Regulation of embryo development in Norway spruce by *WOX* transcription factors

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(photo: early embryo in control)

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Abstract

In seed plants, the apical-basal axis of the plant body is established during early embryogenesis. Major regulatory genes of the apical-basal axis formation belong to the *WUSCHEL-RELATED HOMEOBOX* (*WOX*) gene family of transcription factors. The spatiotemporal expression pattern and the molecular role of the *WOX* genes has mainly been studied in the angiosperm model plant Arabidopsis (*Arabidopsis thaliana*). Similar information in conifers is limited. The aim of my thesis has been to characterize *WOX* genes in Norway spruce (*Picea abies*) and to elucidate the function of *WOX* genes expressed during embryo development.

We cloned 11 *WOX* homologs from Norway spruce and examined their phylogenetic relationship to *WOX* genes from other species. The phylogenetic analyses showed that the major diversification within the *WOX* gene family took place before the gymnosperm-angiosperm split. *PaWOX8/9*, *PaWOX2* and *PaWOX3*, which are expressed in embryos, were selected for further studies.

PaWOX8/9 and *PaWOX2* are highly expressed in early and late embryos, and *PaWOX3* is highly expressed in mature embryos. Functional studies were performed in RNAi lines where the genes were down-regulated. Embryos in *PaWOX8/9* RNAi lines showed a disturbed apical-basal patterning caused by abnormal orientation of the cell division plane at the basal part of the embryonal mass. In *PaWOX2* RNAi lines, vacuolated cells differentiated on the surface of the embryonal mass and the embryos failed to form a proper protoderm. Down-regulation of *PaWOX3* disturbed lateral margin outgrowth in cotyledons and needles.

Taken together, our results indicate that *WOX8/9*, *WOX2* and *WOX3* exert evolutionarily conserved functions during embryo development. We can therefore conclude that the regulatory networks of embryo development are at least partly conserved between angiosperms and gymnosperms.

Keywords: apical-basal polarization, conifers, Norway spruce, phylogeny, somatic embryogenesis, stem cells, *WUSCHEL-RELATED HOMEOBOX* genes.

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Dedication

To my parents.

Don't do what you should not do, and do not desire what you should not desire.
Mencius
无为其所不为，无欲其所不欲。
孟子

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 Hedman, H.*, **Zhu, T.***, von Arnold, S., and Solhlberg, J. J. (2013). Analysis of the *WUSCHEL-RELATED HOMEOBOX* gene family in the conifer *Picea abies* reveals extensive conservation as well as dynamic patterns. *BMC plant Biol BMC Plant Biol,* 13, p. 89. * the first two authors contributed equally.

II **Zhu, T.**, Moschou, P.N., Alvarez, J.M., Sohlberg, J.J. and von Arnold, S. (2014). *WUSCHEL-RELATED HOMEOBOX 8/9* is important for proper embryo patterning in the gymnosperm Norway spruce. *J Exp Bot 65(22), 6543-6552*.

III **Zhu, T.**, Moschou, P.N., Alvaréz, J.M., Sohlberg, J.J. and von Arnold, S. 2014. *WUSCHEL-RELATED HOMEOBOX 2* is important for protoderm development in the gymnosperm Norway spruce. (Manuscript).

IV Alvaréz, J.M., Sohlberg, J.J., Engstöm, P., **Zhu, T.**, Englund, M., Moschou, P.N. and von Arnold, S. *WUSCHEL-RELATED HOMEOBOX 3* regulates lateral organ formation in shoots in Norway spruce. (Submitted).

The contributions of Tianqing Zhu to the papers included in this thesis were as follows:

I Tianqing Zhu took part in planning the work and analysis of the results. Shared the laboratory work with H. Hedman.

II Tianqing Zhu was highly involved in planning the work and performed all laboratory work except for the tracking. Wrote the first draft of the paper.

III Tianqing Zhu was highly involved in planning the work and performed all laboratory work except for the tracking analyses. Wrote the first draft of the paper.

IV Tianqing Zhu took part in discussions and performed the tracking analysis

1 Introduction

Around the world, forests are regarded as one of the solutions to problems of waning sources of oil and ongoing climate change. Forests are a renewable source of both raw materials for industry and climate-neutral energy. The world population is expected to increase to 9.6 billion by 2050 (Gerland *et al.*, 2014). The population growth will increase the global demand for 'food, fiber and fuel'.

Forest covers 9.4% of the earth's surface (or 30% of the total land area). About 55% of the Swedish land area is covered by productive forest of which 42% consists of Norway spruce (*Picea abies*) and 39% consists of Scots pine (*Pinus sylvestris*) (Swedish Statistical Yearbook of Forestry, 2014). Conifer species are growing naturally in almost all parts of the world. In the northern hemisphere, the conifers are restricted to subtropical high altitude, temperate and boreal zones. The extant conifers include six to eight families, with a total of 65-70 genera and 600-650 species (Gibson & Gibson, 2007). Many coniferous species are of great commercial importance, e.g. timber for constructions, raw material for pulp and paper production, fibers for producing textiles and as bioenergy.

In 1994 the Swedish government decided that high forest production and forest sustainability with conserved biological diversity are equally important goals in the Swedish forestry. Forest tree breeding is an important tool for maintaining the balance between the demand for wood products and forest sustainability. Breeding of conifers is a slow process in which economically significant characteristics are continuously improved. The improvement is obtained through well known quantitative techniques involving recurrent crossing, testing and selection. Selected trees are then used for further breeding and mass-propagation. When the superior genotypes have been selected, they are too old to be propagated vegetatively by cuttings. Instead, branches from the selected trees are grafted and allowed to grow up to new trees in seed orchards. Seeds from these seed orchards are then used for reforestation.

Consequently, parts of the genetic gain achieved in the breeding program are lost. The possibility to propagate the selected trees vegetatively in large scale would make it possible to capture the genetic gain more efficiently. By using somatic embryos this goal can be achieved.

Somatic embryogenesis is defined as a process in which a bipolar structure, resembling a zygotic embryo, develops from a somatic cell. The first documentation of somatic embryogenesis was with carrot (*Daucus carota*) cell suspension cultures (Steward *et al.*, 1958, Reinert 1959). Since then, different methods have been developed for propagating a large number of species via somatic embryos. Somatic embryos are also widely used to study the regulation of embryo development.

The embryonic phase is crucial as it is when the plant body pattern is specified. A deeper understanding of the genetic regulation would help to improve the culture conditions for propagating economically important conifers via somatic embryos. In addition, knowledge about the genetic regulation of embryo development in conifers would also be interesting from an evolutionary point of view. Today our knowledge about how embryo development is regulated in conifers is very limited. In contrast, the genetic regulation of embryo development has been thoroughly studied in the angiosperm model plant Arabidopsis (*Arabidopsis thaliana*). However, since angiosperms and gymnosperms separated approximately 300 million years ago, genetic regulation and patterning during embryo development differ significantly between the two groups. Therefore, knowledge obtained from angiosperms about embryo development is not directly transferable to gymnosperms.

We use somatic embryos in Norway spruce as a model system for studying embryogenesis in conifers. The model system includes a stereotyped sequence of developmental stages, resembling zygotic embryos, which can be partially synchronized by specific treatments, making it possible to collect a large number of somatic embryos at specific developmental stages. Compared to the development of zygotic embryos within the seed, which takes five months, the development of mature somatic embryos is much faster. The process from differentiation of early somatic embryos to production of mature embryos occurs within five to seven weeks (Bozhkov & von Arnold, 1998).

My Ph.D. project aims to study the genetic regulation of cell specification during embryogenesis in Norway spruce, and in particular to analyze the expression pattern and the functions of homeodomain transcription factors belonging to the *WUSCHEL RELATED HOMEOBOX* (*WOX*) gene family which is crucial for the embryonic patterning formation, stem-cell maintenance and organ formation in angiosperms.

1.1 Embryo patterning in seed plants

Embryogenesis is the process by which the embryo is formed. The basic body plan is established during this period. A large number of genes are precisely expressed and perform their functions to direct proper embryo patterning.

1.1.1 Angiosperms

A schematic overview of embryo development in Arabidopsis is presented in Figure 1A. The developmental process can, for convenience, be separated into three phases: i) proembryogeny (post fertilization to proembryo), which occurs after fertilization but before organogenesis; ii) early embryogeny (globular-stage to heart-stage transition), which is a process of organogenesis and morphogenesis; iii) late embryogeny, during which organ expansion and maturation are completed (Goldberg *et al.*, 1994).

Proembryogeny begins after double fertilization. The zygote is elongated and divides transversely, generating an apical daughter cell and a basal daughter cell (Fig. 1A.a and b). The cell fate is already decided at this stage.

The apical daughter cell undergoes a series of periclinal and anticlinal divisions to generate the embryo proper. The embryo reaches the 8-cell-stage (octant-stage) after two more divisions (Fig. 1A.c). The upper four cells will subsequently give rise to the apical part of the seedling. The lower four cells will form the root. The basal daughter cell divides transversely to generate the hypophysis and the suspensor which connects the endosperm to the embryo (Fig. 1A.c). The suspensor provides nutrients to the growing embryo and will be degraded by programmed cell death (PCD) before embryo maturation (Goldberg *et al.*, 1994).

Early embryogeny begins with the transition from the 8-cell-stage to the 16-cell-stage (dermatogen-stage). The 8 cells in the embryo proper divide tangentially to separate the inner and the outer layer (Fig. 1A.d) (De Smet & Beeckman, 2011). The outer cells form the protoderm and continue to divide periclinally. Meanwhile the inner cells will give rise to the various concentric tissue layers, *e.g.* the provasculature and the ground tissue (Mayer *et al.*, 1991).

The cell divisions are less coordinated during the globular stage. The hypophysis is the uppermost cell of the suspensor (Fig. 1A.e). It differentiates to form part of the root cap. The cells in the embryo proper continue to divide so that the embryo gradually reaches the triangular stage and then the heart stage when the cotyledons grow out (Fig. 1A.f and g). Transition to the heart stage coincides with the initiation of the root primordium followed by the shoot primordium. Organ expansion and maturation are completed during late

embryogeny (Fig 1.A h and i). Maturation of the embryo is followed by germination.

Monocots have a more complex embryo structure in the mature seed than dicots. Following fertilization, the first cell division is asymmetric and leads to an apical and basal cell. The apical cell develops into the embryo proper and the basal cell forms the suspensor. The stages of embryogenesis in monocots include the proembryo, transition coleoptilar and leaf-1 stages. In contrast to dicots, embryos in monocots have only a single cotyledon.

1.1.2 Gymnosperms (Conifers)

Consistent with the description of Arabidopsis embryo development, embryo development in gymnosperms can also be separated into three phases: proembryogeny, early embryogeny and late embryogeny (Fig. 1B).

Common for many gymnosperms, but rare in most angiosperms is that the zygote undergoes several rounds of nuclear duplication that are not followed by cytokinesis (Fig. 1B.a, b and c). This is followed by cellularization to form two tiers of cells and an eight-celled proembryo (Cairney & Pullman, 2007). These tiers will divide to form four tiers (Fig. 1B.d). The first and second tiers will multiply to form the embryonal mass. The third and fourth tiers will elongate and undergo limited cell division to form the embryonal suspensor.

Early embryogeny begins with the elongation of the embryonal suspensor (Fig. 1B.e). The embryonal mass and the suspensor are separated by a layer of conifer-specific cells called embryonal tube cells. The conifer suspensor consists of several files of non-dividing cells, originating by anticlinal cell division of the basal cells in the embryonal mass. The suspensor elongates and pushes the differentiating early embryo deeper into the female gametophyte. The first histogenesis event occurs during the formation of the early embryo (EE) (Fig. 1B.f). The cells in the surface layer of the embryonal mass divide mainly anticlinally but also tangentially (Hardev, 1978). The surface layer of cells in the embryonal mass functions as a protoderm (Ingouff *et al.*, 2001). The EE further develops into a late embryo (LE) which is the stage before the formation of the cotyledons (Fig. 1B.g).

Late embryogenesis is a period of histogenesis and organogenesis. Early during this stage, the root and shoot meristems (RAMs and SAMs) are delineated and the plant axis established. The maturing embryo (ME1) is characterized by the initiation of cotyledons (Fig. 1B.h). In many gymnosperms multiple cotyledons are differentiated during late embryogeny. Embryos with multiple cotyledons retain a radial symmetry. The ME1 develops into ME2 (almost fully matured embryos) and ME3 (fully matured embryos) (Fig. 1B.i and j). As in angiosperms, the suspensor of gymnosperms

is a transient structure which is dismantled at the beginning of late embryogeny by PCD (Filonova *et al.*, 2000a).

1.1.3 Somatic embryogenesis in Norway spruce

Currently, embryogenic cell lines of conifers are established from juvenile tissue (zygotic embryos and seedlings). The primary explants are incubated on medium supplemented with plant growth regulators (PGRs), auxin and cytokinin, to initiate embryogenic cultures. The developmental pathway of somatic embryos of Norway spruce is schematically presented in Fig. 1C.

In the presence of PGRs, the cultures proliferate as proembryogenic masses (PEMs), which can pass through a series of three characteristic stages distinguished by cellular organization and cell number (PEM I, II and III) (Fig. 1C.a). There are two types of cells in the PEMs: small, rounded and highly cytoplasmic meristematic cells and elongated, highly vacuolated cells.

To stimulate differentiation of somatic embryos, the cultures are transferred to medium lacking PGRs (Fig. 1C.b). There is a general over-representation of genes involved in response to stress during the transition from proliferation to differentiation of early embryos (EEs) (Vestman *et al.*, 2011).

Further development and maturation of somatic embryos is stimulated by transferring the cultures to medium supplemented with abscisic acid (ABA). EEs differentiate after one week on maturation medium (Fig. 1C.c). The protoderm has already been specified during this stage (Ingouff *et al.*, 2001). LEs develop after two to three weeks on maturation medium (Fig. 1C.d). At the developmental switch to late embryogeny, several genes of importance for cell organization, developmental processes, and other biological processes are up- or down-regulated (Vestman *et al.*, 2011). During late embryogeny, the second wave of PCD eliminates terminally differentiated embryo-suspensor cells (Filonova *et al.*, 2000a). The root and shoot apical meristems are delineated and the plant axis established (Fig. 1C.e). ME1s develop after 5 weeks on maturation medium. ME2s and ME3s develop after about 8 weeks on maturation medium (Fig. 1C.f and g). Finally, after partial desiccation, the embryos are germinated on medium lacking PGRs.

Although the early proembryogeny stages in somatic embryos and zygotic embryos are not really comparable, the subsequent developmental pathways are morphologically similar. Compared to zygotic embryos, there are several advantages in using somatic embryos for studying embryo development. First, unlike zygotic embryos which develop under several layers of tissues, somatic embryos can be observed directly. Second, somatic embryos propagated from the same embryogenic cell line have identical genetic background, which makes molecular studies more accurate. Finally, the development of somatic

embryos can be synchronized by specific treatments (Filonova *et al.*, 2000b), which makes it possible to collect a large number of somatic embryos at specific developmental stages. Therefore, the process of somatic embryogenesis has proved to be a valuable tool for studying embryo development in conifers.

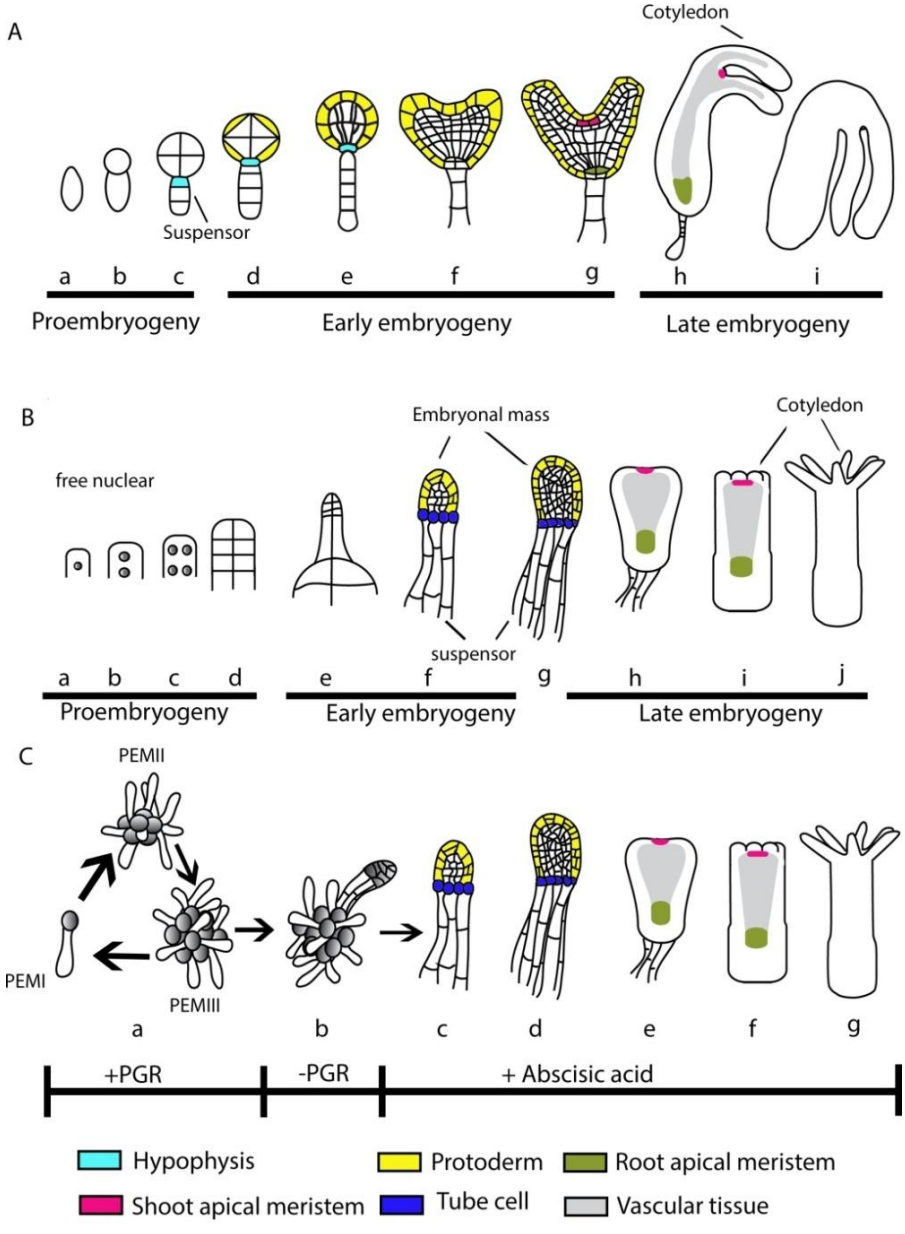

Figure 1. Schematic overview of embryo development. A) Zygotic embryo development in the angiosperm Arabidopsis. a, zygote; b, 2-cell-stage; c, octant stage; d, dermatogen stage; e, globular stage; f, triangular stage; g, heart stage; h, torpedo stage and i, mature embryo. The developmental pathway of zygotic embryo in Arabidopsis is based on the original publication by Mayer *et al.* (1991) and Goldberg *et al.* (1994). B) Zygotic embryo development in the gymnosperm Norway spruce. a, b and c, free nuclear stages; d, proembryo after cellularization; e, differentiating early embryo; f, early embryo (EE); g, late embryo (LE); h, maturing embryo (ME1); i, almost fully matured embryo (ME2) and j, fully matured embryo (ME3). The developmental pathway of a zygotic embryo in Norway spruce is based on the original publication by Hardev (1978). C). Somatic embryo development in Norway spruce. The process involves two broad phases. The first phase, which occurs in the presence of auxin and cytokinin (+PGR), is represented by proliferating proembryogenic masses (PEMs), cell aggregates which can pass through a series of three characteristic stages distinguished by cellular organization and cell number (a. PEM I, II and III). The second phase encompasses, b, development of somatic embryos from PEM III and is triggered by withdrawal of auxin and cytokinin (–PGR). When embryos have differentiated, their further development into c, EE; d, LE; e, ME1; f, ME2 and g, ME3 is stimulated by abscisic acid. The developmental pathway of somatic embryos in Norway spruce is based on the original publication by Filonova *et al.* (2000a).

1.1.4 Auxin regulation of embryo development

Auxin regulate many aspects of plant growth and development, including embryo development. Regulated transport of the hormone auxin plays critical roles in the establishment of the apical-basal axis (Vanneste & Friml, 2009). A family of PINFORMED (PIN) proteins mediates the polar auxin transport. In Arabidopsis, from the 2-cell-stage until the globular-stage, the AtPIN7 protein, which is apically localized in the basal lineage and transports auxin from the basal to the apical region of the embryo. This flow creates an auxin maximum in the embryo proper (Friml *et al.*, 2003) which is required for the formation of the hypophysis. During these stages, AtPIN1 is apolar on the plasma membrane and mediates a uniform auxin distribution within the apical cell lineage. At the globular stage, AtPIN7 switches its polarity and localizes basally. As a consequence, the transport of auxin is reversed towards the suspensor cells and results in an auxin maximum in the hypophysis. The hypophysis is the founder cell of the root meristem (Friml *et al.*, 2003). In accordance, mutants that are unresponsive to auxin show severe defects in embryonic root formation (Dharmasiri *et al.*, 2005; Hamann *et al.*, 2002). In the heart-stage embryo, auxin is transported by AtPIN1, which is localized in the protoderm to the cotyledon primordia (Benkova *et al.*, 2003). Auxin signaling is also important for domain specification (Jiang & Feldman, 2005; Aida *et al.*, 2004). In Norway spruce, *PaPIN1* has a similar expression pattern

as *AtPIN1*, and exerts a role in auxin transport and embryo pattern formation (Palovaara *et al.*, 2010b).

The phytotropin 1-N-naphtylphthalamic acid (NPA) is an auxin transport inhibitor. In Norway spruce, treatment of NPA during early embryogeny results in fused cotyledons and irregular cell divisions in the area of the root meristem, which suggests an essential role of polar auxin transport in correct patterning of both apical and basal parts of conifer embryos (Larsson *et al.*, 2008).

1.1.5 Molecular regulation of embryonic pattern formation

Compared to angiosperms, the understanding of the regulation of embryogenesis in gymnosperms is limited (von Arnold & Clapham, 2008; Cairney & Pullman, 2007; von Arnold *et al.*, 2002). The embryonic patterning in angiosperms and gymnosperms is different regarding the establishment of the apical-basal polarity, the development of the suspensor and the number of cotyledons. However, of the identified 295 Arabidopsis genes related to embryogenesis, about 85% have very strong sequence similarity to an EST in the loblolly pine (*Pinus taeda*) data-base (Cairney & Pullman, 2007; Tzafrir *et al.*, 2003). As current knowledge is mostly derived from Arabidopsis, it is reasonable to use the Arabidopsis embryogenesis as a comparison for the conifer embryogenesis.

Auxin transport is crucial for the establishment of the apical-basal polarization of the embryo. The *MONOPTEROS* (*MP*) gene encodes AUXIN RESPONSE FACTOR 5 (ARF5) which activates auxin-responsive target genes (Hardtke & Berleth, 1998). The loss-of-function *mp* mutant shows aberrant hypophysis division resulting in a rootless phenotype (Weijers *et al.*, 2006). The *BODENLOS* (*BDL*) gene encodes IAA12 which inhibits the activation of MP-dependent genes. The *bdl* mutant, which has a more stable IAA12 than the wild-type, has a similar phenotype as the loss-of-function *mp* mutants (Hamann *et al.*, 2002).

At the heart-stage embryo, the cotyledons initiate at the flanks of the apical embryo domain (Benkova *et al.*, 2003). The SAM is specified between the cotyledons (Mayer *et al.*, 1998). The establishment of the SAM requires the expression of the *SHOOT MERISTEMLESS* (*STM*), a member of the *KNOXI* transcription factor gene family. The *CUP-SHAPED COTYLEDON* (*CUC*) genes *1* and *2*, which belong to the NAC gene family, redundantly regulate the initiation of SAM and the separation of cotyledons (Aida *et al.*, 1997). The *MP* and *PIN* genes are required for the activation of *CUC2* in cotyledon boundaries and the repression of *CUC1* in cotyledons (Furutani *et al.*, 2004). The auxin

maxima sites in the heart-stage embryo are required to control *CUC* gene expression during cotyledon initiation and separation.

In Norway spruce, the expression of *PaHBK2* and *PaHBK4*, which are members of the *KNOX1* gene family, as well as the expression of *PaNAC01*, which is closely related to *AtCUC* genes, are dependent on polar auxin transport (Larsson *et al.*, 2012a; Larsson *et al.*, 2012b). Based on their expression pattern, it has been suggested that *PaHBK2* and *PaHBK4* exert functions related to SAM differentiation during early embryogeny, and *PaNAC01* is important for the formation of separated cotyledons.

The differentiation of the protoderm is the first event of radial patterning. The *GL2*-type homeobox genes, *ARABIDOPSIS THALIANA MERISTEM L1* (*AtML1)* in Arabidopsis (Abe *et al.*, 2003) and *PICEA ABIES HOMEOBOX 1* (*PaHB1)* in Norway spruce (Ingouff *et al.*, 2001) have a role in maintaining the identity of protoderm cells. In addition, for normal embryo development in both Arabidopsis and Norway spruce, expression of *LTP* genes, which encode lipid transfer proteins (LTPs), must be restricted to the protodermal cells (Sabala *et al.*, 2000; Vroemen *et al.*, 1996).

1.2 *WUSCHEL-RELATED HOMEOBOX* (*WOX*) genes

The *WUSCHEL-RELATED HOMEOBOX* (*WOX*) genes encode a family of plant-specific transcription factors. Transcription factors are proteins that can promote or repress the expression of specific genes by binding to specific DNA sequences.

Members of the *WOX* gene family are characterized by the presence of a highly conserved DNA binding homeodomain which consists of 60 to 66 amino acids. Studies of the *WOX* genes have shown that they play crucial roles in cell differentiation in Arabidopsis, petunia, maize (*Zea mays*) and rice (*Oryza sativa*) (Rebocho *et al.*, 2008; Dai *et al.*, 2007; Nardmann *et al.*, 2004; Schoof *et al.*, 2000). By promoting or repressing cell differentiation, *WOX* genes participate in embryonic patterning, stem-cell maintenance and organ formation.

The first identified member of the *WOX* gene family was *WUS* in Arabidopsis (Mayer *et al.*, 1998). Expansion of the *WOX* genes occurred after the separation of mosses from other land plants (Zhang *et al.*, 2010; Nardmann *et al.*, 2009; Deveaux *et al.*, 2008). The *WOX* genes have evolved both at the protein and the transcription level to generate new functions for development which were important for the emerging land plants. All *WOX* genes examined show very specific expression patterns, both spatially and temporally, which are important for their functions (Ueda *et al.*, 2011). Analysis of the tissue

specific expression of *WOX* genes is of interest to elucidate similarities and differences in the regulatory mechanisms of plant development also in species outside the angiosperms.

1.2.1 Phylogeny of the *WOX* gene family

The *WOX* gene family is present only in the 'green' lineage comprising land plants and green algae. According to phylogenetic analysis, the *WOX* gene family has been divided into three clades: the ancient clade, the intermediate clade and the modern clade (van der Graaff *et al.*, 2009).

1 Modern clade

The modern clade includes the most recently evolved *WOX* genes. *AtWUS* and *AtWOX1-7* encode proteins which share a short motif called the WUS box (TLXLFPXX, where X can be any amino acid), which is one of the characteristics of the modern clade (WUS clade). The WUS box is responsible for the conserved repressive activity of WOX proteins (Lin *et al.*, 2013a; Lin *et al.*, 2013b). At the carboxy-terminal end of the WUS, WOX5 and WOX7 proteins, an ERF-associated amphiphilic repression (EAR) motif was also defined as L-[ED]-L-[RST]-L. It is involved in transcriptional repression (Paponov *et al.*, 2009). *WUS* is expressed in the SAM in ferns, angiosperms and gymnosperms (Nardmann & Werr, 2012). The Arabidopsis gene *AtWOX2* is expressed in specific domains from the first cell division of the proembryo until the end of embryogenesis (Haecker *et al.*, 2004).

2 Intermediate clade

The intermediate clade separated before the modern clade and after the ancient clade. *AtWOX8*, *AtWOX9*, *AtWOX11* and *AtWOX12* belong to this clade. Of the plant species with fully sequenced genomes, only Arabidopsis has both *WOX8* and *WOX9*. *AtWOX8* [also named *STIMPY-LIKE*, (Wu *et al.*, 2007)] and *AtWOX9* [also named *STIMPY*, (Wu *et al.*, 2005)] are preferably expressed during embryogenesis and are highly related to the establishment of the apical-basal body axis. The rice *WOX11* ortholog is expressed in the proliferating regions of both SAM and RAM and may act as an integrator of auxin and cytokinin signaling in crown root development (Zhao *et al.*, 2009).

3 Ancient clade

Members of the ancient clade are the most conserved *WOX* genes. The only *WOX* gene found in the green algae belongs to this clade (Deveaux *et al.*, 2008). Compared to *WOX* genes in the modern and the intermediate clades, our knowledge about *WOX* genes belonging to the ancient clade is very limited. The *WOX* genes in *Ostreococcus tauri* (*OtWOX*) and *Physcomitrella patens* (*PpWOX01-03*), all belonging to the ancient clade, have been shown to be

constitutively expressed in all tissues and at all developmental stages analyzed (Deveaux *et al.*, 2008). *PpWOX01-03* genes are required for the initiation of cell growth specifically during stem cell formation (Sakakibara *et al.*, 2014). In Arabidopsis, there are two *WOX* genes belonging to the ancient clade: *AtWOX13* and *AtWOX14*. They are expressed in most organs (roots, shoots and reproductive organs), although the expression pattern is limited to certain cells within the organ (Deveaux *et al.*, 2008). Both *AtWOX13* and *AtWOX14* prevent premature differentiation (Romera-Branchat *et al.*, 2012).

1.2.2 *WOX* genes and embryo pattern formation

AtWOX2, *AtWOX8* and *AtWOX9* are highly related to the establishment of the apical-basal body axis in the embryo (Wu *et al.*, 2007; Wu *et al.*, 2005). The spatial-specific expression pattern of these genes during embryo development is important for the localization of auxin and the formation of the main body axis (Breuninger *et al.*, 2008). *AtWOX2* and *AtWOX8* are co-expressed in the egg cell and the zygote. After the first cell division, *AtWOX2* marks the apical descendants of the zygote. Expression of *AtWOX2* can be detected in all cells in the 4-cell embryo proper, and after that the expression becomes restricted to the apical domain. Loss of *AtWOX2* function leads to an incorrectly separated protoderm layer at the octant stage (Breuninger *et al.*, 2008). *AtWOX8* marks the basal cells after the first cell division of the zygote. After the hypophysis has divided, *AtWOX8* expression ceases in the descendants of the hypophysis, but remains present in the suspensor. After the heart stage, no *AtWOX8* mRNA could be detected (Haecker *et al.*, 2004). Expression of *AtWOX9* is detected after the first division of the zygote (Wu *et al.*, 2007; Haecker *et al.*, 2004). Haecker *et. al.* (2004) reported that expression of *AtWOX9* is restricted to the hypophysis after three rounds of cell divisions. At the heart stage, the expression of *AtWOX9* expands into the central domain and it is expressed at about the same position as *AtWOX2*. Later, Wu *et. al.* (2007) showed that *AtWOX9* is also expressed in the SAM during embryo development. Unlike *AtWOX2* and *AtWOX8*, *AtWOX9* is widely expressed in different proliferating tissues, *e.g.* in vegetative SAMs, the upper portion of RAMs, leaf primordia and floral meristems (Wu *et al.*, 2007; Wu *et al.*, 2005; Haecker *et al.*, 2004). *AtWOX9* and *AtWOX8* share redundant functions in the establishment of the apical-basal axis (Wu *et al.*, 2007; Haecker *et al.*, 2004).

In maize, *ZmWOX2A* is active in the zygote and marks the apical cell fate and pre-patterns the position of SAM. It is first expressed early in maize embryos in the apical domain of the embryo proper and then laterally on the adaxial side of the embryo proper, where it becomes restricted to the outer cell

layer. *ZmWOX2A* transcripts are detected when the SAM has been established in the leaf-1 stage (Nardmann *et al.*, 2007).

Maize has three *ZmWOX9* genes but no *WOX8* gene. *WOX8* is also absent in the genomes of rice and poplar (*Populus tremula*). *ZmWOX9A, B* or *C* is not expressed in the zygote (Chandler *et al.*, 2008). However, following the coleoptile stage, *ZmWOX9A, B* and *C* are differentially expressed in the suspensor: *ZmWOX9C* is expressed in the outer cell layers of the suspensor and *ZmWOX9A* and *ZmWOX9B* are expressed throughout the suspensor (Nardmann *et al.*, 2007). In general, analysis of *ZmWOX2A* shows that it has conserved similar functions to those of *AtWOX2* in Arabidopsis. However, additional *WOX9* paralogs as well as an apparent lack of any *WOX8* ortholog suggests functional diversification between maize and Arabidopsis.

1.2.3 *WOX* genes and cell specification

Besides the role in the establishment of the apical-basal axis during embryogenesis, *WOX* genes play key roles for initiation of meristem and stem cell maintenance.

In Arabidopsis, expression of *AtWUS* and *AtWOX5* marks the stem cell organizing centre (OC) of the SAM and the QC in the RAM (Sarkar *et al.*, 2007; Mayer *et al.*, 1998). Expression of the *AtWUS* transcription factor in the OC represses stem-cell differentiation and induces expression of CLAVATA3 (CLV3) which is a secreted peptide signal. CLV3 will bind to its receptor CLV1 and repress *AtWUS* at the transcriptional level (Brand *et al.*, 2000; Schoof *et al.*, 2000). This kind of WUS-CLV loop also exists between some other modern clade *WOX* genes and *CLV-like* genes (Ohmori *et al.*, 2013; Osipova *et al.*, 2012; Sarkar *et al.*, 2007). *AtWOX5* performs a similar function to *AtWUS* but in the RAM and they are functionally equivalent (Sarkar *et al.*, 2007). In maize, *ZmWOX5B* marks the QC and is expressed in a central basal domain of the embryo proper subtended by vacuolated suspensor cells, and *ZmWUS* marks the stem cell in the OC of the SAM (Nardmann *et al.*, 2007). However, studies in gymnosperms so far suggest that only a single *WUS/WOX5* is expressed in the embryos. The SAM-specific expression of *WUS* and RAM-specific expression of *WOX5* seem to be special for angiosperms (Nardmann *et al.*, 2009).

The *WOX3* sub-clade includes. the *PRESSED FLOWER* (*PRS*) gene in Arabidopsis, the duplicated genes *NARROW SHEATH1* and *2* (*NS1/NS2*) in maize, and the duplicated genes *NARROW LEAF2* and *3* (*NAL2*/*NAL3*) in rice. *PRS* in Arabidopsis and *NS1/NS2* in maize are important for the recruitment of founder cells from lateral domains of SAMs and lateral outgrowth of leaves (Shimizu *et al.*, 2009; Nardmann *et al.*, 2004; Matsumoto & Okada, 2001;

Scanlon *et al.*, 1996). *NAL2*/*NAL3* in rice also regulates leaf width (Ishiwata *et al.*, 2013). Functional equivalence has been experimentally demonstrated between *WUS/WOX3* (Shimizu *et al.*, 2009) and partially between *WOX3/WOX4* (Ji *et al.*, 2010).

AtWOX1, which belongs to the *WOX3* sub-clade, is expressed between the adaxial and abaxial domain in leaves in Arabidopsis. It acts redundantly with *PRS* to regulate the lateral-specific blade outgrowth and margin-specific cell fate (Nakata *et al.*, 2012). The *WOX1* homologs *MAEWEST* (*MAW*) in petunia (Vandenbussche *et al.*, 2009), *STENOFOLIA* (*STF*) in barrel clover (*Medicago truncatula*) and tobacco (*Nicotiana sylvetris*) (Tadege *et al.*, 2011) are important for lateral outgrowth of the leaf blade.

In poplar and empress tree (*Paulownia tomentosa*), no *WOX3* homologs could be identified. It was suggested that the *WOX3*-mediated function of leaf blade outgrowth regulation is carried out by other genes in the modern *WOX* clade in poplar (Liu *et al.*, 2014).

2 Aim of this study

The aim of this study has been to increase our understanding of the molecular regulation of early embryo development in conifers. We have specifically analyzed the expression pattern and the functions of homeodomain transcription factors belonging to the *WUSCHEL RELATED HOMEOBOX* (*WOX*) gene family, which are crucial for embryonic patterning, stem-cell maintenance and organ formation in angiosperms.

Specific aims were to investigate:

- the phylogeny of the *WOX* gene family in Norway spruce;
- the importance of *PaWOX2* and *PaWOX8/9* during early embryo development;
- the importance of *PaWOX3* during late embryo development and germination.

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3 Results and Discussion

3.1 Analysis of *WOX* gene family in Norway spruce (I)

Members of the *WOX* gene family play important roles in determining cell fates during all stages of plant development (Mayer *et al.*, 1998; Laux *et al.*, 1996). The number of *WOX* genes present in the genome of different species correlates to some degree with the complexity of the species body pattern. We characterized members of the *WOX* gene family in Norway spruce and performed phylogenetic analysis.

3.1.1 Identification of Norway spruce *WOX* (*PaWOX*) genes

We have isolated 11 *PaWOX* genes using degenerate primers targeting the homeodomain. The isolated homeodomain was extended by genome walking. The whole genomic sequence was obtained for all genes except for *PaWOX13* which had extremely long introns.

In order to get a rough estimate of in which tissues the different genes are expressed, we isolated samples from different stages and tissues, i.e. PEMs, EEs, LEs, ME3s, shoot tips, root tips, cambium dissected from branches, young needles from elongationg shoots and two year or older needles. The expression of the *PaWOX* genes in different tissues of Norway spruce was analysed using quantitative real-time PCR (qPCR).

By qRT-PCR, we found that all genes, except *PaWUS*, *PaWOX8C* and *PaWOX8D*, were expressed in a few or all tissues analyzed. The lack of expression of *PaWUS*, *PaWOX8C* and *PaWOX8D* might suggest that these genes are inactive pseudogenes. However, there is nothing in the genomic sequences of these loci to support this notion.

3.1.2 *PaWOX* genes are represented in all three main clades

For phylogenetic analysis, we included homeodomain sequences from the Norway spruce genes, as well as from genes from green algae, bryophytes, lycophytes, ferns, gymnosperms, and angiosperms (Nardmann *et al.*, 2009; Wu *et al.*, 2005). We used two different phylogenetic methods and both nucleotide and protein alignments to infer the phylogeny. The analyses showed good support for the three major clades of *WOX* genes: the ancient clade, the intermediate clade, and the modern clade. The *PaWOX* genes group within the main clades of the angiosperm genes suggesting that the diversification took place before the angiosperm/gymnosperm split (Fig. 3).

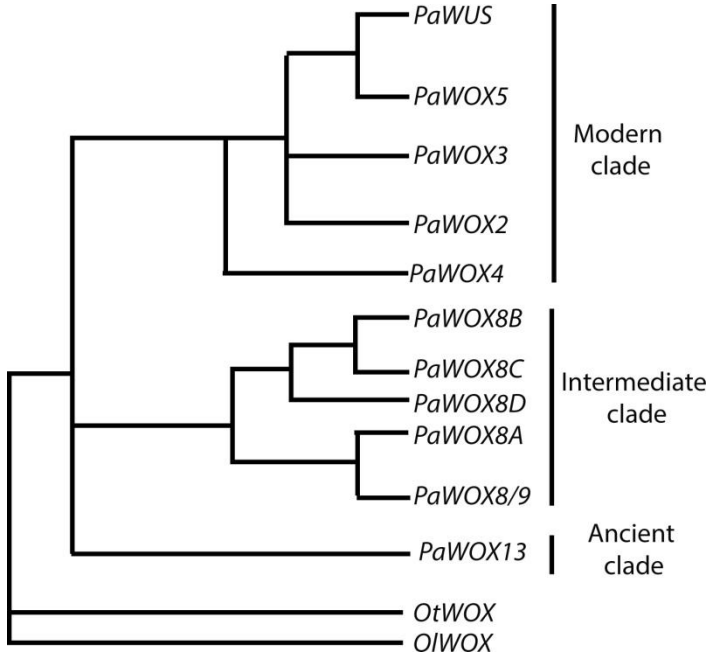

Figure 2. Sketch of a phylogenetic tree presenting the three main clades of *PaWOX* genes. Green algaes *Ostreococcus tauri,* Ot and *O. lucimarinus*, Ol are used as outer group.

1 Modern clade

There is almost a complete set of sequences in Norway spruce that are orthologous to the Arabidopsis sequences in the modern clade. The only Arabidopsis sequences we could not find orthologs of were *AtWOX1*, *AtWOX6* and *AtWOX7*. *AtWOX7* is most likely the result of a very recent duplication in the Arabidopsis lineage. The lack of sequences orthologous to *AtWOX1* and *6* might be because we were unable to amplify them with our primers; alternatively, there has been either an angiosperm-specific diversification or a gymnosperm-specific gene loss.

The support for the orthology of *PaWOX2* and *AtWOX2* was strong in the full WOX gene tree but not as strong in the modern clade sub-tree. *PaWOX2* expression was detected only in PEMs and LEs. This expression pattern of *PaWOX2* is in accordance with the expression of *AtWOX2*, which gives further support for the orthology of *PaWOX2* and *AtWOX2* (Haecker *et al.*, 2004).

The support was good for the orthology of *PaWOX3* and *AtWOX3*. The highest transcript level of *PaWOX3* was detected in mature embryos and shoot tips, which is in accordance with the expression reported for *AtWOX3* and a maize *WOX3* ortholog (Shimizu *et al.*, 2009; Matsumoto & Okada, 2001).

The monophyly of the *WOX4* clade was weakly supported by the full *WOX* gene tree. However, *AtWOX4* was the closest Arabidopsis homolog. The highest expression of *PaWOX4* was detected in the cambium, which might suggest that the function of *WOX4* is conserved as a regulator of the cambial meristem in Norway spruce as it is in Arabidopsis.

The support for the orthology of *PaWUS* and *AtWUS* was strong, though we have not been able to detect any transcript of *PaWUS* in the analyzed tissues. The support for *PaWOX5* to group with *AtWOX5* was not very strong. *PaWOX5* mRNA was only detected in mature embryos, shoot tips and root tips. In contrast to other analysed gymnosperms (Nardmann *et al.*, 2009), we found a good resolution for the *WUS/WOX5* sub-clade including all *WUS* and *WOX5* orthologs from the different species. Our results together with a previous report (Nardmann *et al.*, 2009), suggest that the duplication leading to distinct *WUS* and *WOX5* genes predates the gymnosperm-angiosperm split. However, this does not mean that the division of function between the apical meristems of shoots and roots was present.

2 Intermediate clade

In the intermediate clade, there were no clear Norway spruce orthologs to the angiosperm sequences. The five isolated *PaWOX* genes belonging to the intermediate clade could be more closely related to *AtWOX11* and *12*, closer to *AtWOX8* and *9*, or they could be as sisters to the angiosperm sequences.

PaWOX8/9 appeared to be the most widely expressed *PaWOX* gene in the intermediate clade. It is expressed in all analyzed tissues but is most highly expressed during embryogenesis. The expression pattern of *PaWOX8A* is similar to the expression pattern of *PaWOX2*. The transcript of *PaWOX8B* was detected only in PEMs, which is a very special tissue. The data so far available show that the expression of the intermediate clade genes, both in Norway spruce and Arabidopsis, are more limited to embryo development than the genes in other clades (Skylar *et al.*, 2010; Winter *et al.*, 2007; Wu *et al.*, 2005).

We could identify at least two different sequence variants of *PaWOX8B-D*, which we believe are allelic. Interestingly, there are at least four EST

sequences in loblolly pine (accession numbers DR690333, DT636589, DR692518, DT634290) belonging to the intermediate clade. All of these four loblolly pine sequences were derived from embryogenic tissues (Cairney & Pullman, 2007; Tzafrir *et al.*, 2003).

Taken together, it seems that there has been an expansion of genes in the intermediate clade and the embryo-specific functions of these genes are universal within the *Pinaceae* family; though whether this is a feature also of other gymnosperm lineages is still an open question.

3 Ancient clade

Only one Norway spruce gene grouped into the ancient clade, *PaWOX13*, which is in accordance to what has been reported for other gymnosperms (Nardmann *et al.*, 2009). *PaWOX13* was expressed in all tissues analyzed. In a similar way, the ancient clade genes *AtWOX13* and *AtWOX14* from Arabidopsis are expressed in most tissues, although there is no clear expression data from the embryo stage (Deveaux *et al.*, 2008). This suggests the expression patterns of the ancient clade genes are similar between Norway spruce and Arabidopsis.

3.2 The role of *PaWOX* genes during embryogenesis in Norway spruce (II and III)

Apical-basal polarity is established at the beginning of embryogenesis through the establishment of small and densely cytoplasmic meristematic cells of the embryonal mass and terminally differentiated vacuolated, expanding suspensor cells (Larsson *et al.*, 2008; Smertenko *et al.*, 2003). The basally situated cells in the embryonal mass have been suggested to be distal stem cells (Zhu *et al.*, 2014), which divide anticlinally to give rise to one meristematic cell in the embryonal mass and one vacuolated cell in the suspensor. The border between the embryonal mass and the suspensor cells is clear and the embryonal mass displays a distinct protoderm with a smooth surface (von Arnold & Clapham, 2008). The specification of the protoderm is essential for normal patterning during embryo development in both angiosperms and gymnosperms (Watanabe *et al.*, 2004; Ingouff *et al.*, 2001; Sabala *et al.*, 2000; Dodeman *et al.*, 1997).

3.2.1 Transcript levels of *PaWOX2 and PaWOX8/9* during embryo development. (II and III)

To obtain higher resolution of the expression of *PaWOX2* and *PaWOX8/9*, we analyzed the expression of these genes in PEMs, EEs, LE1s, LE2s, ME1s, ME2s and ME3s (Fig. 3). The transcript level of *PaWOX2* was low in PEMs

and increased when EEs were formed. The highest transcript level of *PaWOX2* was observed in LE1s. Thereafter, the transcript level of *PaWOX2* decreased successively to become almost undetectable in MEs. The transcript level of *PaWOX8/9* was high in PEMs, EEs, LEs, but significantly lower in MEs. The expression of *PaWOX2* and *PaWOX8/9* in embryos suggests that *PaWOX2* and *PaWOX8/9* are involved in regulating embryo development.

In order to study the function of *PaWOX2* and *PaWOX8/9*, we constructed RNAi lines using both *β*-estradiol inducible and constitutive promoters. Two controls have been used: untransformed control (U-control) and transformed control (T-control, transformed with a GUS reporter).

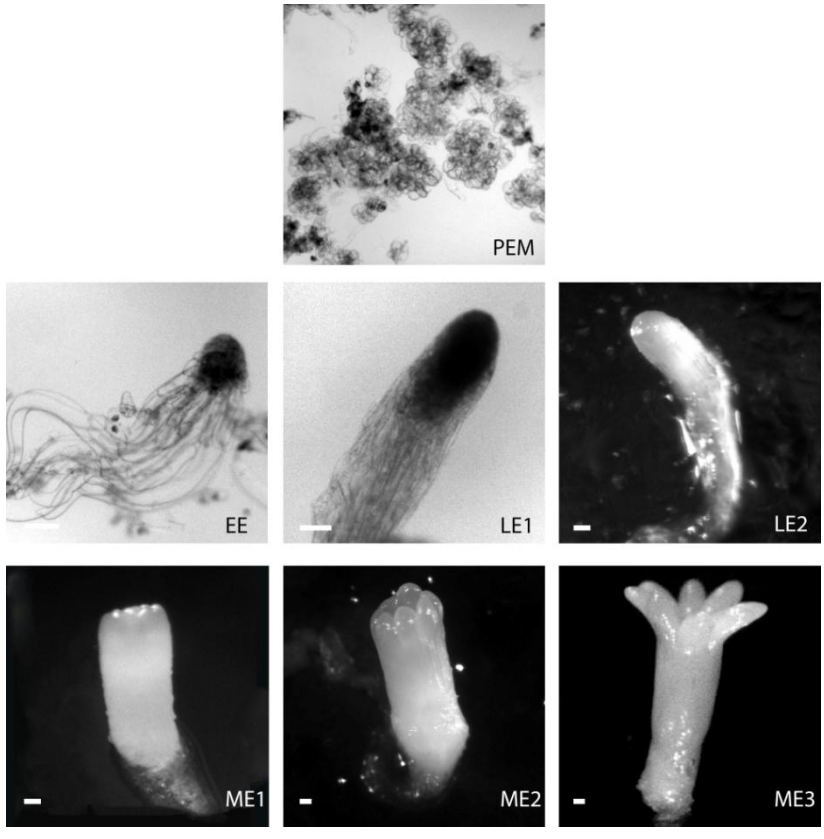

Figure 3. The developmental stages during embryo development in Norway spruce. Proliferating proembryogenic masses (PEMs), three and seven days after transfer to pre-maturation medium lacking PGRs; early embryo (EE), after one week on maturation medium; early late embryo (LE1) and late embryo before the formation of cotyledons (LE2) after one and two weeks on maturation medium, respectively; maturing embryo (ME1), characterized by the initiation of cotyledons after 5 weeks on maturation medium; ME2 (almost fully maturated) and ME3 (fully maturated) after about 8 weeks on maturation medium. Scale bar=100 µm.

3.2.2 *PaWOX8/9* is required for apical-basal organization in early and late embryos. (II)

More than 50% of the EEs in all *PaWOX8/9* RNAi lines had aberrant morphology. These embryos lacked a strict border between the embryonal mass and the suspensor. Vacuolated cells differentiated both from the basal cells in the embryonal mass and from the upper part in the embryos. Furthermore, the suspensor cells were observed to show higher variation in length and width in *PaWOX8/9i* embryos than in control embryos. In aberrant EEs, the basal cells in the embryonal mass divided anticlinally, periclinally and inclined. The apical-basal polarization was disturbed by the aberrant cell division planes. These aberrant cell division planes were observed only in embryos from the *PaWOX8/9* RNAi lines.

It is known that the cell fate of stem-cell daughters is positional dependent, and the position of the daughter cells is spatially determined and requires highly regulated cell division (Scheres *et al.*, 1995; Vandenberg *et al.*, 1995). Normal anticlinal cell divisions in the basal cells in the embryonal mass allow the embryos to develop along the apical-basal axis. However, the inclined or periclinal divisions in the basal cells in the embryonal mass gave rise to two daughter cells, which both remained in the basal part of the embryonal mass resulting in radial growth of the basal part of the embryonal mass. As a consequence, the LEs acquired a cone-shaped morphology. LEs with cone-shaped morphology were rarely observed in embryos from control cultures, while more than 50% of the LEs in the *WOX8/9i* lines showed a cone-shaped morphology.

These results suggest that i) the orientation of cell division planes in the stem cells in the basal part of the embryonal mass affects the shape of EEs; ii) *PaWOX8/9* directly or indirectly regulates the cell division orientation, and is important for the cell fate determination required for normal apical-basal organization in early embryos. A similar function has been shown in Arabidopsis, where *AtWOX8* and *AtWOX9* regulate cell morphology and division pattern in both the basal and the apical lineages from the single-cell embryo stage (Breuninger *et al.*, 2008).

3.2.3 *PaWOX2* is required for normal protoderm development in early and late embryos. (III)

On average 50% of the aberrant EEs in the 35S:*WOX2i* lines showed a characteristic aberrant phenotype. This aberrant phenotype could also be observed in LEs. The embryonal mass in these EEs and LEs lacked a smooth surface, and vacuolated cells, typical of the suspensor, differentiated in the embryonal mass. These observations suggest the absence of protoderm in the

embryonal mass, which was never observed in the control. The frequency of aberrant EEs in the inducible RNAi line XVE-*WOX2i.12* was significantly increased after *β*-estradiol treatment, but only when the treatment was performed before the formation of the LE2s. This suggests an important role of *PaWOX2* during the formation of LEs.

The protoderm will develop into epidermis, which is characterized by the secretion of lipids and waxes into its outer cell wall (Javelle *et al.*, 2011). The continuous hydrophobic layer forms the cuticle. A cuticularized layer is formed early during embryo development, already after differentiation of the protoderm (Yeats & Rose, 2013). By staining LE1s with Oil Red, it was found that the cuticularized layer was very thin or absent at the LE1s from 35S:*WOX2i* lines. The differentiation of the protoderm is regulated by *GL2*-type homeobox genes *ATML1* in Arabidopsis (Ito *et al.*, 2002) and *PaHB1* in Norway spuce (Ingouff *et al.*, 2001). Furthermore, LTPs are involved in secretion or deposition of extracellular lipophilic material (Sterk et al. 1991). Proper functioning of the protoderm in Norway spruce embryos requires a specific expression pattern of *GL2*-type and LTPs genes (Ingouff *et al.*, 2001; Sabala *et al.*, 2000). The assumption is that the disturbed cuticularization in *PaWOX2* RNAi embryos reflects that not all cells in the outer cell layer have attained protoderm identity and can synthesis and secret lipids to the outer surface.

Taken together, we show that *PaWOX2* is important for protoderm development specifically in EEs and LE1s, which is in accordance with the transient expression pattern of *PaWOX2*. In Arabidopsis *wox2* mutant embryos, the protoderm formation failed as a result of incorrect cell division plane orientation in the upper part of the embryo (Breuninger *et al.*, 2008). These observations suggest a conserved function of *WOX2* genes in protoderm development. In addition, the definition of the protoderm is regulated in a similar way in seed plants.

3.2.4 Defects during the development of MEs in *PaWOX* RNAi lines. (II and III)

Down-regulation of *PaWOX8/9* during the whole maturation process resulted in heart-shaped MEs. The elongation of the embryos along the apical-basal axis decreased, and instead the embryos expand horizontally. Sections of these embryos showed that the cortical cells in heart-shaped embryos were much larger than in normal MEs. The heart-shaped MEs observed in the *PaWOX8/9* RNAi lines resemble those of the *AtWOX9* mutant *stip-1* in Arabidopsis which fail to elongate along the apical-basal axis and cells expand horizontally (Wu *et al.*, 2007).

The EEs and LE1s from *PaWOX2* RNAi lines lacked a distinct border between the embryonal mass and the suspensor, and the suspensor cells were shorter. This phenotype suggests a role of *PaWOX2* in the correct development of suspensor cells, which is consistent with its expression in the suspensor (Palovaara *et al.*, 2010a).

The cultures of *PaWOX2* RNAi lines continued to proliferate on maturation medium. About 50% of the EEs formed in *PaWOX2* RNAi lines had an aberrant morphology. Furthermore, about 50% of the EEs failed to develop normally. The low recovery rate results in a low yield of MEs. In Arabidopsis, approximately one-third of all *wox2* mutant embryos fail to divide properly in the upper part at the octant stage (Breuninger *et al.*, 2008). However, most of these mutants recover eventually (Breuninger *et al.*, 2008; Haecker *et al.*, 2004). The results of our study suggest a more essential role for *PaWOX2* during early embryo development in Norway spruce than that of *AtWOX2* in Arabidopsis. Expression of *AtWOX2* is limited to the upper tier (Haecker *et al.*, 2004). In contrast, *PaWOX2* is expressed throughout the EE, including its suspensor (Palovaara *et al.*, 2010a). This broader expression pattern of *PaWOX2* compared to *AtWOX2* might explain why the down-regulation of *WOX2* causes more severe defects in Norway spruce. However, there are other explanations, for example, redundant function of other *WOX* genes and differences between somatic embryogenesis and zygotic embryogenesis. Further studies are needed before we will know if *WOX* genes in conifers have redundant functions.

In order to elucidate when during embryo development *PaWOX2* and *PaWOX8/9* are important for formation of MEs, XVE-*WOX2i* and XVE-*WOX8/9i* lines were treated with *β*-estradiol either during the whole maturation period or from the third week on maturation medium when LEs had developed. *β*-estradiol treatment did not have an effect on the maturation process either in XVE-*WOX2i* or in the XVE-*WOX8/9i* lines when the treatment started at the third week on maturation medium. However, the effects of the *β*-estradiol treatment were significant when the treatment was started before the formation of LEs.

In conclusions, our results suggest that i) *PaWOX2* and *PaWOX8/9* are important for normal development of early and late embryos, ii) *PaWOX2* might exert a more essential function in Norway spruce than *AtWOX2* in Arabidopsis, and iii) down-regulation of *PaWOX8/9* inhibits the elongation of the embryo along the apical-basal axis.

3.2.5 Time-lapse tracking analysis during embryogenesis (II and III)

In order to compare the development of embryos from control and *PaWOX* RNAi lines, we performed time-lapse tracking analyses. Three developmental pathways could be distinguished: (i) normal embryo maturation (Fig. 4.A); (ii) embryo degeneration, in which the cells on the surface layer of the embryonal mass became vacuolated and new embryogenic tissue was initiated from the degenerated embryo (Fig. 4.B); and (iii) embryo degeneration-regeneration, in which new LEs differentiated from the first selected embryo followed by development of maturing embryos (Fig. 4.C).

Most of the EEs, whether normal or cone-shaped, from the U-control culture developed into normal mature embryos following the normal developmental pathway. 30% to 50% of embryos from the T-control, as well as from the other transgenic lines, developed into ball-shaped embryos. This frequency is significantly higher than in the U-control, in which only about 20% of the embryos developed into ball-shaped embryos. We assume the increasing frequency of ball-shaped embryos is a side-effect of the transformation.

The specific developmental pathway in *PaWOX2* RNAi lines was embryo degeneration. Embryos following the degeneration pathway differentiated vacuolated cells on the surface of the embryonal mass (Fig. 4.B, a). Consequently, the original EE degenerated and new PEM-like embryogenic tissue differentiated from the degenerated embryo (Fig. 4.B, b). EEs, which developed into MEs (Fig. 4.B, c), differentiated from the new embryogenic tissue. However, we can't exclude that some of the EEs went through a new degeneration process. This kind of degeneration can also explain the over-proliferation of the 35S:*WOX2i* cultures after transfer to maturation medium.

The most striking difference between the cone-shaped embryos from *PaWOX8/9i* lines and the U-control was the degeneration-regeneration developmental pathway of LE1s. In this pathway, several new embryos differentiated from the first selected embryo (Fig. 4.C, b-e). Unlike the degeneration pathway in embryos from the 35S:*WOX2i* lines, in the degeneration-regeneration pathway, there were no vacuolated cells differentiating from the degenerating embryo and the regenerated tissue was already at the LE2 stage when it could be observed (Fig. 4.C, b). The degeneration-regeneration pathway is more like a process of re-patterning. It is known that a strict balance between cell division and cell differentiation is essential for normal embryo development and that randomization of cell division planes in embryos leads to drastic morphological defects (Laux *et al.*, 1996; Traas *et al.*, 1995; Berleth & Jurgens, 1993). It is likely that the division-plane alterations in the stem cells at the basal part of the embryonal mass

change the cell identity, which results in the generation of several LE2s instead one (Fig. 4.C, a).

Taken together, the results of time-lapse tracking support the ideas that i) *PaWOX2* is important for the definition of the protoderm and ii) *PaWOX8/9* is important for apical-basal embryo patterning formation.

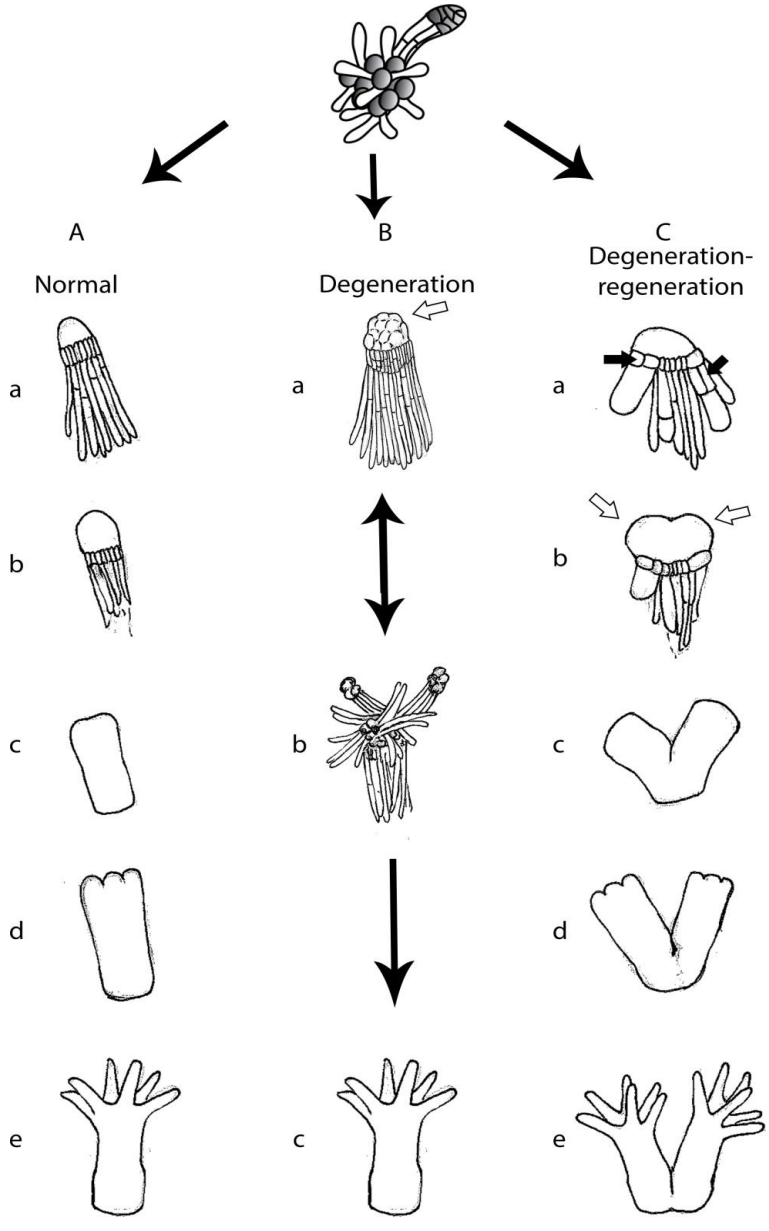

Figure 4. Schematic figure presenting embryo developmental pathways. Time-lapse tracking experiments were performed with EEs, which were isolated after one week on maturation medium and transferred to fresh maturation medium. EEs were sampled from control cultures as well as from 35S:*WOX8/9i* and 35S:*WOX2i* lines. When tracking embryos from 35S:*WOX8/9i* lines, both normal and cone-shaped embryos were selected. The development of each embryo was followed for over two weeks. Three typical developmental pathways were observed: A) Normal development, most EEs from the controls developed normally; a. EE, b. LE, c. ME1, d. ME2 and e. ME3. B) Degeneration, this developmental pathway being unique for EEs from the 35S:*WOX2i* lines; a. EE, note that the cells on the surface layer of the embryonal mass were vaculated (⇧, vacuolated surface layer), b. initiation of embryonic tissue from the degenerated embryo, c. ME3; C) Degeneration-regeneration, this developmental pathway being mostly observed for the cone-shaped embryos from 35S:*WOX8/9i* lines; a. EE, note the aberrant cell division plane (⬆, aberrant cell division plane), b, LEs, note that new LEs differentiated from the first selected embryo (⇧, embryonic tissue), and the new LEs continued to develop into c. ME1, d. ME2 and e. ME3.

3.2.6 *PaWOX8/9* affects the transcript abundance of cell-cycle-regulating genes. (II and III)

Asymmetric cell division is at least partly controlled by cell-cycle-regulating genes (Weimer *et al.*, 2012). The observed aberration in the plane of cell division in *PaWOX8/9i* lines implies that *PaWOX8/9* might influence the expression of cell-cycle-regulating genes. Therefore, we examined the transcript level of ten cell-cycle-regulating genes [*PaRBRL*, two *E2F* family genes (*PaE2FABL*), five *CYCLIN-LIKE* (*PaCYCLs*) genes, *PaMPK6L* and *PaESP*] in proliferating PEMs in the U-control and line 35S:*WOX8/9i.4*. Five out of the ten cell-cycle-regulating genes examined showed significant differences in transcript level between the U-control and the *PaWOX8/9* RNAi line. This suggests that *PaWOX8/9* directly or indirectly controls the transcript level of some cell-cycle-regulating genes.

We could not detect any changes in the transcript level of the tested cell-cycle-regulating genes in EEs from *PaWOX2* RNAi lines. This suggests that *PaWOX2* may not control cell division at the transcriptional level as *PaWOX8/9* does, and that *PaWOX2* and *PaWOX8/9* are involved in early embryo patterning through different pathways.

3.3 *PaWOX3* conserves its function in lateral organs initiation (IV)

In angiosperms, *WOX3* genes are involved in the recruitment of founder cells from lateral domains of shoot meristems, which form lateral regions of

the leaves (Shimizu *et al.*, 2009; Nardmann *et al.*, 2004; Matsumoto & Okada, 2001; Scanlon *et al.*, 1996). In order to elucidate if *PaWOX3* has similar functions as *WOX3* genes in angiosperms, the expression pattern of *PaWOX3* during embryo development was analyzed and thereafter *PaWOX3* RNAi lines were established for functional studies.

3.3.1 Expression of *PaWOX3* is high in mature embryos and shoot tips.

We analyzed the expression of *PaWOX3* during embryo development and in germinated embryos, by qRT-PCR analyses. We found the highest level of *PaWOX3* transcripts in mature embryos and in shoot tips. The spatial expression of *PaWOX3* was analyzed by histochemical GUS assay. GUS signals were detected at the base of the cotyledons and in the lateral margins. Results obtained by GUS staining and qPCR analyses were in good agreement with results obtained by *in situ* mRNA hybridization in the MEs. These results suggest that *PaWOX3* is active in developing lateral margins.

The orthologs of *PaWOX3* in Arabidopsis (*PRS*) are expressed at the margins of cotyledon primordia (Haecker *et al.*, 2004) and later at the apices and the lateral margins of cotyledons (Nardmann *et al.*, 2004). The maize *PRS* ortholog *NS* shows a similar expression pattern (Matsumoto & Okada, 2001). These similar expression patterns imply that the function of *WOX3*-related genes might be conserved between angiosperms and gymnosperms.

3.3.2 *PaWOX3* is required for normal cotyledon and needle development

Tracking analyses of LE1s from control and *PaWOX3* RNAi lines was performed on maturation medium for 15 days. No deviation from normal embryo development could be observed, which was expected since *PaWOX3* is expressed only at very low level at the earliest stages of embryo development.

The first aberrant phenotype that was identified in the *PaWOX3* RNAi lines was observed in the MEs. Cotyledons in MEs from *PaWOX3* RNAi lines were usually shorter and thicker, and had less pointed tips than those from control lines. After germination, normal cotyledons had a flattened morphology. In contrast, one third of the cotyledons in germinated embryos from *PaWOX3* RNAi lines had a rounded shape. Furthermore, these cotyledons were folded in the middle apical part. At the same time as the aberrant morphology was observed in the cotyledons, *PaWOX3* was being expressed in the cotyledons. Furthermore, needles from *PaWOX3* RNAi plants were round-shaped instead of flattened. Interestingly, the needles from *PaWOX3* RNAi plants also lacked sawtooth hairs which are common on normal needles. Sections of the needles showed that the round-shaped needles lacked the lateral outgrowths, which the normal flattened needles had.

In addition to the aberrant morphology of cotyledons and needles, the root length was reduced in plants from three out of four *PaWOX3* RNAi lines. In angiosperms, only a decrease of the number of lateral roots, but not the root length, has been reported in rice *nal2/nal3* mutant plant (Cho *et al.*, 2013). In this aspect, *PaWOX3* appears to differ from its angiosperm orthologs.

Arabidopsis *PRS* and maize *NS1/NS2* perform a conserved function in the recruitment of founder cells from lateral domains of shoot meristems that form lateral and marginal regions of leaves and flower organs (Shimizu *et al.*, 2009; Nardmann *et al.*, 2004; Matsumoto & Okada, 2001; Scanlon *et al.*, 1996). Our results suggest that this function of *WOX3* genes is largely conserved between gymnosperms and angiosperms. However, the *WOX3* gene might perform a broader function in Norway spruce.

4 Conclusions

The *PaWOX* genes group within the main clades of the corresponding angiosperm genes, suggesting that their diversification took place before the angiosperm/gymnosperm split, approximately 300 million years ago. There are clear orthologs of both *WUS* and *WOX5* that are present in the gymnosperm Norway spruce. Furthermore, there has been an expansion in the number of genes in the intermediate clade and the embryo-specific functions of these genes have been retained within the *Pinaceae* family.

PaWOX8/9 is highly expressed during early and late embryo development. *PaWOX8/9* regulates the establishment of the apical-basal embryo pattern by controlling the orientation of the cell division plane, which in turn controls cell fate determination during early embryo development. This function is evolutionarily conserved between gymnosperms and angiosperms. In addition, *PaWOX8/9* is required for the regulation of cell-cycle genes.

PaWOX2 is transiently expressed during early embryo development. *PaWOX2* is required for proper protoderm formation, which is essential for normal patterning during embryo development. In addition, *PaWOX2* is important for the expansion of suspensor cells. We suggest that *WOX2* exerts a conserved role in protoderm development in gymnosperms and angiosperms. However, the *WOX2* gene might perform a broader function in gymnosperms than in angiosperms.

PaWOX3 is highly expressed in mature embryos at the base of each cotyledon and in the lateral margins separating the cotyledons into an adaxial and an abaxial side. Reduced expression of *PaWOX3* causes aberrant cotyledon and needle morphology. We suggest that *WOX3* exerts a conserved function in margin outgrowth in lateral organs in both gymnosperms and angiosperms.

5 Future perspective

In conifers, knowledge about the molecular regulation of embryo development and patterning formation is very limited. My thesis presents the *WOX* gene family in Norway spruce. Based on the current results, I believe that further studies of these *WOX* genes and their related regulators would bring deeper understanding on the molecular network during embryo patterning formation in conifers.

Genes belonging to the *WOX4* sub-family are supposed to have functions related to cambium development. It would be of great interesting to study the cambium development in the conifer species Norway spruce. *PaWOX5,* but not *PaWUS,* is expressed in both the root tip and the shoot tip. The hypothesis is that *PaWOX5* exerts the function of maintaining stem cells in both RAM and SAM. However, studies on *PaWOX5,* so far, could not elucidate the function of this gene. In addition, the redundancy among the *PaWOX* genes might also be an interesting subject, especially from an evolutionary point of view.

RNA interference has proven to be a useful tool in functional studies. The genome database of Norway spruce, which was recently published, can enhance such studies. However, further improvement of techniques is needed for obtaining a deeper insight into the embryo patterning, for example by developing molecular markers for specific cell types and tissues.

The epigenetic mechanism plays an important role in the molecular network in Norway spruce. It is believed that the epigenetic 'memory' affects phenology and is important for adaption to stressful condition in Norway spruce. However, the molecular mechanism behind the epigenetic memory is still unknown. Investigation of epigenetic mechanism in conifers would become a great source of knowledge in plant breeding.

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