et al. 1989)
MgPreCot	*P. elliotti* (Liao et al. 1995)
MgPreCot	*P. monticola* (Percy et al. 2000)
MgPreCot	*P. nigra* (Salajová et al. 1992; Salajová et al. 1999)
MgPreCot	*P. palustris* (Nagmani et al. 1993)
MgClePol	*P. patula* (Jones et al. 1995)
PreCot	*P. pinaster* (Lelu-Walter et al. 1999; Park et al. 2006; Lelu-Walter et al. 2006)
MgPreCot	*P. pinaster* (Miguel et al. 2004)
MgPreCot	*P. pinea* (Carneros et al. 2009)
MgClePol	*P. radiata* (Cerda et al. 2002; Montalbán et al. 2012)
MgPreCot	*P. radiata* (Montalbán et al. 2012)
PreCot	*P. radiata* (Hargreaves et al. 2009)
ME	*P. radiata* (Find et al. 2014)
MgPreCot	*P. strobus* (Finer et al. 1989; Klimaszewska et al. 1997; Garin et al. 1998)
MgClePol	*P. strobus* (Klimaszewska et al. 2001; Park et al. 2006)
ME	*P. strobus* (Garin et al. 1998)
MgClePol	*P. sylvestris* (Keinonen-Mettälä et al. 1996; Häggman et al. 1999; Lelu-Walter et al. 1999; Park et al. 2006)
PreCot	*P. taeda* (Becwar et al. 1990)
MgPreCot	*P. taeda* (Li et al. 1996; Li et al. 1998; Pullman et al. 2002)
MgClePol	*P. roxburghii* (Mathur et al. 2000)
MgPreCot	*P. roxburghii* (Arya et al. 2000)

1.4.1 Somatic embryogenesis in *Pinus* species

In most species like *Pinus* that have cleavage polyembryony, embryogenic cultures are initiated from immature zygotic embryos prior to cotyledon development (Table 1). The immature zygotic embryos are in a very sensitive stage of development, and therefore whole megagametophytes are often used as explants. It has been suggested that embryogenic cultures arise from the continuation of the zygotic cleavage process (Bozhkov et al. 1997; Park et al. 2006). In contrast, in species like *Picea* that do not have cleavage

polyembryony, mature cotyledonary embryos are most commonly used as explants. In Norway spruce it has been shown that differentiated cortical cells are initially stimulated to dedifferentiate before embryogenic tissue differentiate (Mo et al. 1996).

Many *Pinus* species are recalcitrant to develop somatic embryo development. The frequency of initiated embryogenic cell lines from immature zygotic embryos is commonly very low, and only a few of the established embryogenic cell lines respond to maturation conditions. Often the maturing embryos stop developing, dedifferentiate or develop abnormal phenotypes (Klimaszewska et al. 2007 with ref.). A number of experimental approaches have been taken to improve the protocols for initiation of embryogenic tissue, differentiation of early embryos and development of late and cotyledonary embryos as well as for plant regeneration in *Pinus*. Several factors have been tested including different compositions and concentrations of basal media (e.g. differences in macro- microelements, gelling agents, polyethylene glycol, activated charcoal) and the types and concentrations of plant growth regulators. Although improvements have been obtained for the different steps, further studies are required to better understand somatic embryo development in *Pinus*.

Within the framework of the European project Somatic Embryogenesis in Pines (SEP), 325 embryogenic cell lines of Scots pine from immature seeds from 15 half sib families were established (Burg et al. 2007). The initiation frequency varied from 0.2 to 11 % depending on collection date and family. A high yield of high quality cotyledonary embryos was obtained from only 11 of the established cell lines (von Arnold, personal communication). To elucidate whether genetic instability induced by *in vitro* stress varies among families and if genetic instability influences the adaptation to *in vitro* conditions and embryo development, the genetic stability of four variable nuclear microsatellite loci in embryogenic cell lines and cotyledonary somatic and zygotic embryos were compared. The following results were obtained (Burg et al. 2007): (i) A high mutation rate in microsatellites was observed during establishment of embryogenic tissue and the mutation rate varied significantly among families. (ii) The genetic instability of the families correlated positively with their embryogenic potential. (iii) The genetic instability correlated negatively with the frequency of cotyledonary embryo formation. It was suggested that the relatively high mutation rates found for some families might reflect the plasticity of the families to adapt to stress, which is important for widely distributed species such as Scots pine. Interestingly, the allelic distribution of microsatellites in clones derived from somatic embryo plants of Norway spruce

was not significantly different from that of the parents (Helmersson et al. 2004).

In addition to that the developmental stage of the immature zygotic embryo and the choice of culture medium are critical for establishing embryogenic cultures in *Pinus* species, the genotype of the parent trees can be of great importance. For example, the initiation frequency of embryogenic tissue in loblolly pine could be increased by about 45% by carefully selecting the appropriate parents (MacKay et al. 2001; MacKay et al. 2006). Furthermore, in Scots pine it has been shown that the parental effects vary at different stages during somatic embryogenesis (Niskanen et al. 2004). The maternal effect was strong for initiation of embryogenic cultures and during the maturation stage, while a paternal effect was detected only for the proliferation stage.

1.5 Global gene expression during embryo development

Gymnosperms and angiosperms are thought to have diverged from a common ancestor about 300 million years ago (Smith 2010). Angiosperms diversified to about 250,000 species (so far reported). In contrast, gymnosperms are represented by only 700 species, of which conifers are represented by 50 genera and 550 species (Raven et al. 1999). Conifers have exceptionally large genome sizes (between 6.5 and 37 GBP) compared to most other plants species, including many angiosperm tree species such as eastern cottonwood (*Populus detoides*, 540 MBP), rose gum (*Eucalyptus grandis*, 600 MBP) and bardi bush (*Acacia victoriae*, 2 GBP) (Ahuja and Neale 2005 with ref.). Furthermore, within the *Pinaceae* family the genome sizes are among the largest and vary between 18 and 37 GBP. The size and complexity of these genomes have presented technical challenges, and for a long time prevented whole-genome sequencing projects from getting started. However, advances in sequencing technologies and bioinformatics have now made it easier to handle these large genomes, and to date the whole genomes of white spruce (*Picea glauca*) (Birol et al. 2013), Norway spruce (Nystedt et al. 2013) and loblolly pine (*Pinus taeda*) (Zimin et al. 2014) have been sequenced, and whole-genome sequencing of Scots pine as well as that of other species are in the pipeline.

A lot of progress has been made, including faster and cheaper DNA sequencing, in next generation sequencing (NGS) technologies (Buermans et al. 2014). NGS technologies generate millions of DNA fragments in a single sequencing run (Mutz et al. 2013). However, the different NGS technologies yield different read lengths and read numbers per run, and therefore, the choice

of technology depends on the nature and the experimental design of the project. The 454 *Sequencing* (Roche) was the first NGS platform available as a commercial product (Shendure et al. 2008), and it generates longer reads (200-300 bp) than to e.g. PyroMark ID (Qiagen, 40bp), Genome analyzer (Ilumina/Solexa, 25-35 bp), SOLiD (Applied Bioscience, 35-100 bp) (Mutz et al. 2013). The 454 S*equencing* may be the technique of choice for e.g. *de novo* sequencing, where longer reads are more suitable because it facilitates the assembly generation.

1.5.1 Angiosperms

Various approaches have been taken for elucidating how the development of embryos in plants is regulated, and several transcriptome analyses of somatic and zygotic embryogenesis in angiosperms have been reported. Metabolic network dynamics have been analysed during embryo development in Arabidopsis by using microarray technologies (Zeng et al. 2007). Several Arabidopsis seed-specific genes were identified by performing Affymetrix GeneChip experiments (Le et al. 2010). Furthermore, transcript datasets for early stages of somatic embryo development in Arabidopsis have been generated through high-throughput Illumina HiSeq (Wrickramasuriya and Dunwell 2015). In comparative transcriptome analyses of somatic and zygotic embryos in chocolate tree (*Theobroma cacao*) (Maximova et al. 2014) and cotton (*Gossypium hirsutum*) (Jin et al. 2012) it has been shown that many genes involved in ethylene metabolism and response were more highly expressed in somatic than in zygotic embryos. Furthermore, the expression of genes involved in the synthesis of auxin, polyunsaturated fatty acids and secondary metabolites was higher in somatic embryos than in zygotic embryos of the chocolate tree (Maximova et al. 2014). Transcriptome analysis of early developing maize (*Zea mays*) seeds have identified metabolic activities specific in endosperm and in embryo (Lu et al. 2013). In addition, a number of transcription factors and imprinting genes were specifically expressed in embryo or endosperm. By performing whole transcriptome profiling during initiation of somatic embryos in maize, it was shown that the expression of stress-related genes and genes involved in hormone transport was highly up-regulated (Salvo et al. 2014).

1.5.2 Conifers

Several transcript profiling studies during embryo development have been reported also for conifers. About 68,700 expressed sequence tags (ESTs) were regenerated from somatic and zygotic embryos in loblolly pine (Cairney et al.

2006). Out of 295 genes important for the embryo development in Arabidopsis, 85% had very strong sequence similarity to an EST in the loblolly pine database (Carney and Pullman 2007). Putative processes associated with early somatic embryo development in Norway spruce were identified by using microarray analyses (Vestman et al. 2011). The results showed the involvement of stress-related processes and auxin-mediated processes. de Vega-Bartol et al. (2013) performed a microarray analysis during development of zygotic embryos of maritime pine (*Pinus pinaster*), and showed the involvement of epigenetic regulation and transcriptional control related to auxin transport and response. Furthermore, their results indicated that important events during embryogenesis seem to be coordinated by putative orthologs of major developmental regulators in angiosperms. In a comparative transcriptome analysis of early somatic embryos of Brazilian pine (*Araucaria angustifolia*), Elbl et al. (2014) showed the importance of auxin signalling. Several metabolic events were detected in proliferating embryogenic cultures of Japanese larch (*Larix leptolepis*) (Zhang et al. 2012).

Taken together, most of the global scale transcriptome analyses of embryo development in plants, and especially in conifers, have so far generated a lot of data and identified candidate processes and genes, whose roles now have to be evaluated in future functional studies.

2 Aims of this study

It is difficult to propagate many species belonging to *Pinus* via somatic embryos. A main problem is that a high frequency of the somatic embryos develops into abnormal cotyledonary embryos. The overall aim of this thesis has been to gain knowledge and a better understanding about the pathways controlling the successive developmental stages leading to cotyledonary embryos. This knowledge will be valuable for improving the culture conditions in order to propagate *Pinus* species on a large scale via somatic embryos.

The work had the following specific objectives:

- To elucidate the developmental pathway of somatic embryos in Scots pine and identify deviations from the normal path.

- To gain knowledge about totipotence and embryogenic potential in conifers, with the goal to make it possible to establish embryogenic cultures from more differentiated tissues.

- To identify genes and putative processes involved in the initiation of cleavage polyembryony, and in the development of a dominant embryo in *Pinus*.

3 Results and Discussion

3.1 Development of somatic embryos in Scots pine (I, II)

The successive developmental stages leading to cotyledonary somatic embryos must be understood fundamentally for optimal management of somatic plant regeneration systems. In order to gain more information about the developmental pattern of somatic embryos in Scots pine, we initially analysed the developmental pathway of somatic embryogenesis in eight cell lines. Two contrasting cell lines were chosen for more detailed analysis; cell line 12:12 giving rise to cotyledonary embryos with normal morphology, and cell line 3:10 giving rise to cotyledonary embryos with abnormal morphology. It has been possible to regenerate normal plants from cell line 12:12 but not from cell line 3:10 (von Arnold, personal communication).

We started with developing a staging system where the development of the somatic embryos was separated into 10 consecutive stages. The developmental stages are presented in figure 1 in paper I and II.

Up to stage 2, when early embryos composed of an embryonal mass in the apical part and a suspensor in the basal part had differentiated, the developmental pattern was similar in cell line 12:12 and 3:10. In cell line 12:12, the majority of the stage 2 embryos proceeded in their development into normal stage 3 embryos. The suspensor cells started to degrade at stage 4, and were eliminated around stage 7. In contrast, many stage 2 embryos in cell line 3:10 developed into abnormal stage 3 embryos, which had a cone-shaped embryonal mass, differentiated excess suspensor cells (supernumerary suspensor cells), and lacked a strict border between the embryonal mass and the suspensor. On average, more than 30% of the early embryos in cell line 3:10 carried supernumerary suspensor cells compared to less than 10% in cell line 12:12. It has been shown that the developmental programs of the embryonal mass and the suspensor are closely coordinated, and an imbalance

causes embryo defects and mortality (Smertenko et al. 2003; Bozhkov et al. 2005). Accordingly, the radial growth of the embryos in cell line 3:10 continued during the whole maturation process, resulting in many stunted cotyledonary embryos with shortened or aborted hypocotyl.

Auxin-regulated pattern formation has been studied by treating embryos with well characterized polar auxin transport (PAT) inhibitors such as NPA (1-N-naphtylphthalamic acid). For instance, NPA-treatment during development of somatic embryos of Norway spruce led to abnormal cell divisions and decreased programmed cell death (PCD) (Larsson et al. 2008). As a consequence, an imbalance between the embryonal mass and the suspensor was established, where many of the NPA-treated embryos developed a cone-shaped embryonal mass and supernumerary suspensor cells. In order to test if disturbed PAT could be one of the reasons for the formation of supernumerary suspensor cells in Scots pine somatic embryos, we analysed how embryo morphology was affected when cell line 12:12 and 3:10 were treated with NPA. The proportion of somatic embryos with supernumerary suspensor cells increased in cell line 12:12 after treatment with NPA, suggesting that the unbalanced ratio between the embryonal mass and the suspensor is caused by disturbed PAT. In cell line 3:10, the frequency of degenerating embryos increased significantly after NPA-treatment, suggesting that the PAT is already very disturbed in cell line 3:10, and that a further reduction of the PAT leads to embryo degeneration.

In Norway spruce, the suspensor cells are degraded by PCD (Filonova et al. 2000). In order to assess if PCD is suppressed in Scots pine somatic embryos carrying supernumerary suspensor cells, the number of cells undergoing PCD was analysed by TUNEL-assay in cell line 3:10. The pattern of TUNEL-positive cells was similar in normal somatic embryos and in embryos with supernumerary suspensor cells. This suggests that the imbalance between the embryonal mass and the suspensor is a consequence of an overproduction of suspensor cells rather than suppressed PCD.

3.1.1 Characterization of embryogenic cultures developing normal cotyledonary somatic embryos

Initially we performed extensive tracking experiments of embryos in cell line 12:12. Based on the tracking studies, we documented that a large proportion of early embryos degenerated on maturation medium. In the embryos that developed normally, the cells in the embryonal mass remained intact while the suspensor cells were gradually degraded. Among the embryos that degenerated, there was one dominating degeneration pattern that was followed

during all developmental stages analysed. The embryonal mass of the embryos disintegrated and/or cells in the embryonal mass became vacuolated (degeneration pattern i). To obtain a better understanding of the degeneration process, we stained the embryos with Sytox® Orange nuclei acid which is impermeable to living cells but penetrates membranes in dead or dying cells, and binds to double stranded DNA or RNA in late apoptotic and necrotic cells. In embryos at stage 3 the basal cells of the embryonal mass died first and after that the cell death process gradually proceeded towards the apical part of the embryonal mass, in a similar way as subordinate embryos are degraded by PCD in the seed (Filonova et al. 2002). Thus it seems that aberrant early embryos in a normal cell line are eliminated in a similar way as subordinate embryos during zygotic embryogenesis.

3.1.2 Characterization of embryogenic cultures developing abnormal cotyledonary somatic embryos

A high frequency of embryo abnormalities has been documented in seeds of Scots pine derived from trees that grow in the northern parts of Sweden (Dogra 1967). Dogra (1967) suggested that these abnormalities are caused by stress-induced persistent polyembryony, where the dominant embryo loses its dominance and no suppression or elimination of the subordinate embryos can occur. The abnormalities described by Dogra (1967) are strikingly similar to the defects observed in cotyledonary somatic embryos of cell line 3:10. Furthermore, lobing or partial cleavage is a phenomenon that has been reported in many gymnosperms (Singh 1978). It is common in zygotic embryos with retarded development, and it results from unequal growth rates between different domains in the embryonal mass (Dogra 1967; Singh et al. 1978). Lobing can also result in cleavage polyembryony if the lobes independently develop their own embryonal tube cells (Singh 1978). However, if zygotic embryos with retarded development lobe abundantly they degrade (Dogra 1967). We observed that a large proportion of stage 2 to 4 embryos in cell line 3:10 developed lobes; however, we also observed that these lobing embryos often degenerated. These observations suggest that somatic embryos in cell lines similar to 3:10 fail to develop embryos comparable to dominant zygotic embryos, but rather remain at a stage of persistent polyembryony.

In order to elucidate the origin of the abnormalities observed in embryogenic cultures of Scots pine, we made extensive tracking experiments of cell line 3:10. Similarly as in cell line 12:12, the time-lapse tracking experiments of cell line 3:10 showed that only a low proportion of early embryos developed into cotyledonary embryos. The majority of the early

embryos degenerated. However, the dominant degeneration pattern, in cell line 3:10, was different from that in cell line 12:12. In cell line 3:10, the embryos degenerated into less organized embryos, in which elongated, vacuolated cells differentiated from the embryonal mass and meristematic nodule-like structures were present in the suspensor (degeneration pattern ii). Frequently, new embryos started to differentiate from the nodule-like structures, and subsequently also these embryos started to degenerate into less organized embryos, causing a continuous loop of embryo degeneration and embryo differentiation. As revealed by Sytox® Orange staining, the degenerating embryos in cell line 3:10 showed clusters of dead cells in the apical part of the embryonal mass. This indicates that abnormal embryos in cell line 3:10 are partly degenerated before they differentiate new embryos, contrastingly to the abnormal embryos in cell line 12:12 that are eliminated.

3.2 Initiation of embryogenic cultures in conifers (II, III)

3.2.1 Picea

Seed germination marks the end of the embryonal development and rapid repression of embryonic genes is observed as the seeds start to absorb water (Tai et al. 2005). In Arabidopsis, the *LEAFY COTYLEDON1* (*LEC1*) and *LEC2* genes, as well as *FUSCA3* (*FUS3*) are required to maintain the embryonic stage, but they must be down-regulated to allow germination (Braybrook et al. 2008). Furthermore, *ABSCISIC ACID INSENSITIVE3* (*ABI*3) and its ortholog *Viviparous-1* (*VP1*) in maize promote embryo maturation (To 2006). Histone deacetylases (HDACs) are involved in the suppression of embryogenic properties after germination by repressing embryonic genes like *LEC1* and *ABI*3 (Tanaka et al. 2008). When treating seeds from Arabidopsis with the HDAC inhibitor trichostatin A (TSA), germination is inhibited simultaneously as the expression of *LEC* genes and *ABI1* are activated, and embryo-like structures start to differentiate (Tanaka et al. 2008). Furthermore, ectopic expression of *LEC1* in Arabidopsis stimulates differentiation of embryo-like structures in seedlings (Lotan et al. 1998). The molecular mechanisms involved in the transition from a differentiated vegetative cell to a cell with embryogenic competence have been studied in Arabidopsis but are largely uncharacterized in conifers.

In order to estimate the possibilities to activate the embryogenic potential in vegetative cells in conifers by manipulating the expression of a *LEC1*-type gene, we isolated a *LEC1*-type gene in Norway spruce (*PaHAP3A*) and examined its expression during development of somatic embryos. The

expression of *PaHAP3A* was high in proliferating embryogenic cultures and in early and late somatic embryos, but low in mature somatic embryos. In contrast, the expression of the Norway spruce homologue to *VP1* (*PaVP1*) was low during early embryo development, but high in late and mature embryos. Embryogenic cultures exposed to TSA during the maturation treatment continued to proliferate, and no mature embryos were formed. During the whole maturation treatment the expression level of *PaHAP3A* remained high and that of *PaVP1* remained low.

Embryogenic cultures from *Picea* species, including Norway spruce, are usually initiated from differentiated cells in mature embryos, after the cells have been stimulated to dedifferentiate (Mo et al. 1996). The initiation frequency of embryogenic cultures in Norway spruce is usually high, commonly between 50-80% depending on genotype (Högberg et al. 1998). However, as the embryos germinate, the potential to initiate embryogenic cultures decreases successively (Bonga et al. 2010; Klimazewska et al. 2010). In order to find out if TSA-treatment affects the embryogenic potential in Norway spruce, we germinated cotyledonary somatic embryos for 10 days on medium supplemented with TSA, before stimulating initiation of embryogenic tissue. On average 35 % of the germinated control embryos, not treated with TSA, differentiated embryogenic tissue. The germination progression was partially inhibited when the embryos were exposed to TSA. However, on average 85% of the TSA-treated embryos differentiated embryogenic tissue, which is similar to the initiation frequency from non-germinated cotyledonary embryos. We further tested if TSA-treatment could affect the embryogenic potential of embryos that had already germinated for 10 days. The germinating embryos were exposed to TSA for 5 days before they were stimulated to initiate embryogenic tissue. The initiation frequency was on average 22% among the TSA-treated germinating embryos, which was significantly higher than the average initiation frequency of 5% in the germinating control embryos. Thus, TSA-treatment during germination both retards germination and maintains the embryogenic potential. These results are in accordance with what has been previously shown in Arabidopsis (Tanaka et al. 2008), suggesting that TSA affects germination and the embryogenic potential in a similar way in Arabidopsis and Norway spruce.

Overexpression of *PaHAP3A* did not increase the embryogenic potential in germinated somatic embryos of Norway spruce (Uddenberg et al. 2016), which is in accordance with what previously has been shown in germinated embryos of white spruce overexpressing a *HAP3A* gene (Klimazewska et al. 2010).

Taken together, TSA-treatment affects the expression of *PaHAP3A* during maturation treatment, and maintains the embryogenic potential in germinating embryos. However, if the embryogenic potential has been lost, it is not possible to regain it, and overexpression of *PaHAP3A* does not increase the embryogenic potential in germinated embryos.

3.2.2 Pinus

Embryogenic cultures of several *Pinus* species are initiated from isolated megagametophytes containing immature zygotic embryos at the stage of cleavage polyembryony (Keinonen-Mettälä et al. 1996; Häggman et al. 1999; Burg et al. 2007; Lelu-Walter et al. 2008). It has been suggested that the cleavage process continues during both the initiation phase and in proliferating embryogenic tissue (Bozhkov et al. 1997; Park et al. 2006), and that the low quality of the cotyledonary embryos is caused by this continuation of the cleavage process (Klimaszewska et al. 2007). It has previously been reported that somatic embryos are successively formed from one or several zygotic embryos that protrude from the micropylar end of the megagametophyte (Finer et al. 1989; Becwar et al. 1990; Liao et al. 1995; Pullman et al. 2003; Lara-Chavez et al. 2011). These somatic embryos will multiply and form proliferating embryogenic tissue.

In order to find out, in more detail, how embryogenic cultures of Scots pine are initiated, we analyzed the embryogenic tissue during the initiation phase. The very first embryogenic tissue that protruded at the micropylar end was composed of a degenerating embryo. Dead cells were detected in the suspensor as well as in the elongated vacuolated cells that differentiated from the embryonal mass. As the embryogenic tissue started to proliferate, it consisted of embryogenic cell aggregates (stage 1a) as well as early embryos. A large proportion of dead cells was detected in both the embryogenic cell aggregates and in the early embryos. Our observations show that the initial protruding zygotic embryo(s), starts to degenerate before new embryogenic cell aggregates are formed, indicating that the initiation of embryogenic cultures in Scots pine is not a direct continuation of the cleavage process. New embryos, which differentiated from the cell aggregates, degenerated into less organized embryos similar to somatic embryos that degenerate according to degeneration pattern (ii). This suggests that the continuous loop of embryo degeneration and differentiation, which is characteristic for cell lines giving rise to abnormal cotyledonary embryos, starts already at the stage of initiation. As mentioned before only 3.4% (11 out of 325) of the established embryogenic cell lines in Scots pine gave rise to normal cotyledonary embryos. Furthermore, it has to be

kept in mind that the method we used for analyzing the first tissue protruding from the megagametophyte is destructive, and therefore, it has not been possible to estimate if this specific tissue would give rise to a normal or an abnormal cell line. Based on our results we conclude that the risk to establishing abnormal cell lines in Scots pine, and probably in most *Pinus* species, is high when the embryogenic cultures are initiated from immature zygotic embryos at the cleavage stage.

So far, it has not been possible to initiate embryogenic tissue from mature zygotic embryos of Scots pine (Keinonen-Mättälä et al. 1996; Häggman et al. 1999; Burg et al. 2007; Lelu-Walter et al. 1999; unpublished). To test if TSA-treatment could affect initiation of embryogenic tissue in Scots pine, we analyzed the embryogenic potential in cotyledonary zygotic embryos and cotyledonary somatic embryos of cell line 12:12 and 3:10 after treatment with TSA. The TSA-treatment did not stimulate differentiation of embryogenic tissue from cotyledonary zygotic embryos or from cotyledonary somatic embryos from cell-line 12:12. However, embryogenic tissue was initiated from more than 70% of the cotyledonary embryos from cell line 3:10 after TSA-treatment, while no embryogenic tissue was formed from embryos not treated with TSA. The embryogenic cultures derived from the TSA-treated embryos showed the same developmental and degeneration pattern, as well as the same phenotype of cotyledonary embryos as in the original cell line 3:10. In Norway spruce, we found that TSA-treatment could maintain the embryogenic potential but not restore the embryogenic potential once it had been lost. As expected, our results showed that the embryogenic potential was lost already in cotyledonary zygotic embryos of Scots pine and in cotyledonary somatic embryos from cell line 12:12, while the abnormal cotyledonary embryos in cell line 3:10 still retained the embryogenic potential. However, it remains unclear if the continuous loop of embryo degeneration and embryo differentiation in embryogenic cultures of Scots pine would persist even if the embryogenic tissue would be initiated from normal cotyledonary embryos.

3.3 Transcriptome profile analysis of early stages during embryogenesis in Scots pine (IV, unpublished)

3.3.1 Zygotic embryogenesis

We have shown that a high proportion of early embryos in abnormal cell lines starts to degenerate, giving rise to a continuous loop of embryo degeneration and embryo differentiation. We speculate that the abnormal cell lines do not develop embryos comparable to dominant zygotic embryos, but rather remain

at a stage of persistent cleavage polyembryony. In order to improve the protocols for somatic embryogenesis in Scots pine as well as in other *Pinus* species, it is crucial to understand how the cleavage process is regulated and how the development of a dominant embryo and the suppression of the subordinate embryos is controlled.

To our knowledge, there are no large-scale studies available about the molecular regulation of early zygotic embryo development in *Pinus*. We therefore performed a genome-wide high-throughput transcriptome sequencing of the earliest stages during zygotic embryogenesis in Scots pine. Zygotic embryos and megagametophytes were collected at four different developmental stages. The four developmental stages are presented in figure 1 in paper IV. The seed transcriptome sequencing was performed using the sequencing technique 454-Roche which generated 6.6 million raw reads that were assembled *de novo* into 121,938 transcripts. Of these transcripts, approximately 80,000 had a detectable expression level, and of these, 36,036 contained open reading frames.

Conifers and other gymnosperms are difficult experimental systems owing to the few sequenced genomes and few functionally characterized proteins. Most data sets that are available today are derived from angiosperms and are far from optimal for the annotation of *Pinus* species. For instance, the best studied plant species, Arabidopsis, is an annual angiosperm with a very small genome (135 MBP) (The Arabidopsis Genome Initiative 2000) whereas Scots pine is a gymnosperm tree with a very large genome (24.6 GBP) (Grotkopp et al. 2004). Furthermore, embryogenesis in Arabidopsis differs in many ways from embryogenesis in *Pinus* species, for instance in not having cleavage polyembryony. It is therefore not surprising that many of our identified coding transcripts either lacked a homologue in Arabidopsis or were annotated to a gene in Arabidopsis with unknown function. As a consequence, our study has been based on only a limited part of the seed transcriptome.

Our approach in this study was, despite the limitations, to identify genes and putative processes involved in the initiation of cleavage polyembryony and in the development of a dominant embryo.

For identification of candidate genes, we began by performing pairwise comparisons between the transcripts showing the largest differences in abundance between embryos and megagametophytes at each developmental stage. Among the differentially expressed transcripts, 22 candidates were carefully selected, based on interesting expression profiles, and on their annotation to genes already known to be involved in embryogenesis in plants. It should be noted that from hereon for convenience I shall refer each Scots

pine gene to the Arabidopsis gene that it shares most sequence similarity with. By quantitative RT-PCR analysis we analyzed the expression of the selected genes in three to four biological replicates. Based on the expression profiles of different genes we identified putative processes that might be involved in the initiation of the cleavage process and the development of a dominant embryo. Some of the putative processes are presented in Table 2.

Table 2. *Putative processes occurring during early zygotic embryo development. The relative transcript level of selected transcripts during early embryo development was analyzed by quantitative RT-PCR analysis (Paper IV). Following stages were included: E1, a single embryo before cleavage; E2, an embryo at the stage of cleavage; E3DO, a dominant embryo; E3SU, subordinate embryos; E4, a dominant embryo just before cotyledon differentiation. Based on the mRNA abundance of the transcripts during different embryo developmental stages we have suggested that they are involved in stage-specific processes. + indicates the developmental stage with the highest abundance of each transcript*

Process	Developmental stage					Transcript
	E1	E2	E3DO	E3SU	E4	
Cleavage polyembryony	+					*SERK1*
						TT7
						EXPB1
Repression of development of the dominant embryo	+	+				*DFL1*
						CYP78A7
Apical-basal polarization			+		+	*ANAC009*
						FAMA
Radial polarization		+	+	+	+	*PDF2*
	+					*LTP4*
Differences between dominant and subordinate embryos	+					*HAP3A*
		+		+		*AIL5*
			+		+	*VP1*

Cleavage polyembryony

The Scots pine homologues to *SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1* (*PsSERK1*), *TRANSPARENT TESTA 7* (*PsTT7*), and the expansin gene *EXPB1* (*PsEXPB1*) all had a significantly higher expression in early embryos (E1) than at later stages. Arabidopsis *SERK1* is important for the competence of a cell to form an embryo (Hecht et al. 2001). The Arabidopsis *TT7* encodes the enzyme flavonoid 3´hydroxylase which converts kaempferol to quercetin (Schoenbohm et al. 2000; Lewis et al. 2001). It has been shown that quercetin inhibits auxin transport and elongation growth (Jacobs and

Rubery 1988, Lewis et al. 2001). Furthermore, the loosening of the cell wall is required for cell separation (Chen et al. 2001). We assume that the high expression of *PsSERK1*, in early embryos at the stage before cleavage, is important for starting the cleavage process by stimulating the four apical cells to differentiate into four separate embryos; that the down-regulation of *PsTT7*, at the stage of cleavage, activates auxin transport and allows further development of the four embryos; and that up-regulation of the expansin gene *PsEXPB1* allows the embryo to cleave into four tiers, by loosening up the cell walls.

Repression of development of dominant embryo

In early embryos (E1) and in embryos at the stage of cleavage (E2), the Scots pine homologues to *DWARF IN LIGHT 1* (*PsDFL1*) and *CYTOCHROME 78A7* (*PsCYP78A7*), had an expression that was significantly higher than at later stages. The gene product of *DFL1* inhibits cell elongation in shoots and hypocotyls in Arabidopsis (Nakazawa et al. 2001), and overexpression of a *CYP78A* family member in rice (*Oryza sativa*) reduces the size of the embryo (Yang et al. 2013). Although the knowledge of the functions of these genes is limited, it is tempting to assume that a high expression of the genes at the cleavage stage restricts the development of the dominant embryo.

Apical-basal polarization

The Scots pine homologues to *NAC DOMAIN CONTAINING PROTEIN 9 (PsNAC009)* and *FAMA* (*PsFAMA*) had a significantly higher expression in dominant embryos (E3DO and E4) than in early embryos (E1 and E2) and in subordinate embryos (E3SU). An early embryo develops along the apical-basal axis to establish the shoot and root meristem. This patterning requires a highly regulated spatio-temporal cell division. In Arabidopsis, *ANAC009* regulates the cell division plane in the root cap (Willemsen et al. 2008) and *FAMA* regulates the switch between cell division and cell differentiation in stomata (Ohashi-Ito and Bergmann 2006). The high expression of *PsNAC009* and *PsFAMA*, in dominant embryos might reflect how important a correct cell division pattern is in the basal cells in the embryonal mass during the development of a dominant embryo.

Radial polarization

In early embryos (E1), the Scots pine homologue to *PROTODERMAL FACTOR2* (*PsPDF2*) was expressed at a significantly lower level than in embryos at later stages. Contrastingly, the Scots pine homologue to a lipid

transfer protein gene (*PsLTP4*) was significantly more highly expressed in early embryos (E1) than in embryos at later stages. Differentiation of the protoderm is essential for normal patterning during embryo development (Goldberg et al. 1994). The Norway spruce *HOMEOBOX 1* (*PaHB1*) gene, belonging to the same HD-ZIP IV family as *PROTODERMAL FACTOR2 (PDF2)* in Arabidopsis, is important for protoderm specification in somatic embryos of Norway spruce (Ingouff et al. 2001). Furthermore, the expression of a putative lipid transfer protein (*LTP*) gene, *Pa18*, is specifically expressed in the protoderm in developing somatic embryos of Norway spruce (Sabala et al. 2000). It is tempting to assume that the increase in expression of *PsPDF2* from the cleavage stage is correlated with the specification of the protoderm and that down-regulation of *PsLTP4* is a consequence of the expression of the gene being restricted to the protodermal cells. Interestingly, the expression profile of both genes is similar in dominant and subordinate embryos, indicating that subordinate embryos have a normal radial polarization.

Differences between dominant and subordinate embryos
The expression of *PsHAP3A* is low in dominant and subordinate embryos, suggesting that both types of embryos have entered the maturation phase. However, the high expression of a putative *AINTEGUMENTA-like 5* gene (*PsAIL5*) which is supposed to maintain embryonic identity (Tsuwamoto et al. 2010), in subordinate embryos but not in dominant embryos, indicates that the transition from the morphogenic phase to the maturation phase is disturbed in subordinate embryos. Furthermore, *PsVP1* is up-regulated in dominant embryos (E3DO and E4) but not in subordinate embryos, which supports that the subordinate embryos have not reached the maturation phase.

Taken together, we have identified genes whose expression profiles correlate with important processes during early zygotic embryo development in Scots pine. We therefore suggest that: (*i*) *PsSERK1*, *PsTT7* and *PsEXPB1* might be important for the cleavage process; (*ii*) *PsDFL1* and *PsCYP78A7* might be important for the repression of the development of a dominant embryo; (*iii*) *PsANAC009* and *PsFAMA* might be important for the apical-basal polarization of the embryo; (*iiii*) *PsPDF2* and *PsLTP4* might be important for the radial polarization of the embryo.

3.3.2 Somatic embryogenesis

In order to further identify differences between normal and abnormal embryogenic cell lines of Scots pine, the expression levels of *PsSERK1*,

PsTT7, *PsEXPB1*, and *PsCYP78A7* were analyzed by quantitative RT-PCR, during six consecutive developmental stages of somatic embryos from cell line 12:12 and 3:10.

Table 3. *Expression pattern of PsCYP78A7, PsSERK1, PsTT7, and PsEXPB1 during early somatic embryo development in cell line 12:12 giving rise to cotyledonary embryos with normal morphology, and cell line 3:10 giving rise to cotyledonary embryos with abnormal morphology. The relative transcript levels of PsSERK1, PsTT7, and PsEXPB1 were analysed by quantitative RT-PCR in three biological replicates. Six consecutive developmental stages were tested: S1a – Stage 1a, proliferating embryogenic cell aggregates; S1b – Stage 1b, slightly globular structures with densely packed cells covered by a smooth surface; S1c – Stage 1c, early embryos that start to differentiate; S2 – Stage 2, early embryos; S3- Stage 3, late embryos; S4- Stage 4, late embryos just before cotyledon differentiation. Plus signs indicate the relative expression level of each transcript during different developmental stages. Different number of + indicate significant differences in the expression level, where "+(+)" indicates that the expression level is significantly different neither from + nor from ++*

Transcript	Cell line	Developmental stage					
		S1a	S1b	S1c	S2	S3	S4
CYP78A7	12:12	++	++	+	+	+	+
SERK1		+	++	+	+	+	+
TT7		++	+++	+	+(+)	+	+
EXPB1		+	++	n.d.	++	+	+
CYP78A7	3:10	++	+	+	+	+	+
SERK1		++	+	+	+	n.d	n.d
TT7		+(+)	++	++++	+(+)	++(+)	+
EXPB1		+	++	+++	++	+(+)	+

No expression detected indicated by n.d

The expression profile of *PsCYP78A7*was similar in both cell lines, but for *PsSERK1, PsTT7* and *PsEXPB1* the expression profiles were different (Table 3). In cell line 12:12, the significantly highest expression of *PsSERK1* was detected at developmental stage 1b, and *PsTT7* was significantly down-regulated when early embryos start to differentiate at stage 1c. Expression of *PsEXPB1* was detected at all developmental stages except early embryos at stage 1c. Contrastingly, in cell line 3:10, *PsSERK1* was significantly highest expressed at developmental stage 1a, and *PsTT7* was significantly down-regulated in early embryos at stage 2. Furthermore, the significantly highest expression of *PsEXPB1* was detected at stage 1c.

PsSERK, PsTT7 and *PsEXPB1* were all identified in the transcriptome profile analysis of early zygotic embryos (3.3.1) as genes involved in the

putative process of cleavage polyembryony. We find it very interesting that the expression profile of these genes differed between the normal cell line 12:12 and the abnormal cell line 3:10.

4 Conclusion

A high frequency of early and late somatic embryos in abnormal cell lines carries supernumerary suspensor cells, which results in an unbalanced ratio between the embryonal mass and the suspensor. The presence of supernumerary suspensor cells is at least partly caused by disturbed polar auxin transport.

A high proportion of early and late somatic embryos degenerate in both normal and abnormal cell lines of Scots pine. During normal somatic embryo development, the degenerating embryos are eliminated in a similar way as subordinate embryos are eliminated in the seed. In contrast, during abnormal embryo development the degenerating embryos are not eliminated, instead the degenerated embryos start to differentiate new embryos.

During initiation of embryogenic tissue from early zygotic embryos at the stage of cleavage polyembryony, the zygotic embryos are initially degenerated before an embryogenic culture is established. Therefore, the initiation of embryogenic cultures does not seem to be a direct continuation of the cleavage process. Our results indicate that there is a high risk that cell lines initiated from early zygotic embryos become abnormal.

The expression of *PaHAP3A* is high during early somatic embryo development of Norway spruce, but decreases during late embryogeny. Treatment with the deacetylase inhibitor trichostatin A (TSA) during maturation of somatic embryos inhibits the maturation progression of embryos and maintains the expression of *PaHAP3A*. TSA-treatment of germinating and germinated embryos maintains existing embryogenic potential, but does not restore the embryogenic potential if it has already been lost.

Based on the study of global gene expression, during the earliest stages of zygotic embryo development in Scots pine, we suggest that: (i) the cleavage process is initiated when the four apical cells of the embryo are stimulated to differentiate into four separate embryos; auxin transport is activated; and loosening of the cell-walls allows the four tiers to cleave longitudinally; (ii) the growth of the embryos is restricted directly after cleavage, preventing one of the embryos from becoming dominant; (iii) correct apical-basal polarization is important for the development of a dominant embryo; (iiii) the subordinate embryos have a normal radial polarization.

5 Future perspectives

How somatic and zygotic embryos develop in *Pinus* species is still poorly understood. However, the studies reported in this thesis are an important base for further research in embryology of *Pinus*. The transcriptome study provides an enormous source of data which can be used for further investigations of the molecular regulation of early embryo development. Especially interesting are the novel transcripts, i.e. transcripts with no homologue in Arabidopsis or other angiosperm species, which might be unique to species belonging to *Pinus* (or conifers). Functional studies of these genes would greatly expand our understanding of the regulation of embryo development in *Pinus* species.

Functional studies of the candidate genes, identified in paper (IV), are at present conducted in embryogenic cultures of Norway spruce. In the next step it will be important to perform functional studies in Scots pine. This requires that an efficient protocol for transforming embryogenic cultures of Scots pine is developed. None of the standard protocols used for transformation of conifers has so far been applicable to Scots pine.

Since there is a high risk of establishing cell lines giving rise to abnormal cotyledonary embryos when the embryogenic cultures are initiated from zygotic embryos at the stage of cleavage polyembryony, it would probably be better to initiate embryogenic cultures from more differentiated tissues. To accomplish this, further research is needed for elucidating how to regain embryogenic potential in older tissues.

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