Changes in Global Gene Expression and Auxin Dynamics During Embryo Development in Conifers

Daniel Vestman
Faculty of Natural Resources and Agricultural Sciences
Department of Plant Biology and Forest Genetics
Uppsala

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Cover: Section of microarray used for Norway spruce
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Abstract

Conifers are of great economical importance, but our knowledge about how the development of conifers is regulated is limited. The over-all aim of this study has been to gain a better understanding of the molecular regulation of embryo development in conifers. This can in turn lead to improved methods in forest tree biotechnology and give insights in the evolution of land-plants. We have used somatic embryos for studying early stages during embryo development, with a focus on global changes in gene expression and auxin dynamics.

Global changes in gene expression during early embryo development in Norway spruce (*Picea abies*) was analyzed by using a microarray spotted with 12,536 cDNA clones. This allowed us to pinpoint important molecular events like the establishment of different domains and boundary regions, "nurse cells" having a megagametophyte signaling function and a switch from embryonal to vegetative development.

Utilizing the same microarray production used for Norway spruce, we studied comparable stages during Scots pine (*Pinus sylvestris*) early embryo development, and identified processes such as the initiation of pattern formation. A comparison with the Norway spruce microarray revealed a possible delay in the onset of programmed cell death and polar auxin transport. These are differences that could account for problems with recalcitrance in Scots pine.

Two auxin responsive reporter constructs were used to monitor the dynamics of auxin during embryo development in Norway spruce. We could show that auxin is first synthesized and accumulated in the suspensor of early embryos, and that the site of synthesis is shifted to the embryonal mass in late embryos while polar auxin transport relocates the auxin in a basal direction. In maturing embryos, auxin is synthesized in the basal part and distributed throughout the embryo by polar transport.

Taken together, this study has revealed important processes during embryo development in Norway spruce and Scots pine and also shown how auxin is dynamically distributed during embryo development in Norway spruce.

Keywords: Conifer, early embryogeny, late embryogeny, microarray, Norway spruce, Scots pine, somatic embryogenesis, polar auxin transport, GH3.

Author's address: Daniel Vestman, Swedish University of Agricultural Sciences,
Uppsala BioCenter, Department of Plant Biology and Forest Genetics,
P.O. Box 7080, 750 07 Uppsala, Sweden
E-mail: Daniel.Vestman@slu.se

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List of Publications

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

I Vestman, D., Larsson, E., Uddenberg, D., Cairney, J., Clapham, D., Sundberg, E., von Arnold, S. (2010). Important processes during differentiation and early development of somatic embryos of Norway spruce as revealed by changes in global gene expression. *Tree Genetics & Genomes* 7(2), 347-362.

II Vestman, D., Abrahamsson, M., Larsson, E., Cairney, J., Sundberg, E., Clapham, D., von Arnold, S. Global changes in gene expression during differentiation of early somatic embryos and development of late embryos in Scots pine. *Manuscript*

III Vestman, D., Larsson, E., Sundberg, E., von Arnold, S. Local auxin activity is crucial during embryo development in conifers. *Manuscript (preliminary)*

Paper I is reproduced with the permission of the publisher.

The contribution of Daniel Vestman to the papers included in this thesis was as follows:

I Ran all the analysis of the microarray data. Performed all qRT-PCR analysis. Participated in analyzing data and writing manuscript.

II Ran all the analysis of the microarray data. Performed all qRT-PCR analysis. Participated in analyzing data and manuscript writing.

III Participated in designing the project. Performed large part of the cloning and transformation work. Analyzed the results. Participated in writing the manuscript.

1 Introduction

Conifers are economically important to many countries as a source of material for the building and/or the paper and pulp industry. Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*) together account for more than 80 % of the Swedish growing stock (Skogsindustrierna, 2010).

Traditional forest tree breeding is a slow process where certain phenotypes are selected and used for crossings in continuous cycles. For Norway spruce and Scots pine, every cycle of selection takes 20-25 years. Selected trees with sought after properties are also used for mass propagation. Branches from such trees are grafted onto main stems and allowed to grow into trees in seed orchards where their seeds are collected to be used in forest plantation (Högberg et al., 1998).

Somatic embryogenesis (SE) is an attractive method to combine breeding and propagation programs. SE cultures are initiated from seeds of selected trees and induced to form embryos that develop into seedlings for planting. The SE cultures are also cryogenically stored so that genotypes that turn out to be suitable for forest production can be reused for clonal mass production. By in this way using the actual selected genotype for mass propagation instead of its seeds, loss of genetic gain is avoided and time is saved.

1.1 Embryogenesis in plants

Embryogenesis in plants is the developmental process from fertilization to the germination of a seedling. This normally takes place within a seed which provides protection and contains maternal tissue that supplies nutrients for the growing embryo. Features common for all plant embryogenesis are the early establishment of an apical-basal axis and the subsequent establishment of a radial symmetry in right angle to the apical-basal axis. This is followed by the establishment of several organs, like the shoot apical meristem (SAM) and the root apical meristem (RAM). These two spatially opposite meristems will be

responsible for producing a large part of the adult plant. As the cotyledon development commences, eudicots gain a bilateral symmetry while for instance many conifers conforms to a radial cotyledon symmetry. The lineages of gymnosperms and angiosperms separated from each other approximately 300 million years ago (Smith et al., 2010) and their respective processes of embryogenesis displays both similarities and differences. Features common among gymnosperms, but otherwise rare, include biparental organelle inheritance, polyembryony, multiple cotyledons and a haploid megagametophyte (reviewed by Vuosku et al., 2009).

The eudicot weed Arabidopsis serves as a model organism for plant studies in general (reviewed by Laux et al., 2004 and De Smet et al., 2010). The Arabidopsis embryo is relatively small and the cells divide in a tightly controlled manner, making it possible to build an accurate model of the morphogenic events. Studies of marker genes for an apical and basal cell fate show that the apical-basal polarity is established already in the newly created zygotic cell. This cell elongates and divides into an apical and a basal daughter cell. Divisions of the apical cell create an eight-cell globular embryo while the basal cell divides into the hypophysis and the extra-embryonal suspensor. The eight-cell state is formed after another round of cell division. Here, four regions with different developmental fates can be distinguished: The top half of the embryo proper will form the SAM and most of the cotyledons. The bottom four cells of the embryo proper will form the hypocotyl, part of the cotyledons, the root and part of the RAM. The central part, the hypophysis, will give rise to the distal RAM, the quiescent center and the root cap stem cells. The basal suspensor continually divides in the horizontal plane, pushing the developing embryo in to the lumen of the ovule. The differentiation of these distinct morphological patterns is initiated from transcriptional programs in precursor cells and refined as the embryo develops.

1.2 Somatic embryogenesis

Somatic embryogenesis is the process when an embryo is formed from somatic cells without the need for a fertilization event to occur. This was first described to have occurred in suspension culture of carrot (*Daucus carota*) (Steward, 1958). Since the development of somatic embryos resembles that of zygotic embryos, it can be used to study embryology. As an *in vitro* system, it allows researchers to control the progression of embryogenesis, enabling the collection of developmentally synchronized tissues for study. Somatic embryos are especially useful when studying plants like conifers that have a long generation time and also suffer from irregular flowering, making extensive

sampling difficult. Furthermore, SE can be used for large scale propagation, something already in commercial use for e.g. Nordmann fir (*Abies nordmanniana*). However, induction of somatic embryogenic cultures from other tissues than zygotic embryos is in most cases not possible, and in some species induction is not possible at all. The inability to induce an embryogenic culture from a certain species, tissue, developmental stage or genotype is called recalcitrance. More knowledge about the regulation of embryo development in general and somatic embryo development in particular, can help us overcome difficulties with recalcitrance and lead to improved methods of clonal propagation.

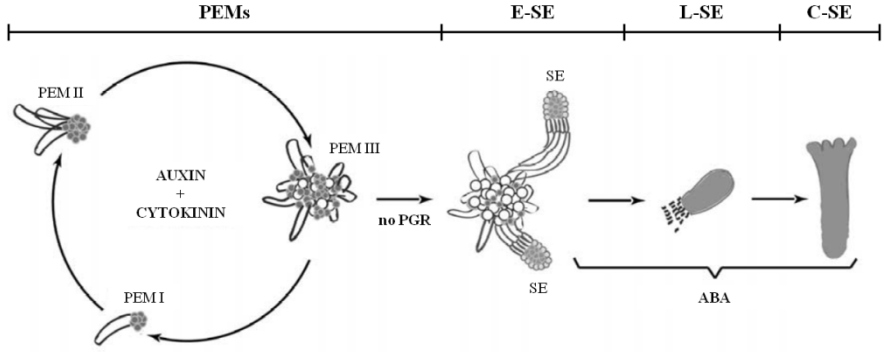

Figure 1. A schematic representation of SE in Norway spruce. The different developmental stages are proembryogenic masses (PEMs), early somatic embryo (E-SE), late somatic embryo (L-SE) and cotyledonary somatic embryo (C-SE). Reproduced with kind permission of Springer Science and Business Media: von Arnold et al., (2002), *Plant Cell, Tissue and Organ Culture*, 69 (3), 233-249.

1.2.1 Norway spruce

Embryogenesis in gymnosperms is usually divided into three successive stages (Singh, 1978). (1) proembryogeny takes place before elongation of the suspensor, (2) early embryogeny which takes place after elongation of the suspensor but before the establishment of any apical meristem, and (3) late embryogeny which start at the point of establishment of the root and shoot meristems, and throughout the following embryo development.

Norway spruce somatic embryos are initiated from mature zygotic embryos before desiccation, cultured on growth medium containing the plant growth regulators (PGRs) auxin and cytokinin. This treatment stimulates the differentiation of embryogenic tissue (von Arnold and Clapham, 2008). When the embryogenic culture has been established, it can be proliferated as proembryogenic masses (PEMs) under conditions similar to those of the

initiation phase (Filonova et al., 2000a). The PEMs go through three morphologically distinguished stages as they grow. A PEM I is made up of dense cytoplasmic cells in a lump, adjoining to a single vacuolated cell (Figure 1 PEMs). The addition of more vacuolated cells places the structure in the PEM II stage, which is notably bipolar in the organization of its two cell types (Figure 1 PEMs). Further proliferation, of primarily the densely cytoplasmic cells, creates the enlarged non-polar PEM III structures. (Figure 1 PEMs). Depletion of PGRs from the medium leads to development of early somatic embryos from the PEM III structures (Figure 1 E-SE). To initiate formation of late embryos and subsequent maturation, the embryos must be grown on ABA-containing medium (Figure 1 L-SE). During maturation, the SEs undergo morphological and biochemical changes, including differentiation of a SAM and a crown of cotyledons, and deposition of storage material (Figure 1 C-SE) (Filonova et al. 2000a).

In angiosperms, the formation of a distinct protoderm early in embryogenesis is essential for the succeeding development. A putative lipid transfer protein (LTP), Pa18, cDNA was isolated from Norway spruce (Sabala et al., 2000). *In situ* hybridization showed that it was first expressed ubiquitously in PEMs but then more extensively in the outer cell layer of SEs. Ectopic expression in transgenic sublines disturbed formation of embryos, the outer cell layer of the embryonal mass (EM) frequently becoming elongated and vacuolated. Similarly, the homeobox gene *Picea abies Homeobox1* (*PaHB1)* was shown to be expressed ubiquitously in PEMs but later restricted to the outer cell layer of Norway spruce somatic embryos (Ingouff et al., 2001). Furthermore, its ectopic expression in transgenic cell lines produced embryos with an EM lacking the normally smooth surface. Taken together these findings show that, similarly to angiosperms, the embryo development of Norway spruce is dependent on the correct formation of a distinct protoderm.

Programmed Cell Death (PCD) is an important process of embryogenesis in many organisms. When PGRs are depleted in a proliferating culture of Norway spruce, PCD eliminates those PEMs that do not embark on the path of early embryogenesis. PCD is also responsible for destruction of the elongated suspensor cells of the embryo (Filonova et al., 2000b). There are two main types of PCD in plants, vacuolar and necrotic cell death (van Dorn et al., 2011). Vacuolar cell death, which is the kind of PCD occurring in the Norway spruce suspensor, is distinguished by removal of the cell contents by lytic vacuoles and the massive release of hydrolases from the ruptured lytic vacuole membrane. A type II plant metacaspase cDNA was extracted from embryogenic cell culture of Norway spruce and named *mcII-Pa*. The metacaspases are related to the caspases, the primary PCD inducers in animals.

In situ hybridization showed accumulation of transcripts in suspensor cells of early embryos and in sites of vascular differentiation in mature embryos. When *mcII-Pa* was silenced with an RNAi construct, embryo formation was blocked (Suarez et al., 2004). Additional indications of caspase-like mechanisms comes from a study showing that the caspase-6 specific peptide substrate VEID-AMC is proteolytically cleaved in early stages of embryo development, a period of extensive cell death. Biochemical inhibition of this proteolysis prevents normal embryo development by disturbing suspensor differentiation and hindering PCD (Bozhkov et al., 2004). While Arabidopsis has nine metacaspases (AtMC1-9), is currently not known how many exist in Norway spruce. However, metacaspases are not only involved in PCD but also take part in such diverse processes as pathogen resistance and cell cycle regulation (Tsiatsiani et al., 2011).

It has been demonstrated in Arabidopsis that polar auxin transport (PAT) during embryo development is essential for the establishment of apical-basal polarity, correct cellular organization and organ differentiation. Larsson et al. (2008) demonstrated the importance of correct PAT during Norway spruce embryo development. They showed that the PAT inhibitor 1-N-naphtylphthalamic (NPA) caused aberrant phenotypes that resemble mutants affecting auxin synthesis, transport and response in Arabidopsis. These phenotypes include cotyledon defects, disruption of polarity and distortion of shoot and root meristems. In conclusion, they proposed that PAT plays an essential role in gymnosperm embryo development, as it does in angiosperm embryo development.

Four *KNOTTED1-like homeobox* (*KNOXI*) gene members have been identified in Norway spruce, *HBK1*, *HBK2*, *HBK3* and *HBK4*/*PaKN4* (Guillet-Claude et al., 2004; Hjortswang et al., 2002; Sundås-Larsson et al., 1998). Arabidopsis has four KNOXI genes: *SHOOT MERISTEMLESS* (*STM*), *BREVIPEDICELLUS* (*BP*), *Kn1-like in Arabidopsis thaliana2* (*KNAT2*) and *KNAT6*. Their main function is in maintaining the activity of the SAM and of its lateral organ and stem boundaries throughout development (reviewed by Hay and Tsiantis, 2010). Arabidopsis plants over-expressing the Norway spruce *HBK*-genes displayed phenotypes typical for *KNOXI* overexpressors in Arabidopsis (Larsson et al., 2012a) Transcriptional studies during the differentiation and development of normal embryos and embryos lacking a SAM due to NPA treatment indicated that *HBK1* and *HBK3* seem to have general functions during embryo development, while *HBK2* and *HBK4* are likely to be important for the formation of a functional embryonal SAM.

The NAC family of transcription factors is a large plant specific gene family involved in a large number of processes. The Arabidopsis NAC genes

CUP-SHAPED COTYLEDON 1 (*CUC1*) and (*CUC2*) are together with *STM* expressed in the incipient SAM as well as in the outlines of developing cotyledons (Aida et al., 1999). This expression is essential for SAM formation and cotyledon separation. Furthermore, Aida et al. (2002) suggest that the expression of *CUC1* and *CUC2* is repressed by auxin. Larsson et al. (2012b) cloned two CUC-like NAC genes from Norway spruce, named *PaNAC01* and *PaNAC02*. *PaNAC01* provided CUC2-function in Arabidopsis *cuc1*/*cuc2* double mutants and was shown to be PAT-regulated and associated with SAM and cotyledon formation. In summary, they suggest a conserved function between *PaNAC01* and CUC1/CUC2.

Stasolla et al. (2004) studied transcription levels in two embryogenic cell lines of Norway spruce, one with normal development and one with a developmental arrest at the PEM to early somatic embryo transition. A microarray containing 2,178 expressed sequence tags (ESTs) from loblolly pine (*Pinus taeda*) was used. They showed that the normal line displayed a distinct transcriptional pattern of general repression followed by an induction. This pattern was not present in the developmentally arrested line. Comparing the sets of differentially expressed genes produced from the arrays of the normal and the blocked lines pinpointed some processes potentially important for correct developmental progression. These included detoxification of reactive oxygen species, transmethylation events and carbohydrate metabolism in connection with cell wall modifications.

1.2.2 Scots pine

The developing embryos of many species of *Pinus*, but not of *Picea*, undergo a process of multiplication within the seed, called cleavage polyembryony. One of the embryos usually become dominant and the other subordinate embryos are degraded by PCD and are reabsorbed by the megagametophyte (Filonova et al., 2002b). Embryogenic cell lines of Scots pine are usually initiated from early zygotic embryos that are in the process of multiplying by cleavage polyembryony and it is thought that the establishment of a proliferating culture is an extension of the polyembryony phenomenon (Bozhkov et al., 1997). Scots pine cell lines can be induced to form embryos through a similar process as described for Norway spruce. Unfortunately, many species in the genus Pinus, including Scots pine, suffer from a low frequency of successful initiations of embryogenic cell lines, and also from poor quality of cotyledonary embryos (Bonga et al., 2010).

Abrahamsson et al. (2011) described the developmental path of embryos from a cell line that produced mostly normal cotyledonary embryos. Over 50% of the somatic embryos carried more than four cotyledons and most of them

had a shoot meristem. This was compared to two cell lines where a high proportion of early and late embryos carried supernumerary suspensor cells and many of the developed cotyledonary embryos had less than four cotyledons and aborted or abnormal hypocotyl. Treatment with the PAT inhibitor NPA during pre-maturation and maturation caused an increase in embryos carrying supernumerary suspensor cells in the normal cell line. Moreover, in both the normal and one of the abnormal cell lines, the proportion of embryos carrying partially or severely fused cotyledons increased after NPA treatment. They suggested that disturbed PAT might lead to stimulated division of meristematic cells at the base of the EM giving rise to extra suspensor cells. The resulting imbalance between the suspensor and the EM might further effect the signaling within the embryo. Additionally, when comparing levels of PCD in Scots pine to levels reported for Norway spruce (Helmersson et al., 2008), they found much higher levels in proliferating embryogenic cultures. It was suggested that the mechanism responsible for eliminating subordinate zygotic embryos by PCD is still active in embryogenic cultures of Scots pine. Also, in Norway spruce it is essential for proper development that PCD increases during differentiation of early embryos, and no such increase was found in Scots pine. They suggested that the unbalance between suspensor and EM and the potential lack of PCD-regulation might be the cause of recalcitrance in Scots pine somatic embryo cultures.

1.3 Auxin control of plant embryogenesis

The phytohormone auxin plays an important role in plant embryo development. It has been most thoroughly studied in Arabidopsis. Mutants that are defective in biosynthesis, transport, perception or response to auxin all display severe phenotypes during embryo development (Möller and Weijers, 2009).

1.3.1 Biosynthesis

The dynamics of auxin sources in plants are complex. There are likely many pathways of indole-3acetic acid (IAA) biosynthesis, and in addition, IAA can be released from several inactive conjugated forms (reviewed by Zhao, 2010). The two main pathways of synthesis are the tryptophan (Trp) -dependent and the Trp-independent pathways. The best understood, the Trp-dependent pathway, follows two routes. One dependent on the *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS* (*TAA1*) and *TRYPTOPHANE AMINOTRANSFERASE RELATED* (*TAR*) *1* and *2*, and one dependent on the *YUCCA* (*YUC*) family of flavin monooxygenases. Mutants with disruptions in either the Trp-dependent or the Trp–independent pathway show similar

phenotypes, indicating that they act non-redundantly to ensure sufficient auxin levels for proper pattern formation.

1.3.2 Transport

The effects of auxin in plant development are dependent on its correct concentration gradients and local maxima. This is largely accomplished by asymmetric distribution of the efflux carrying PIN-FORMED (PIN) proteins. There are eight members of the PIN-family in Arabidopsis and six of these have been functionally characterized. (reviewed by Křeček et al., 2009). Based on their secondary structure the PIN-family is divided into two subfamilies: The long PINs that are responsible for transport of auxin out of the cell, and the short PINs that mediate intracellular compartmentalization and homeostasis of auxin. The localization of PIN proteins is dependent on their state of phosphorylation (reviewed by Möller and Weijers, 2009). Apical membrane localization is caused by phosphorylation, while basal localization is due to dephosphorylation. Several PIN proteins are involved in Arabidopsis embryo development and multiple PIN-mutations have a cumulative effect on the phenotype.

1.3.3 Perception

Auxin is perceived through the action of the plant hormone receptor TRANSPORT INHIBITOR RESPONSE 1 (TIR1). The small IAA molecule mediates the binding of the TIR1 part the SCF^{TIR1} ubiquitin-ligase complex to the AUX/IAA transcriptional regulators. IAA enhances the substrate binding affinity by filling a grove between the proteins and acting as a molecular glue (Tan et al., 2007). This binding induces a ubiquitin dependent proteolysis of the AUX/IAA, which often acts as a transcriptional repressor of Auxin Response Factors (ARFs). The synthetic auxins NAA and 2,4-dichlorophenoxyacetic acid (2,4-D) are also recognized by TIR1 although their molecular structures differ somewhat from that of IAA (Tan et al., 2007).

1.3.4 Auxin reporters

Hagen et al. (1991) demonstrated the use of the soybean *GRETCHEN HAGEN 3* (*GH3*) auxin inducible promoter as a reliable auxin reporter. The GH3 protein belongs to a family of enzymes that synthesize a variety of IAA-amino acid conjugates (Staswick et al., 2005). Reasons for using *GH3* include its rapid induction process where induction can be detected within five minutes (Hagen and Guilfoyle 1985). This induction is also specific in that it is only induced by active auxins (Hagen et al., 1991). Moreover, it is dose-dependent for auxin concentrations and has a generally low expression, making specific

expression easy to detect (Li et al., 1999). Synthetic auxin-inducible promoters, such as *DR5*, have also been constructed using multimers of the auxin responsive element (*AuxRE*) motifs from the *GH3* promoter (reviewed by Chapman and Estelle, 2009).

1.3.5 Auxin dynamics in angiosperm embryo development

The localization of PIN1, 4 and 7 in the Arabidopsis embryo supports the picture of auxin localization obtained by means of the auxin responsive reporter construct *DR5* (Friml et al., 2003). As *DR5* expression is detected in the apical cell of the newly divided zygote and in the apical cells of the eight-cell embryo, PIN7 is localized at the apical membrane of the underlying basal cells. This localization acts to drive auxin towards the apical part. Up to the sixteen-cell stage, PIN1 is localized in the apical proembryo but without apparent polarity. At the globular stage, when *DR5* is expressed in the hypophysis and in the topmost suspensor cell, PIN7 is localized in upper suspensor, but flips its polarity to drive auxin efflux downwards. At this stage PIN1 has gained a basal position in the central cells of the proembryo, while taking an apical position in the newly formed protoderm. These patterns of PIN1 localization are maintained in the transition state, where its apical polarity in the protoderm facilitates the formation of auxin maxima at the points of the emerging cotyledons. The central PIN1 presence keeps auxin transport in a downward direction from the prevasculature. Except for auxin maxima at the emerging cotyledons, the earlier basal maximum is maintained in the transition stage. At this stage, PIN7 is continually located at the basal side of the suspensor cells. A third protein, PIN4, localizes to the hypophysis at the globular stage and shifts to the hypophysis lens shaped daughter cell during the transition state.

PIN-mutants share phenotypes with other auxin related mutants (reviewed by Möller and Weijers, 2009). Already in the one-cell embryo, *pin7* causes transverse instead of longitudinal division of the apical cell. Throughout the subsequent embryo development, *pin1*, *pin3*, *pin4* and *pin7* all cause aberrant cell division in the hypophysis, vascular cells and the RAM. Also, formation and/or separation of cotyledons is impaired.

1.3.6 Auxin dynamics in Norway spruce embryo development

Palovaara et al. (2010) identified at least four PIN genes in conifer EST data bases. However, based on the large number of angiosperm PIN genes, more are expected to be found as more sequence data accumulates. Based on available EST sequences, they cloned a PIN gene from Norway spruce somatic embryos. This gene, *PaPIN1*, was the only one expressed in samples from embryos. *In*

situ hybridization located the *PaPIN1* transcript to the epidermal cell layer of developing embryos. During late embryogeny, *PaPIN1* was expressed in pre-procambial cells and the root apex, and in late cotyledonary embryos, in procambium from the cotyledon tips down to and through the root apex and further down the columella cells in the root cap. This correlated roughly with the staining pattern of an anti-IAA antibody, suggesting that PaPIN1 is involved in PAT. Treatment with the PAT inhibitor NPA disrupted the observed patterns, further linking PaPIN1 to PAT. Phylogenetic analysis clustered *PaPIN1* with the Arabidopsis *PIN3*, *PIN4* and *PIN7*, but localization indicates a functional resemblance to *PIN1*. In globular embryos of Arabidopsis, PIN1 together with PIN4 and PIN7 is expressed with basal polarity in the procambium, driving auxin flux towards the basal embryo pole (Friml et al., 2003). It has been suggested that PAT induces the formation of the procambium in Arabidopsis. Palovaara et al. (2010) suggested that *PaPIN1* mediated PAT could be responsible for inducing formation of procambial cells and RAM in Norway spruce.

Treatment of early embryos with the PAT inhibitor NPA resulted in elevated levels of endogenous IAA of early embryos, and doubled the frequency of early embryos that differentiated (Larsson et al. 2008). The increased number of differentiated embryos was also increased when the treatment was conducted during maturation, although not as drastically. NPA treatment also caused severe phenotypic effects. Early embryos carried supernumerary suspensor cells and meristematic cells in the suspensor, giving the embryos a cone-shape. Some extreme phenotypes were ball-shaped and lacked any perceivable polarity. A wide range of aberrations was observed in maturing embryos, somewhat dependent of when during development that the NPA treatment had been administered. Phenotypes included were: partially or completely fused cotyledons, abnormal or absent SAM, distorted and/or split basal region, intercellular spaces and a less organized root pole and root cap. PCD, which is required for normal Norway spruce embryo development, was repressed in NPA treated embryos. In conclusion, they demonstrated the importance of correct PAT during Norway spruce embryo development and that the aberrant phenotypes caused by NPA resemble auxin-related mutants in Arabidopsis. They propose that the mechanisms of PAT are conserved between angiosperms and gymnosperms.

1.4 Global Gene expression during embryo development

Microarray analysis has proven a useful method for screening transcriptional dynamics of a large number of genes. Affymetrix arrays are probably the most used in plant science but various commercial and academic microarray platforms exist with different coverage and technical solutions (Baginsky et al., 2010). In conifers, most large scale transcriptional investigations have been performed on loblolly pine (*Pinus taeda L.*). However, is has been shown that Norway spruce and Scots pine mRNA are both able to hybridize to loblolly pine arrays and yield valid data (van Zyl et al., 2002). Hi-throughput sequencing of mRNA (RNA-Seq) offers a new alternative to hybridization based methods. RNA-Seq is not dependent on previous knowledge of genome or transcriptome data and is thus useful when working with non-model species.

2 Aims of the study

The over-all aim of this study is to gain a better understanding of the molecular regulation of embryo development in conifers. This can in turn lead to improved methods in forest tree biotechnology and insights in the evolution of land-plants.

Specific aims were to investigate:

- changes in global gene expression during differentiation of early and development of late embryos in Norway spruce.
- changes in global gene expression during differentiation of early and development of late embryos in Scots pine.
- auxin dynamics during embryo development in Norway spruce.

3 Results and Discussion

3.1 Important processes during differentiation and early development of somatic embryos in Norway spruce

We have extended the study of early events during embryogenesis in Norway spruce by using microarray slides spotted with 12,536 cDNA clones from loblolly pine cDNA libraries (somatic and zygotic embryos at different developmental stages and megagametophytes). We have focused on the first stages of embryogenesis: the differentiation of early embryos from proembryogenic masses (PEMs) and the beginning of the development of late embryos. Few such studies have been undertaken and the work which we performed is the first comprehensive analysis of gene expression of a conifer species during early stages of SE.

The cell cultures investigated using the array were collected (1) one week after transfer to PGR containing media, comprising of PEM-structures, (2) 24 h after withdrawal of PGRs, still comprising of PEM-structures, (3) one week after withdrawal of PGRs, when early embryos had started to differentiate and (4) one week after transfer to ABA containing medium, when most embryos had continued to develop into late embryos. Since verified annotations are lacking for most conifer sequences, we have used the nomenclature of the most similar Arabidopsis gene. To verify the microarray methodology, six genes were selected for qRT-PCR using the same material as used on the array. The analysis showed a consistency of differential up- and down-regulations. qRT-PCR was also used to validate the biological relevance of the microarray. The expression of six genes, representing six processes, was investigated in a cell line different from the one used for the array with the conclusion that the experiment could be trusted.

3.1.1 Differentially expressed genes and what they reveal

All differentially expressed genes were mapped to functional GO terms in the category describing biological processes. This revealed a general over-representation of genes involved in responses to stress throughout embryogenesis. In the transition from early to late embryos, genes involved in metabolism were under-represented while those involved in catabolic processes, carbohydrate metabolic and GA-mediated signalling were over-represented.

PCD

24 h after withdrawal of PGRs, genes regulating PCD (like *METACASPASE9*) were up-regulated. The indication of PCD is in accordance with earlier studies of the PCD-machinery in Norway spruce (Bozhkov et al., 2004). PCD-mediated degradation of the suspensor is required for proper development of embryos. The early activation of genes involved in PCD showed that the PCD-dependent degradations necessary for proper differentiation starts as soon as early embryos begin differentiating.

Embryo differentiation

Already 24 h after PGR withdrawal, a gene required for a functional suspensor (*TMP-C*) was up-regulated indicating the immediate switch from proliferation to embryo differentiation. It has been shown that some cell-clusters in Norway spruce tissue cultures exhibit transcriptional traits similar to the maternal megagametophyte, and they have been called nurse cells (Wiweger et al., 2003). Accordingly, we found differentially expressed genes known to be involved in signalling between maternal tissue and embryo, like *NAC-REGULATED SEED MORPHOLOGY 2* (*NARS2,* Kunieda et al., 2008) and *MATERNAL EFFECT EMBRYO ARREST-* (*MEE*) genes (Pagnussat et al., 2005). We assume that nurse cell originating transcripts play an important role in the differentiation of early embryos.

Embryo maturation

The expression of several *LATE EMBRYOGENESIS ABUNDANT PROTEIN-* (*LEA*) genes (like AT3G15670) and an ABA-responsive gene (AT5G13200) increased during the switch to late embryogenesis, showing that maturation had initiated. Importantly, the expression of a *LEAFY COTYLEDON 1-LIKE* (*L1L*) homologue decreased during late embryo development. *LEAFY COTYLEDON 1* (*LEC1*) in Arabidopsis is a master regulator whose expression suppresses the embryonic transition towards vegetative development (reviewed by Braybrook and Harada, 2008). Also, expression of the maturation promoting *ABA*

INSENSITIVE 3 (*ABI3*) gene increased during the same period. This is supported by the work of Uddenberg et al. (2011). They showed that the transcription levels of a Norway spruce LEC1-type *HAP3* gene, *PaHAP3A*, was down-regulated in late development of somatic embryos. This indicates that the switch from embryonic to vegetative development is initiated at the stage of late embryos in Norway spruce.

Auxin

Many transcripts coding for auxin related genes were differentially expressed. At the switch from proliferation to early embryo differentiation, a *SUPERROOT 1* (*SUR1*) homolog was down-regulated, suggesting that IAA synthesis is increased (Mikkelsen et al., 2004), and a putative small auxin-up RNA (SAUR) was up-regulated. This is indicative of an increase in auxin signalling. At the transition to late embryo development, a putative Norway spruce *TIR1* auxin receptor, a homolog of the auxin induced *INDOLE-3-ACETIC ACID INDUCIBLE 11* (*IAA11*) and the auxin-signalling promoting *MYB77* transcripts were up-regulated. This shows that the auxin related mechanisms are further up-regulated as the embryos develop further.

Organ differentiation

One week after withdrawal of PGRs, when early embryos had differentiated, *PROTODERMAL FACTOR2* (*PDF2*) was differentially up-regulated. PDF2 is essential for specification of a protoderm, indicating that pattern formation has been initiated. In addition, several genes involved in cell wall modifications (like pectinesterase) were differentially expressed in the switches to both early and late embryo development. Differentially expressed during the same time were three *LATERAL ORGAN BOUNDARIES* (*LOB*) homologues. *LOB* genes are involved in the formation of lateral organs in Arabidopsis (Shuai et al., 2002), and their expression in early and late embryos indicate the start of cotyledon initiation. A homolog of the *LEUNIG* (*LUG*) gene, in Arabidopsis involved in homeotic floral organ identity (Navarro et al., 2004), was differentially up-regulated in early embryogenesis. We speculate that LUG might play a role in the specification of cell identity in Norway spruce embryos. GA signalling is important in Arabidopsis embryo development for the specification of a SAM and correct cotyledon formation (Silverstone et al., 2007). A homologue of the GA-response regulator *RGA-LIKE 1* (*RGL1*) and two *GA2-oxidase* transcripts were differentially expressed in both early and late embryos, perhaps indicating the initiation of a SAM.

3.1.2 Conclusion

By studying changes in the global gene expression we have been able to pinpoint molecular events in Norway spruce early embryo development. Our results show the involvement of stress related processes, "nurse cells" having megagametophyte signalling functions, early cell fate decisions separating different domains in the early embryo, the auxin-responsive machinery, a switch from embryonal to vegetative development and factors regulating formation of boundary regions.

3.2 Global changes in gene expression during embryo differentiation and early embryo development in Scots pine

The aim of this study was to identify important processes during early stages in Scots pine embryo development. Using a microarray spotted with 12,536 cDNA clones, we studied transcriptional changes during the transition between (1) proliferating embryogenic cultures in the presence of PGRs, (2) embryogenic cultures two weeks after change to a PGR-free medium when early embryos had differentiated and (3) embryogenic cultures two weeks after transfer to solid medium containing ABA when late embryos had developed. The developmental stages studied are comparable to those used in a study of Norway spruce early embryo development by Vestman et al. (2011). The same microarray production used for Norway spruce was also used for Scots pine, allowing us to compare the selected transcriptome of the two species during early embryo development. However, it should be kept in mind that embryogenic cultures of Scots pine are less developmentally synchronized compared to cultures from Norway spruce.

3.2.1 Important processes

PCD

During the switch to differentiation of early embryos in Scots pine, two inhibitors of PCD, *BAX INHIBITOR 1* (*BI1*) and *APOPTOSIS INHIBITOR 5* (*API5*), were differentially up-regulated, indicating that PCD was inhibited. However, during the switch to late embryo development, a *METACASPASE 4* (*MC4*) transcript was differentially up-regulated, suggesting that the PCD machinery had been activated. The role of PCD in somatic embryo development has been studied thoroughly in Norway spruce (Bozhkov et al., 2004 and 2005, Suarez et al., 2004). In Norway spruce, PCD is required for proper degradation of the suspensor cells after early embryos start to

differentiate. Furthermore, the PCD machinery is up-regulated already 24 h after transfer to a media lacking PGRs (Vestman et al., 2011). We conclude that the elevated expression levels of genes involved in inhibition of PCD during differentiation of early embryos followed by the induction of *MC4* in late embryos of Scots pine suggests that PCD is delayed compared to Norway spruce. This is supported by Abrahamsson et al. (2011) who showed that, in contrast to embryogenic cultures of Norway spruce, PCD did not increase during differentiation of early embryos of comparable phenotype in Scots pine embryogenic cultures.

Auxin

During early embryo differentiation in Scots pine, the homologues of both the PAT enhancing *ARABIDOPSIS THALIANA V-PPASE 3* (*ATAVP3*) and the PAT reducing *CYTOCHROME P450 75B1* (*CYP75B1*) were up-regulated. At the switch to late embryo development, an auxin efflux carrier (AT1G71090) and an auxin responsive gene (AT4G38860) were up-regulated, indicating an induction of the auxin responsive machinery. In Arabidopsis it has been shown that controlled PAT is essential for proper establishment of an apical-basal axis in the developing embryo (Friml et al., 2003). Larsson et al. (2008) has shown that a blocked PAT in Norway spruce embryo development leads to abnormal cell divisions and decreased PCD, resulting in both basal and apical abnormalities. In this context, we conclude that the induction of PAT in Scots pine embryogenic cultures seem delayed compared to Norway spruce.

Pattern formation

An increase in the transcripts of homologues of *WRKY2*, *SEUSS* (*SEU*), *LUG* and *NARS2* during the development of early embryos indicates that pattern formation has been initiated. WRKY2 is a transcription factor that directly activates transcription of *WUSCHEL RELATED HOMEOBOX 8* (*WOX8*) which is important for correct asymmetric cell division in the Arabidopsis zygote (Ueda et al., 2011). SEU and LUG work together in regulating several developmental events (Bao et al., 2010) and NARS2 is needed for progression through embryo development in Arabidopsis (Kunieda et al., 2008).

3.2.2 Conclusion

By analyzing global changes in gene expression in Scots pine, we have been able to describe important molecular events that regulate the development of early and late embryos. Comparisons to gene expression in Norway spruce have revealed a possible delay in the onset of PCD and PAT. Both processes are essential for proper embryo differentiation and their delay may represent a

cause for the high level of erroneous development observed in somatic embryos of Scots pine.

3.3 Auxin dynamics during Norway spruce embryo development

The aim of this work has been to localize sites of auxin activity in different cells/tissues during differentiation and development of somatic embryos of Norway spruce, and to elucidate how NPA-treatment affects the auxin response. Hagen et al. (1991) used a soybean (*Glycine max*) auxin responsive promoter (*GmGH3*) to monitor auxin responses *in vivo*. We have isolated a GH3 promoter from Norway spruce and fused it to the GUS reporter gene (*PaGH3::GUS*). Transforming Norway spruce embryogenic cultures with the *PaGH3::GUS* construct and with a *GmGH3::GUS* construct allowed us to successfully study auxin dynamics during embryo development.

3.3.1 Auxin localization in PEMs

In PEM structures of all stages, *PaGH3:GUS* and *GmGH3:GUS* activity was present in both small cytoplasmically dense cells and in elongated vacuolated cells. However, in our study we cannot exclude that the rather high over-all *PaGH3* and *GmGH3* activity may be due to the presence of synthetic auxin 2,4-D in the proliferation medium. 2,4-D is an influx substrate and has been shown to interfere with the signal pattern of the *DR5* auxin reporter in Arabidopsis (Friml et al., 2003). Treatment with NPA had no noticeable effect on the activity of the two GH3 constructs.

3.3.2 Local auxin dynamic during Norway spruce somatic embryogenesis and the effect of PAT inhibition

Early embryos start to differentiate after one week of PEM cultivation on proliferation medium, when the level of PGRs has declined. At this stage, the PEM cells still show a strong *PaGH3* and *GmGH3* expression, while the developing EM shows no *PaGH3 or GmGH3* expression. It is very difficult without time-laps tracking to determine which cells will start to differentiate into embryos. To be able to do that we would need a fluorescent marker which is not destructive to the cells when assessed. Our hypothesis is that early embryos start to differentiate from patches of strong auxin responsiveness, and that the early differentiating embryos are depleted from auxin response.

When early embryos have differentiated from PEMIII structures they show a weak *PaGH3* and *GmGH3* expression in the basal part of the EM and/or in

the suspensor including the tube cells. After blocking PAT with NPA the *PaGH3* and *GmGH3* activity increased and was restricted to only the suspensor. This suggests that auxin is transported from the suspensor to the basal part of the EM during early embryo differentiation. The increased *PaGH3* and *GmGH3* activity in the suspensor after NPA-treatment might also be an effect of an overall increase in endogenous auxin during the differentiation of early embryos in the presence of NPA as was shown by Larsson et al. (2008). Interestingly, in Arabidopsis there is also a basal-to-apical auxin transport during early embryo development. However, this transport results in an auxin response in the whole embryo proper, and not just in the basal part as in Norway spruce. This indicates a difference in auxin distribution between angiosperms and gymnosperms. We suggest that the basal auxin accumulation might be involved in promoting division of stem cells in the basal EM cells, and promoting elongation of tube cells.

At the beginning of late embryogeny, the *PaGH3* and *GmGH3* expression becomes restricted to the basal part of the EM, tube cells and uppermost suspensor cells. Sectioning revealed a maximum in the newly formed root organizing center (ROC) and an expression trailing down through pre-procambial cells towards the suspensor. Following NPA treatment, *PaGH3* expression was detected in the upper part of the EM of the developing embryo. This suggests that auxin during late embryogeny is transported from the apical to the basal part of the embryo. Furthermore, we suggest that the decrease of auxin in the suspensor is indicative of increased PCD, a process necessary for correct development. Interestingly in Arabidopsis, auxin responses are also very strong in the basal part of globular to torpedo stage embryos, including the hypophysis, the founder of the future root meristem (Friml et al., 2003), suggesting a conserved function for auxin during RAM formation.

In mature cotyledonary embryos, *PaGH3* and *GmGH3* were continually expressed in the whole embryo except for the cotyledons, with a stronger expression in the epidermis, and in the basal part surrounding the RAM. Blocking of PAT with NPA intensified the *PaGH3* signal but often reduced the apical distribution of the signal. These changes are consequent with a center of auxin production in the RAM and a reduced movement of auxin towards the apical part. NPA treatment also caused several phenotypes like lack of a SAM, fused or aborted cotyledons, split basal region and ball-shaped embryos, as described by Larsson et al. (2008).

3.3.3 Conclusion

Taken together, auxin is first synthesized and accumulated in the suspensor of differentiating embryos, possibly driving elongation of the suspensor. As the

ROC is initiated in late embryos, the source of auxin is shifted to the EM while PAT drives the flux towards the basal part and uppermost suspensor. As the embryo elongates and cotyledons emerge, auxin synthesis is maintained in the ROC, and PAT directs the auxin in an apical as well as basal direction. We anticipate that auxin is dynamically relocated also in the apical parts of mature embryos, although we have not been able to detect this response with the method used here. The dynamic developmental changes in auxin flux suggest a tightly regulated interplay of synthesis and directed transport. The role of auxin transport proteins will have to be investigated in detail if the intricacies are to be fully explained.

4 Conclusions

The study of global gene expression during early embryo development in Norway spruce pinpointed important molecular events like the establishment of different domains and boundary regions, “nurse cells” having a megagametophyte signaling function and a switch from embryonal to vegetative development.

The study of global gene expression during early embryo development in Scots pine identified processes such as the initiation of pattern formation and a comparison with Norway spruce revealed a possible delay in the onset of programmed cell death and polar auxin transport.

Auxin is first synthesized and accumulated in the suspensor of early embryos, and the site of synthesis is shifted to the embryonal mass in late embryos while polar auxin transport relocates the auxin in a basal direction. In maturing embryos, auxin is synthesized in the basal part and distributed throughout the embryo by polar transport.

5 Future perspectives

It is our hope that the studies reported here will constitute a base for further research in conifer embryology. The global gene expression data presented here can be used a springboard for further investigations into the genetic regulation of embryo development in conifer species. The continuation of the studies of auxin dynamics in Norway spruce embryo development is under way and has the potential to generate important insights.

The understanding of conifer embryology is still in its nascent stage, where most processes are referred to by comparing to the counterparts in a more studied model organism like Arabidopsis. The coming genome sequence of Norway spruce is a big step towards transcending the limitations so far imposed by the large genomes of conifers. Emerging technologies like next generation sequencing might hold the power to further transform genomics and transcriptomics into tools readily available for the conifer researcher. A more fundamental understanding of the genetics governing the development of conifer species can help unleash the potential of genetic engineering to improve traits valuable to the forest industry.

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