Developmental Phase Transitions in Norway Spruce

A Molecular Approach to Identify Regulatory Mechanisms

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Cover: Left image, an inbred *acrocona* plant displaying an apical transition shoot – a gradual conversion from vegetative to female identity of the top shoot. Right image, confocal micrograph of a transgenic somatic embryo, with an ectopic embryo emerging from the hypocotyl region.

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Abstract

Plant development proceeds through distinct phases that are controlled by complex networks of regulatory genetic circuits and fine-tuned by environmental and endogenous cues. Many of these regulatory networks have been unraveled in annual and perennial angiosperms, while they remain predominantly unknown in ecologically and economically important gymnosperm species such as the conifers.

By assessing global gene expression profiles during early somatic embryo development in Norway spruce we identified transcripts potentially associated with the transition from the embryonal to the vegetative phase. A conifer transcript (*PaHAP3A*) homologous to *LEAFY COTYLEDON1* (*LEC1)*, a well-known master regulator of Arabidopsis embryogenesis, was characterized. *PaHAP3A* is active during early embryo development and is down-regulated during embryo maturation. Overexpression of *PaHAP3A* during embryo maturation causes differentiation of ectopic embryos. Together, our results support sub- and/or neofunctionalization between angiosperm and gymnosperm *LEC1*-type genes. Global inhibition of histone deacetylase (HDAC) action during embryo maturation alters the patterns of embryonic gene expression and arrests the maturation progression, while HDAC inhibition during germination retains the embryogenic potential.

In the naturally occurring, early cone-setting Norway spruce mutant *acrocona*, vegetative shoots typically display a transition from vegetative to female identity. By in situ hybridization assays, expression patterns of previously identified and novel MADS-box genes were characterized in female cones from wild-type and *acrocona* plants at distinct developmental stages. Furthermore, an RNA sequencing approach utilizing a population of inbred *acrocona* plants identified a MADS-box gene (*DAL19*) as a potential important factor for the initiation of the seed cone.

Taken together, this thesis presents novel insights into the regulatory networks that control important phases during the life cycle of Norway spruce, indicating both conservation and functional divergence between gymnosperms and angiosperms.

Keywords: Conifers, embryogenic potential, HDAC inhibition, *LEC1*, MADS-box, phase transitions, *Picea abies* var. *acrocona*, somatic embryogenesis.

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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. (2011). Important processes during differentiation and early development of somatic embryos of Norway spruce as revealed by changes in global gene expression. *Tree Genetics and Genomes* 7, 347-362.

II Uddenberg D., Valladares S., Abrahamsson M., Sundström J.F., Sundås-Larsson A., von Arnold S. (2011). Embryogenic potential and expression of embryogenesis-related genes in conifers are affected by treatment with a deacetylase inhibitor. *Planta* 234(3), 527-539.

III Uddenberg D., Reimegård J., Clapham D., Almqvist C., von Arnold S., Emanuelsson O., Sundström J.F. (2013). Early cone setting in *Picea abies acrocona* is associated with increased expression of a MADS box transcription factor. *Plant Physiology* 161(2), 813-823.

IV Carlsbecker A., Sundström J.F., Englund M., Uddenberg D., Izquierdo L., Kvarnheden A., Vergara-Silva F., Engström P. (2013) Molecular control of normal and *acrocona* mutant seed cone development in Norway spruce (*Picea abies*) and the evolution of conifer ovule-bearing organs. *New Phytologist* 200(1), 261-275.

Appendix

Uddenberg D., Abrahamsson M., Sundström J.F., von Arnold S. Overexpression of *PaHAP3A* retards embryo development and stimulates differentiation of ectopic embryos.

Papers I-IV are reproduced with the permission of the publishers.

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

I Taken part in discussing the results and writing the paper. Conducted expression analyses of some of the candidate genes.

II The overall responsibility for planning the study, analysing data and writing the paper. Performed most of the experimental work.

III Highly involved in planning the study, analysing data and writing the paper. Performed most of the experimental work, except for sequencing that was carried out at SciLife lab (KTH).

IV Performed and analysed the phylogenetic work.

Appendix

The overall responsibility for planning the study, analysing the data and writing the manuscript. Performed most of the experimental work.

1 Introduction

Today the world faces a major challenge in meeting the demand for an ever-increasing need for fuel and fiber. Forestry holds a potential key role in meeting these demands. Growing forests and forest products used for wood and fiber-related products are natural carbon sinks that store carbon. Furthermore, forest by-products can be used as a green renewable alternative to the diminishing non-renewable fossil fuels. The world population now exceeds 7 billion and is estimated to reach 9 billion in 2050 (http://esa.un.org/wpp/). The challenge to supply the growing population with forest products in a sustainable way implies a pressing need to increase the productivity and quality of the forests.

Spermatophytes, i.e. seed plants, are commonly classified into two major groups: angiosperms and gymnosperms. Coniferous trees, one of Sweden's most important raw materials, belong to the gymnosperm group of seed plants. The gymnosperm group also includes gnetophytes, ginkgophytes and cycads. The name gymnosperm stems from the Greek word gymnospermos meaning naked seed, and refers to the seeds (or maybe more correct – ovules) being openly positioned on their reproductive structures (Farjon 2008). The angiosperms, flowering plants, encompasses all other species of extant seed plants. Angiosperms have their seeds enclosed within an ovary. Molecular markers and fossil records estimate that angiosperms and gymnosperms separated some 300 million years ago (Smith et al., 2010)

Over the past decades a vast amount of research has aimed at understanding the molecular mechanisms that regulate developmental processes, especially in the angiosperm model plant Arabidopsis (*Arabidopsis thaliana*). However, little is known of the molecular regulation of developmental pathways of conifers, despite their ecological and economic importance.

1.1 Developmental Phases During the Life Cycle of Plants

Most plants undergo distinct transitions between different developmental phases during their life cycle. The sequence of developmental phases covering the lifespan of an organism is referred to as ontogeny and is usually characterized by chronological age (Gatsuk et al., 1980). Phase transitions are under the strict supervision of a precise temporal and spatial genetic machinery and many transitions are consequences of responses to environmental cues such as light intensity and quality, day length, nutrient availability, and temperature, but also to endogenous signals transmitted by hormones (reviewed in e.g. Huijser & Schmid, 2011; Bäurle & Dean, 2006).

The life cycle of a plant generally encompasses four major ontogenetic phases: (1) the embryonic or latent phase, (2) the vegetative phase, (3) the reproductive phase and (4) the post-reproductive phase (Smirnova & Bobrovskii, 2001; Gatsuk et al., 1980). Phase specific traits include leaf shape and size, leaf retention, branching pattern, disease resistance, capacity for producing adventitious roots, and reproductive competence (Huijser & Schmid, 2011; Poethig, 2010). Furthermore, depending on species these traits can occur either in a bimodal fashion, present or absent, or continuously throughout the plant body, increasing or decreasing. The continuous presence of traits can be manifested such that different parts of a plant may exist in different developmental phases, a phenomenon known as heteroblasty (Goebel 1889; recently reviewed in Zotz et al., 2011). Ontogeny can be further categorized, not only by chronological age, but also by the biological criteria that indicate the stage of development. The additional division of phases into stages or periods is especially apparent during vegetative growth, during which plants acquire the competence to produce reproductive structures. Initially during vegetative growth the plants are considered to be juvenile and they mainly utilize their photoautotrophic competence to increase their size and mass. Later during vegetative growth some shoots will take on more adult reproductive characteristics, and in day-length sensitive plants this can be manifested as the competence for flower induction by photoperiodic stimuli (Huijser & Schmid, 2011; Poethig, 2010; 2003). This transition from juvenile to adult shoot development is referred to as vegetative phase change (Poethig 1990). In annuals such as Arabidopsis these phases can be subtle and gradual compared to the more extreme manifestations found in heteroblastic perennials such as *Acacia* species and European ivy (*Hedera helix*) (recently reviewed Zotz et al., 2011). Gradual changes in heteroblasty, as typically detected during reproductive competence, are usually used as criteria to classify the progression of the juvenile-to-adult phase change. However, it is not established how heteroblasty relates to the reproductive competence of the

shoot, and the use of the terms 'juvenile' and 'adult' for describing both heteroblastic changes (e.g. leaf morphology) and the transition from vegetative to reproductive shoots may lead to misperception (Huijser & Schmid, 2011; Zotz et al., 2011 and references within).

In trees such as the conifers the differential development of shoot meristems usually creates a gradient of ontogeny, in which the apical part of the tree can display reproductive characteristics and the basal part remains juvenile. This ontogenetic gradient, which also can be apparent along a single branch, makes trees very attractive systems with regard to phenotypic and molecular studies of developmental phase transitions. In natural stands it is apparent that plants, and especially trees, of the same chronological age have reached different stages of ontogenetic ageing, thus complicating studies concerning the structure and dynamics of a population. Therefore, based on the ontogeny of about 100 plant species Gatsuk and colleagues proposed an even more fine-scaled division of ontogenetic sequences (Gatsuk et al., 1980): the vegetative phase (2) involves a seedling state, a juvenile state and a virginile (adult) state; the reproductive phase (3) a young state, a mature state and an old state; and the post-reproductive phase (4) a subsenile state and a senile state. However, it remains to be determined if these states are intermediate stages or the manifestation of several genetic programs.

1.2 Forest Tree Improvement

Sweden is a richly forested country with forests covering more than half of its total land area. Spruce and pine together represent more than 80 % of the total forest biomass (Skogsindustrierna 2012). The economic importance of wood is manifested in such essential products as paper, pulp, timber and energy, taken together; Sweden is the second largest exporter of these wood products in the world (Skogsindustrierna 2012). Hence, forestry is a multi-billion (SEK) industry and one of the country's most valuable resources.

To meet the increasing worldwide demand for fuels and fiber, it is essential to improve quality and quantity through efficient tree breeding strategies. Companies in the forest industry are today looking beyond classic usages of forest products such as pulp for paper and wood for building, and aspire towards producing novel biomaterials, building materials and fuels. If this ambition is to succeed, refined tree breeding strategies aiming towards more complex traits are needed, if the Swedish forestry sector wants to continue their aspiration to be at the cutting-edge. To breed for more complex traits, however, it is of utmost importance to gain a more thorough understanding of the basic genetic networks that control important developmental phases. The first, and

foremost, reason for forest tree improvement through breeding is to maximize the economic value of the products obtained from the trees included in the breeding programs. However, recent public enlightenment concerning climate change and previous and present exploitation of pristine land areas may have steered some interest towards other prospective uses as well. Future uses that may increase in demand include forests as e.g. prevention of soil erosion, a part of agroforestry or pure amenity forests (Eriksson et al., 2006).

This thesis converges on phase transitions in conifers using Norway spruce (*Picea abies*) as a model. Therefore the next two paragraphs, concerning forest tree breeding and mass propagation, will be presented with regard to Norway spruce.

1.2.1 Forest tree breeding

Forest tree breeding aims to improve important traits according to the principles of quantitative genetics. Which traits to improve, in what order of priority, are critical considerations, as are the genetic variation in traits and the mode of inheritance.

In Sweden, forest tree breeding started already during the 1930's and Sweden has ever since been considered as a pioneer in the field. Skogforsk is the authority responsible for the breeding program of spruce and pine in Sweden. The basic breeding regime of Norway spruce is presented in Figure 1.

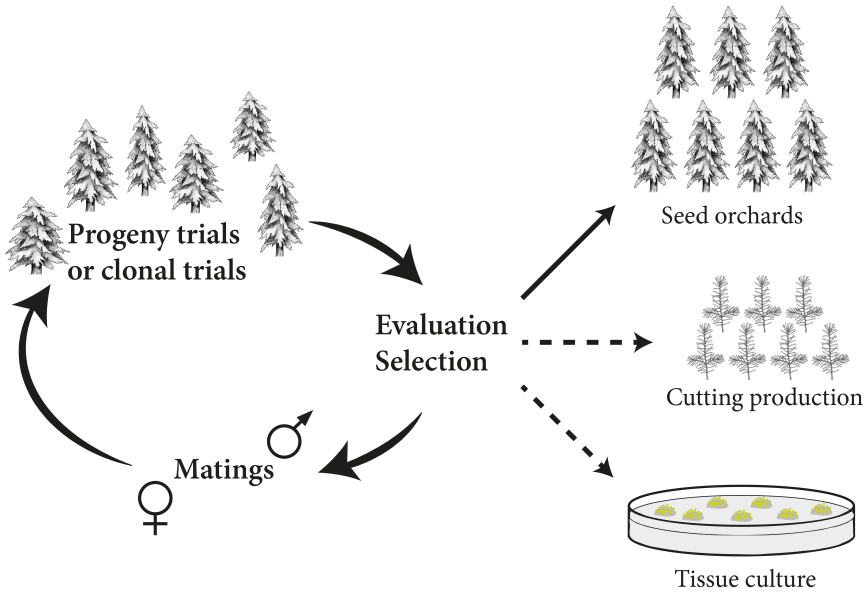

Figure 1. The principal outline of forest tree breeding in Sweden, using Norway spruce as an example. Trees in the breeding population with desirable traits are selected and crossed. Progenies

or cuttings are grown at different locations in field trials for 10-15 years before major assessment of traits. The best trees are then selected and used for further inclusion in the next breeding cycle or used for the establishment of seed orchards. Massпropagation of Norway spruce is typically carried out via sexual reproduction methods in seed orchards but is also possible using vegetative propagation methods such as cuttings and tissue culture. Image after Eriksson et al. (2006).

1.2.2 Mass propagation

After genetic testing of progenies in the breeding program, elite genotypes are selected for mass propagation. Most of the selected genotypes of Norway spruce are propagated via standard sexual reproduction methods in clonal seed orchards. Today, only 69% of all planted Norway spruce seedlings originate from seed orchards (Skogsstyrelsen 2013). Compared to other important tree species this is a very low number and the reason for this is simply that the supply does not meet the demand. One reason for the shortage of stored Norway spruce seeds is that even after reproductive competence is achieved in the reproductive trees, cone-setting is irregular between years. This increases the need for large seed orchard areas and seed storage. Pollen contamination is a serious problem in many coniferous seed orchards, since part of the genetic gain from the breeding is lost. But still, improved forest trees in Sweden today typically display a 10-15 % growth increase compared to their unimproved counterparts. The next generation of trees, derived from seed orchards currently under development, is estimated to yield up to 20-25% improved growth over unimproved trees (Rosvall et al., 2001).

An alternative to sexual propagation via seed orchards is vegetative propagation, widely used for ornamental plants, berries and fruit trees. Genetic gain is a major inducement to use vegetative propagation in other species, since not only the additive genetic variance is exploited, but also the non-additive genetic variance (Eriksson et al 2006). Vegetative propagation of forest trees offers the advantage that pollen contamination and recombination events are avoided. Another possible use of vegetatively propagated material is in parental evaluation trials, whereby one genotype can be tested under different environmental conditions.

There are several different methods of vegetative propagation. The most common methods include cuttings and graftings. Although these techniques are widely used for commercially available angiosperm species, there are problems in implementing them in conifer species such as Norway spruce. One disadvantage is that the techniques are time consuming and include many manual steps, and are thus expensive. Another drawback for cutting propagation is that the mother trees, when they are tested and ready for mass propagation, are usually too old to be propagated via cuttings. Therefore, the

Norway spruce cuttings used in operational forests today originates from bulk propagated elite genotypes.

Another vegetative propagation method is micropropagation – i.e. using plant tissue culture. In many species, multiplication of plants via axillary or adventitious buds is common practice. However, for most conifers the most promising method for large-scale vegetative propagation is via somatic embryos (See sections 1.3.1 and 3.1). The possibility to cryopreserve embryogenic cultures enables the breeders to test genotypes in the field and then thaw elite genotypes and mass propagate them upon customer demand.

1.3 Embryogenesis

Embryogenesis is the first developmental phase of multicellular organisms and begins with defined patterns of cell division of the zygote i.e. the fertilized egg cell. Embryo development in plants is usually divided into two distinct stages, the early morphogenesis stage and the late maturation stage. These stages are under the control of precise genetic and hormonal regulation.

The basic body plan of plants is similar between species. However, the developmental origin can differ among species. In the angiosperm model plant Arabidopsis a seemingly invariant cell division pattern during early embryogenesis enables tracking of lineages back to early stages of embryo development (reviewed in De Smet et al., 2010). However, in most plant species, including the conifers, early cell divisions during embryogenesis do not follow stereotypic patterns, thus complicating tracking of the cell lineage.

1.3.1 Embryo development and patterning

During the first stage of embryo development the basic body plan of the organism is established. In plants, the basic body organization is arranged along two perpendicular polar axes. The main apical-basal axis consists of two distal meristems, the shoot and root apical meristems (SAM and RAM), which are joined together via the hypocotyl. The perpendicular radial axis consists of a succession of concentric tissue layers from the outer epidermal layer, through the ground tissue, to the central vasculature (Ueda & Laux, 2012; Lau et al., 2011).

In many flowering plant species, including Arabidopsis, the beginning of embryogenesis is characterized by elongation of the zygote and subsequent asymmetrical cleavage into a small apical daughter cell and a large vacuolated basal cell. The apical daughter cell further divides in transversal and longitudinal patterns to form the embryo proper, which will develop into most of the plant body. The basal cell divides only transversally to form suspensor

cells, which, apart from its uppermost cell, are terminally differentiated (Ueda & Laux, 2012; West & Harada, 1993). The suspensor is essential during early embryo development and its role includes support and positioning of the embryo, synthesis of plant growth regulators (PGRs) and transport of nutrients to the embryo from the female gametophyte or surrounding cells. The shape of the suspensor varies between species of angiosperms and the suspensor can, as in Arabidopsis, consist of a single file, or be broad with a column of several hundred cells (*Phaseolus*), large and spherical (*Cytisus*) or develop an elaborate branched haustoria (*Tropaeolum* and *Sedum*) (Yeung & Meinke, 1993). The second stage of embryo development – embryo maturation – begins with the arrest of morphogenesis and continues until the seed has reached its metabolically quiescent state and prepares for eventual germination. During the maturation stage a major increase in synthesis and accumulation of storage reserves occurs. (Braybrook & Harada, 2008; Santos-Mendoza et al., 2008; Gutierrez et al., 2007; West & Harada, 1993)

Embryogenesis in gymnosperms, often depicted for the conifers, can be divided into three major stages (Singh 1978): (1) proembryogeny – all stages before the elongation of the suspensor, (2) early embryogeny – all stages after suspensor elongation and before root meristem establishment, (3) late embryogeny – establishment of RAM and SAM and all subsequent histogenic events. Specific conifer features during embryogenesis include the differentiation of tube cells during early embryogeny and the development of multiple cotyledons during late embryogeny. Common, but not general, features among conifer species include the free nuclear stage during proembryogeny, in which nuclear duplication occur without cytokinesis, and cleavage polyembryogeny, the phenomenon in which multiple embryos form during early embryogeny.

Studying development of zygotic embryos in conifers is difficult, due to both the inaccessibility of the embryo within the seed and to the asynchronous development of embryos within one cone. Another obstacle for spruce is the irregular cone-setting between years. These difficulties can be circumvented by the utilization of somatic embryos for studying embryology. A more detailed description of somatic embryogenesis will follow under the Results and Discussion (section 3.1). Somatic embryogenesis differs from zygotic embryogenesis only during the first steps of development, in which embryogenic cultures proliferate as proembryogenic masses (PEMs) in culture medium supplemented with PGRs. After withdrawal of PGRs, early embryos differentiate from PEMs and thereafter the two paths of embryogenesis are similar. The development of somatic embryos in embryogenic cultures can be

synchronized, making it possible to sample and analyze embryos of specific developmental stages.

1.3.2 Molecular regulation of embryo development

Early during embryogenesis, the apical-basal patterning is dependent on distinct expression of transcriptional regulators such as *WUSCHEL RELATED HOMEOBOX* (*WOX*) genes that together with *WRKY DNA-BINDING PROTEIN 2* (*WRKY2*) seem to regulate zygote polarity (Ueda et al., 2011), and the MAPKK kinase *YODA* (*YDA*) which together with its downstream MAP kinases regulate zygote elongation (Bayer et al., 2009; Lukowitz et al., 2004). Directional transport of auxin by e.g. PIN-FORMED (PIN) efflux carriers, and the subsequent local response by transcriptional regulators, have been shown to play major roles in processes such as apical-basal axis specification and cotyledon formation during early embryo development (reviewed in e.g. Ljung, 2013; Grunewald & Friml, 2010; Petrasek & Friml, 2009). Radial patterning of tissue layers mostly depends on cell-to-cell communication that involves peptide signaling and movement of transcription factors (reviewed in Lau et al., 2012).

Recent advances using laser capture microscopy have revealed distinct transcriptional regulation within compartments of the seed during different time points of development (Le et al., 2010). Although many of the identified genes are transcription factors, only a small subset of the genes has been extensively studied. Mutant analyses have revealed a few transcription factors that collectively seem to regulate early features of embryogenesis and seed maturation. These well-studied master regulators comprise the genes *LEAFY COTYLEDON 1* (*LEC1*), *LEC2*, *FUSCA3* (*FUS3*) and *ABSCISIC ACID INSENSITIVE 3* (*ABI3*). The *ABI3* (Parcy, 1997; Giraudat, 1992), *FUS3* (Gazzarrini et al., 2004; Luerssen et al., 1998; Bäumlein et al., 1994) and *LEC2* (Stone, 2001; Meinke, 1994) genes all possess a plant-specific B3 domain and are jointly referred to as the AFL-genes, an acronym of the three founding genes. *LEC1* encodes a HAP3 subunit of the CCAAT-box binding factor (CBF) (Lotan et al., 1998; West, 1994). The corresponding *LEC1/AFL* mutants all display similar pleiotropic phenotypes, including reduced levels of storage compounds, desiccation intolerance or precocious germination. During early embryo development *LEC1*, *LEC2* and *FUS3* are required for correct cell fate determination and patterning events, and later during embryogenesis the *LEC1/AFL* genes combined are essential in the induction and maintenance of seed maturation (reviewed in Santos-Mendoza et al., 2008). Together, these genes have been shown to act directly on promoters of maturation-related genes and to be involved in complex feedback mechanisms also with each

other (To, 2006). A recent systems biology approach utilizing the nowadays-abundant transcriptome data sets available for Arabidopsis, places *LEC1* as the central hub controlling the expression of *FUS3* and *ABI3* (Sreenivasulu & Wobus, 2013). Furthermore, ectopic expression of *LEC1* has been shown to affect both fatty acid biosynthesis and seed storage protein biosynthesis, possibly through the activation of *AFL* genes (Baud & Lepiniec, 2009; Mu et al., 2008; Kagaya, 2005).

Hormonal signaling and crosstalk are crucial for proper embryo development and for the phase transition from embryonic to vegetative development. Abscisic acid (ABA) is a key hormone during maturation and a high ABA to gibberellin (GA) ratio is required for the induction of dormancy (see e.g. Braybrook & Harada, 2008; Gutierrez et al., 2007). ABA signaling is also tightly connected to the expression of *LEC1* and the *AFL* genes (Gutierrez et al., 2007; Finkelstein et al., 2002). Moreover, *FUS3* is up-regulated by LEC1 and LEC2, and in turn directs ABA to GA ratios by feedback regulations involving both activation and repression of GA biosynthesis (Curaba et al., 2004; Gazzarrini et al., 2004). Interestingly, by the use of gain- and loss-of-function mutants Lumba et al. (2012) demonstrated that *FUS3* not only seems to be involved in the embryonic to vegetative phase transition via ABA/GA regulation, but also postembryonically to delay the vegetative phase change through repressing ethylene action (Lumba et al., 2012).

A plant needs a rapid repression of the seed transcriptional program to end embryonic development and to allow onset of germination and the subsequent vegetative phase of growth. Changes in chromatin structure ultimately lead to rapid changes in transcriptional activities of regulatory genes, and are known to occur through several different but interrelated processes. These mechanisms include DNA methylation, histone variant replacement, and histone posttranslational modifications (for reviews see e.g. Margueron & Reinberg, 2011; Feng et al., 2010). Regulators of chromatin state were first identified in *Drosophila melanogaster* and include Polycomb group (PcG) and trithorax group (trxG) proteins. Two main PcG complexes, Polycomb Repressive Complex 1 (PRC1) and PRC2, mediate transcriptional modulation through the deposition of repressive marks on chromatin and have been demonstrated to play instrumental roles in phase transitions and cell fate determination. The highly conserved and well-studied Polycomb Repressive Complex 2 (PRC2) silences genes by catalyzing trimethylation of histone H3 at lysine 27 (H3K27me3), which in turn recruits PRC1 to stabilize the repression via binding to the H3K27me3 mark and catalyzing monoubiquitination of histone H2 at lysine 119 (H2AK119ub). TrxG proteins counteract PcG protein repression by associating target genes with H3K4me2, which leads to

transcriptional activation (reviewed in Bemer & Grossniklaus, 2012; Holec & F. Berger, 2012). Many embryonic genes, including *LEC1/AFL* genes have been identified as targets for H3K27me3 (Kim et al., 2012; X. Zhang et al., 2007). Recently, Berger et al (2011) showed that *LEC2* contains elements that recruit H3K27me3 deposited by PRC2, thus providing a link to PRC2 and the deposition of this repressive mark on the *LEC1/AFL* genes (N. Berger et al., 2011).

Known repressors of seed development include *PICKLE* (*PKL*) and the *VIVIPAROUS1/ABI3-LIKE* (*VAL*) genes (reviewed in Jia et al., 2013; H. Zhang & Ogas, 2009). The *VAL* genes (*VAL1*, *VAL2* and *VAL3*) (also known as *HIGH-LEVEL EXPRESSION OF SUGAR-INDUCIBLE GENE2* (*HSI2*), *HSI2-LIKE1* (*HSL1*) and *HSI2-LIKE2* (*HSL2*) respectively) form a sister clade to the *AFL* genes and encode B3 domain proteins that include homeodomain-like (PHD) and CW-Zf domains frequently found in chromatin factors (Tsukagoshi et al., 2007; Suzuki et al., 2006). Monogenic mutants display wild type characteristics, whereas e.g. *val1 val2* double homozygous mutant seedlings display arrested postembryonic growth and the formation of embryo-like structures around the SAM area (Suzuki et al., 2006). *PKL* encodes a remodeling factor belonging to the CHD3 subfamily of chromatin remodelers (Ogas et al., 1999; Eshed et al., 1999). The primary root of young *pkl* seedlings displays a characteristic swollen, green region denoted the ’pickle root’ and further characterization of *pkl* seedlings revealed that *PKL* repress a whole range of traits associated with the seed, including an accumulation of seed storage reserves (Rider et al., 2004; Henderson et al., 2004). Furthermore, *pkl* seeds display an increased activity of *LEC1*, *LEC2* and *FUS3* (Rider et al 2003), and *PKL* acts to repress *ABI3* and in response to ABA (Perruc et al., 2007). Suzuki and colleagues (2007) also reported an activation of the LEC1/B3-network in *val* mutant seedlings with embryonic phenotype (Suzuki et al., 2006). The epigenetic modes of action of PKL and VAL proteins are slowly starting to be unveiled. PKL promotes the repressive epigenetic mark H3K27me3 through ATP-dependent chromatin remodeling, distinct from PRC2 (Ho et al., 2013; H. Zhang et al., 2012). The exact role of PKL as a chromatin remodeler is not yet determined. However, recent studies have shown that PKL in some respects seem to antagonize PcG proteins, which have led to the suggestion that PKL confer a trithorax mode of action (Gentry & Hennig, 2013 and references within). However, PKL can also repress PcG targets as well as non-PcG targets, also implying a general role of PKL in nucleosome positioning (Ho et al., 2013). The action of the *VAL* genes is hypothesized to be mediated through the B3 DNA binding domain (Sph/RY) (Suzuki et al., 2006). Recently Zhou et al (2013) demonstrated that histone

deacetylase 19 (HDA19) interacts with *VAL2* to repress *LEC1/AFL* gene expression (Zhou et al., 2013). This has led to the speculation that the *VAL* gene repression of *LEC1/AFL* genes includes a VAL-mediated recruitment of a histone deacetylase (HDAC) to target genes with a Sph/RY motif recognized by the B3 domain and chromatin marks that are recognized by the PHD and CW-Zf domains (Jia et al., 2013). However, the VAL proteins are also strongly associated with the action of AtBMI1 that mediates H2Aub on target genes, which in turn leads to maintained PRC2-mediated repression via H3K27me3 (Yang et al., 2013). This is particularly interesting since this contradict the usual hierarchy seen in metazoan systems, where H3K27me3 (PRC2) usually precedes H2Aub (PRC1). Repression of the LEC1/B3 network is also strongly linked to hormonal activity. The *pkl* phenotype during seed maturation is augmented by inhibited GA biosynthesis, and adult *pkl* plants resemble GA signaling mutants, e.g. active GA accumulation and reduced responsiveness to GA (Henderson et al., 2004; Ogas et al., 1997). The embryonic seedling phenotype of *val* mutants is enhanced by inhibition of GA biosynthesis and the down-regulation of the *AtGA3ox1* gene in the *val1 val2* double mutant also suggest GA signaling as an important constituent for embryonic repression (Suzuki et al., 2006).

Little is known about the molecular regulation of conifer embryogenesis. At least partly, this can be attributed to the lack of mutant analyses, which is due to the prevalence of large genome sizes (up to 200-400 times the size of the Arabidopsis genome) in combination with the fact that until very recently conifers lacked a sequenced reference genome (Nystedt et al., 2013). Long regeneration times, and the sheer adult size of most conifers, also contribute to complicate functional studies. During recent years, however, progress in establishing somatic embryogenesis in different conifer species has provided a powerful tool for studying embryogenesis, in which transgenic studies are feasible. Although, many of the present studies concerning molecular regulation of conifer embryogenesis have mainly been of a comparative nature, in which angiosperm model systems such as Arabidopsis have been used as reference for identifying important regulators of developmental processes.

An integral part of embryogenesis is the degradation of cells and tissues by programmed cell death (PCD). In Norway spruce two waves of PCD have been identified to occur during somatic embryogenesis. The first encompasses the degradation of PEM cells during proliferation and the second is responsible for the degradation of suspensor cells during early embryo differentiation (Filonova et al., 2000). Activation of proteases cleaving the caspase substrate Val-Glu-Ile-Asp (VEIDase) is essential for PCD and proper differentiation of somatic embryos (Bozhkov et al., 2004). Furthermore, the elimination of

suspensors during embryogenesis is dependent on the translocation of the metacaspase mcIIPa from the cytoplasm to the nuclei (Bozhkov, 2005; Suárez et al., 2004). Cells expand through increased water and solute uptake channeled by aquaporins, which are active in regions of cellular expansion such as the suspensor. The *PtNIP1;1* gene, from loblolly pine (*Pinus taeda),* encodes a NIP member (noduline-like protein) of the aquaporin protein superfamily, functionally capable of acting as a water channel (Ciavatta et al., 2001). In addition, a *PtNIP1;1* promoter-GUS transgenic approach demonstrated that the expression of the aquaglyceroporin is present in PEMs and once differentiation of embryos begin, the promoter expression is confined to embryonal tube cells and suspensors (Ciavatta et al., 2002).

An important patterning event during conifer embryogenesis includes the establishment of the RAM and SAM. Several members of the Norway spruce *WOX* gene family have recently been identified (Hedman et al., 2013). Spatio-temporal expression patterns suggest that they, like their angiosperm counterparts, are important regulators of developmental processes in conifers, and *PaWOX2* and *PaWOX8*/9 are specifically active during conifer embryogenesis (Hedman et al., 2013; Palovaara & Hakman, 2009). Polar transport of auxin is essential for correct patterning and apical-basal axis formation in conifer embryos (Larsson et al., 2007). Furthermore, chemical inhibition of auxin transport suggests that *PaWOX2* and *PaWOX8/9* are regulated by polar auxin transport (Palovaara et al., 2010; Palovaara & Hakman, 2009). Other genes important for proper patterning of the SAM, and shown dependent on polar auxin transport, include the *KNOX1-like genes HBK2* and *HBK4* (Larsson, Sitbon, et al., 2012) and the *CUP-SHAPED COTYLEDON-like* gene *PaNAC01* (Larsson, Sundström, et al., 2012). Tahir et al. (2006) showed that an *ARGONAUTE* family member, *PgAGO,* from white spruce (*Picea glauca*), is active in cells of both the SAM and RAM and that RNA-mediated *PgAGO* suppression results in abnormal embryo development and a poorly organized SAM (Tahir et al., 2006).

A stepwise peripheral patterning takes place during embryo development in Norway spruce (Ingouff et al., 2003; 2001). Two Norway spruce homeobox genes (*PaHB1* and *PaHB2*), belonging to the homeodomain-glabra2 (HD-GL2) family, are expressed in PEMs. The expression of *PaHB1* becomes restricted to the protoderm in the embryonal mass of early embryos (Ingouff et al., 2001), while *PaHB2* is expressed in the underlying cortical layers in late embryos (Ingouff et al., 2003). *PaHB1* encodes a protein highly similar to a well-studied protoderm marker *ARABIDOPSIS MERISTEM LAYER 1* (*ATML1*). It has also been shown that the *Pa18* gene, encoding a putative lipid transfer protein, is important for proper function of the protoderm (Sabala et al 2000).

Embryo maturation is promoted by ABA, and B3-domain transcription factors play instrumental roles in mediating the ABA response in plants (Gutierrez et al., 2007). A Norway spruce homolog to the B3-domain gene *ABI3*, *Picea abies VIVIPAROUS1* (*PaVP1*), shares similar expression patterns to *ABI3*, suggesting a similar role during embryo maturation in conifers (Footitt, 2003).

1.3.3 Establishment of embryogenic cultures

Somatic embryogenesis is the process by which a somatic cell can ultimately differentiate into a somatic embryo. The regeneration of a plant embryo from a single somatic cell was first described in carrot (*Daucus carota*) over 50 years ago (Steward et al., 1958). The process is believed to involve a dedifferentiation event in a single cell, which is induced by stress or high doses of auxin. Stresses are well-known inducers of altered cell fates and dedifferentiation processes in plants and it is well established that different abiotic stresses (osmotic, dehydration, temperature and heavy metal ions) can be used to induce somatic embryogenesis (Kikuchi et al., 2013; 2006; Ikeda-Iwai et al., 2003). Tissue culture, which usually includes wounding and translocation of the explant from its body into a tissue culture environment, is itself also a major stress-inducing factor that may lead to dedifferentiation (reviewed in Grafi et al., 2011). Many experiments have shown that plants inhabit the ability to regenerate a variety of tissue types and even whole plants from already differentiated organs (Sugimoto et al., 2011 and references to older literature therein). Therefore, it is commonly believed that all plant cells harbor an innate ability for totipotency, i.e. the capacity to regenerate a complete adult organism. Noteworthy, however, is that recent findings suggest that not all plant cells by definition are totipotent, but rather display characteristics of transdifferentiation, i.e. an irreversible switch from one differentiated cell type into another (Sugimoto et al., 2011; 2010; Atta et al., 2009).

In order for somatic embryogenesis to occur, a cell needs to be competent to respond to a certain induction signal. Auxin is believed to be the potential induction signal required for somatic embryo formation (Braybrook & Harada, 2008). Today it is not known what causes the intracellular environment to respond to auxin and thus gain the competence to undergo somatic embryogenesis.

It has been well documented that ectopic activity of certain transcriptional regulators causes the induction of somatic embryogenesis. In Arabidopsis and other angiosperm species *LEC1* (Lotan et al., 1998), *LEC2* (Stone, 2001), *BABY BOOM* (Boutilier et al., 2002)et and *WUSCHEL* (Zuo et al., 2002) are

among some genes whose ectopic expression promote somatic embryo formation without exogenously supplemented auxin. Over-expression of other genes such as *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1* (Hecht et al., 2001) and *AGAMOUS-LIKE15* (Thakare et al., 2008; Harding et al., 2003) enhances the competence for somatic embryogenesis under auxin-inductive conditions.

So far we know little of the molecular interplay between stress, chromatin regulation, transcription factors and hormones. However, many studies indicate a strong connection between somatic embryogenesis, *LEC1/AFL* gene expression and the pivotal role of auxin (reviewed in Braybrook & Harada, 2008; Jia et al 2013). *lec1*, *lec2* and *fus3* mutants are all found to be compromised in their ability to differentiate embryogenic tissue when cultured on medium supplemented with the synthetic auxin 2,4 dichlorophenoxyacetic acid (Gaj et al., 2005), thus *LEC1/AFL* gene expression seems to be highly important for somatic embryo formation. Further direct links between auxin and the *LEC1/AFL* genes include: (1) induced auxin biosynthesis by LEC1 (Junker et al., 2012) and LEC2 (Stone et al., 2008; Braybrook, 2006), (2) up-regulated *FUS3* promoter activity in embryos by auxin (Gazzarrini et al., 2004), (3) and enhanced ectopic embryo maturation phenotype of the *LEC1*-overexpressing *lec1-tnp* mutant by exogenous addition of auxin (Casson & Lindsey, 2006).

Open chromatin is a supposed hallmark of totipotent stem cells (reviewed in Verdeil et al., 2007). Yadav et al. (2009) found that stem cells of the SAM in Arabidopsis displayed enhanced expression of chromatin-modifying genes (Yadav et al., 2009) Histone deacetylation events are crucial during plant embryogenesis and functional inhibition by chemical inhibitors or by histone deacetylase mutants affects seed associated genes as well developmental progression (Zhou et al., 2013; Chen et al., 2010; Tanaka et al., 2007; Tai et al., 2005). Tanaka and co-workers demonstrated that suppressed activity of the genes encoding two HDACs, HDA6 and HDA19, led to arrested post-germination growth and embryo-like structures on true leaves (Tanaka et al., 2007). Recently, He et al (2012) also showed a possible link between histone methylation and dedifferentiation. The mutants *curly leaf/swinger* and *embryonic flower2* (CLF/SWN and ELF2 are core components of the PRC2 complex) failed to produce callus from aerial tissues on inductive media concomitantly as H3K27me3 deposition patterns were shown to be altered at several gene loci, thus implying a role of proper PcG function and dedifferentiation (He et al., 2012).

1.4 Reproductive development

The vegetative to reproductive phase transition in seed plants is connected to shoot architectural changes and the appearance of reproductive organs. The capability of daylength-sensitive plants to differentiate reproductive organs in response to photoperiodic stimuli is usually taken place during the end of the vegetative phase (Huijser & Schmid, 2011; Amasino, 2010). The decision to produce reproductive structures needs an accurate timing in order to avoid unnecessary energy consumption that negatively affects the plant. This decision is orchestrated by a complex genetic network that utilizes information from both environmental and endogenous signals (Andrés & Coupland, 2012; Amasino & Michaels, 2010). When a plant undergoes the transition to the reproductive phase, the vegetative SAM can change its identity and become an inflorescence meristem (IM). The subsequent fate of the IM is species specific and includes either a direct conversion into a floral meristem (FM) or a conversion to lateral meristems that later takes on an FM identity (Huijser & Schmid, 2011).

1.4.1 Reproductive structures

Seed plants are defined by the presence of an ovule that can develop into a seed after fertilization. Although the ovule is the hallmark of seed plants, the evolutionary origin of the ovule itself and its associated structures remains elusive (Mathews & Kramer, 2012; Rudall et al., 2011). Another shared feature between angiosperms and gymnosperms is that micro- and megasporangium are situated on separate organs.

Differences related to the appearance and organization of the reproductive organs include that gymnosperms typically have spatially separated micro- and megastrobili, whereas angiosperm reproductive organs usually are organized in a bisexual flower. Despite the vast diversity in shape and coloration of angiosperm flowers there is still remarkable uniformity in the basic floral architecture.

Most angiosperm flower organs are arranged in consecutive circles, or whorls, in which the reproductive stamens and carpels are surrounded by a sterile perianth. In Arabidopsis the sterile perianth consists of an outer whorl of four sepals and a second whorl of four petals, and the third and fourth reproductive whorls are made up of six stamens surrounding two fused carpels that form the ovule-bearing gynoecium. However, there are exceptions to this basic floral architecture within the angiosperm group. Stamens and carpels are found in all extant angiosperms, but many taxa lack distinct petals and sepals. Several species among the basal angiosperms and monocots have instead two

whorls or a spiral arrangement of undifferentiated tepals surrounding the stamens and carpels (Litt & Kramer, 2010).

Reproductive organs of the gymnosperms are manifested as cones and are either of male or female identity (seed or pollen cones). They also lack the sterile perianth (sepals, petals or tepals) that surrounds the reproductive organs in angiosperms. Norway spruce belongs to the Pinaceae family of conifers, which are mostly monoecious and carry their seed and pollen cones on the same individual. However, some Pinaceae species such as *Pinus johannis* are nearly dioecious (Flores-Renteria et al., 2013). Other gymnosperm species, such as the cycads and Ginkgo, are dioecious with separated female and male trees.

Shoot development in Norway spruce begins in early spring, when vegetative buds flush and start their elongation growth. During early summer an unknown signal determines if the shoot will take on a vegetative or reproductive identity. For comprehensive descriptions on reproductive structures in Norway spruce see also (Carlsbecker, 2002; Sundstrom, 2001). Seed cones usually precede pollen cones as the first reproductive structures to emerge after reproductive competence have been acquired; although later during ontogeny both types coexist on the same individual. Seed cones typically appear on strong leading shoots in the upper part of the tree, at apical positions on lateral branches but never at apical positions of scaffold branches. The seed cone is an elongated axis of spirally arranged sterile bracts with partly fused reproductive ovuliferous scales (each containing two ovules). The development of a seed cone encompasses two calendar years in Norway spruce. During late summer of the first year the ovuliferous scale/bract structures start to differentiate and after dormancy during the following spring the cone is ready to be pollinated. Fertilization takes place during the same year, directly after pollination. The seeds mature within the cone during summer and autumn and are then detached from the cone during the second winter, counting from the initiation of the seed cone. Pollen cone development takes one calendar year and they are typically produced at basal positions of annual shoots. However, they can also be found on lateral branches at apical or lateral positions. Similar to ovuliferous scale/bracts of seed cones, microsporophylls are also spirally arranged along the cone axis. Each microsporophyll contains two microsporangia with several pollen mothercells. Pollen cones usually initiate during spring or early summer, and pollen is released after winter dormancy.

There are still many unresolved questions regarding the relationship between the ovule-bearing structure of gymnosperms and the angiosperm carpel, as well as the nature of the flower axis and the multiple occurrences of

hermaphroditism (Mathews & Kramer, 2012). Molecular and fossil data trace the origin of seed plants (*Spermatophyta*) back to the Middle Carboniferous, about 330 million years ago. The four extant gymnosperm lineages (*Acrogymnospermae*) diverged from the crown seed plants some 30 million years later and the origin of crown angiosperms is estimated to the Late Triassic, around 220 million years ago (Smith et al., 2010). The origin of conifer reproductive organs has previously been assessed by comparing the modern female ovuliferous scale to fossil records of extinct conifers (Florin 1951; Clement-Westerhoff 1988). Florin speculated that the evolution of the seemingly simple structure of the modern ovuliferous scale of modern conifers was the outcome of successive reductions of reproductive short-shoots (a complex structure of multiple interweaved sterile and fertile scales) present in Paleozoic conifers (Florin 1951). The theory of the ovuliferous scale as a complex structure is further supported by the presence of multiple vascular strands connecting the ovuliferous scale with the vasculature in modern conifer species. The reduction process is thought to have happened through a planation process, i.e. distribution of structures into a single plane. Thus, younger fossil records of more modern conifers should display an intermediate shoot structure between modern and Paleozoic conifers. Fossils displaying bilateral symmetry have been found and are exemplified by Lower Permian Voltziales (Florin 1951; Clement-Westerhoff 1988).

1.4.2 Molecular regulation of reproductive development

The timing of the transition from vegetative to reproductive growth is coordinated by responses to endogenous and environmental signals, which ultimately lead to flowering. There are several molecular pathways that have been identified to control flowering time. These pathways comprise the vernalization pathway (prolonged exposure to cold), the photoperiod pathway (day length and light quality), the GA pathway (requirement of GA for normal flowering), and the autonomous pathway (endogenous regulators independent of photoperiod and GA). Another pathway that recently has been described to control flowering time across angiosperm species includes age-related factors (an endogenous pathway including vegetative phase-change and miRNAs), and species specific pathways include an ambient temperature response and an accumulation of carbohydrate assimilates (mainly sucrose) in the apex (for recent reviews on flowering see Song et al., 2013; O'Maoiléidigh et al., 2013; Andrés & Coupland, 2012; Srikanth & Schmid, 2011; Amasino & Michaels, 2010). A simplified overview of the regulation of flowering in Arabidopsis is presented in Figure 2.

Recently, emerging genetic evidence from Arabidopsis and other angiosperm species show that the molecular regulation of the juvenile-to-adult vegetative phase change and the transition to reproductive development converge in an age-related pathway (reviews: Song et al., 2013; Poethig, 2013; Huijser & Schmid, 2011). Two microRNAs (miRNAs), miR156 and miR172, together with their targets, play pivotal roles in controlling these plant phase changes (Poethig, 2013; Wu et al., 2009). miR156 targets *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) transcripts (Schwarz et al., 2008; Wu & Poethig, 2006; Klein et al., 1996), and miR172 mainly targets *APETALA 2-like* transcripts, including *APETALA2* (*AP2*) itself and probably also some *SPL* transcripts (Jung et al., 2011; Mathieu et al., 2009; Jung et al., 2007; Aukerman & Sakai, 2003). Furthermore, miR156 is active during the early vegetative phase of the life cycle and acts to repress reproductive development, whereas miR172 activity increases as the plant ages and acts to promote flowering. Hence, miR156 and miR172 act in an antagonistic fashion and their complementary action during plant development seems to determine the juvenile-to-adult transition in angiosperm plants (reviewed in Song et al., 2013; Poethig, 2013; Huijser & Schmid, 2011; Bergonzi & Albani, 2011). The regulatory control of developmental transitions facilitated by miR156 and miR172 is also conserved in perennial woody angiosperms. Overexpression of miR156 in transgenic *Populus x canadensis* reduced the expression of miR-targeted *SPL* genes and subsequently prolonged the juvenile phase of growth (J.-W. Wang et al., 2011). However, conservation of this regulatory system remains to be elucidated in gymnosperms.

Once a plant has obtained its reproductive competence, it monitors temperature and photoperiod to coordinate flowering to seasonal changes. The idea of a mobile substance (florigen) that is activated in the leaf and then translocated to the SAM where it signals the differentiation of a floral primordium is an old concept, which have eluded scientist for more than 70 years (reviewed in Zeevaart, 2008; Corbesier & Coupland, 2006). In Arabidopsis, long days promote flowering through the protein function of the floral integrator *FLOWERING LOCUS T* (*FT*). The FT protein (suggested to be the elusive florigen) is synthesized in the leaf vasculature and later transported through the phloem to the SAM where it integrates signals to promote flowering (Corbesier et al., 2007). Photoperiod, temperature, plant age and GA have all been reported to influence the action of FT on flowering (reviewed in Song et al., 2013; Andrés & Coupland, 2012; Amasino, 2010). Once translocated to the SAM, the FT protein together with *FLOWERING LOCUS D* (*FD*) activates flowering genes (Abe, 2005; Wigge, 2005).

In the shoot apex FT/FD induce flowering by activating FM identity genes such as *APETALA1* (*AP1*) and other floral integrators such as *SUPRESSOR OF CONSTANS1* (*SOC1*), both members of the MADS-box family of transcription factors (Andrés & Coupland, 2012; Srikanth & Schmid, 2011; Amasino, 2010). *SOC1* has a central role in integrating flowering signals from photoperiodic-, vernalization-, GA-, autonomous and age-related pathways (Immink et al., 2012; J. Lee & I. Lee, 2010). *SOC1* was identified as the earliest gene expressed gene in apical meristems in response to inductive photoperiod in Mustard, and in Arabidopsis mutations in *SOC1* cause late flowering and overexpression is sufficient for photoperiod independent flowering (Borner et al., 2000; Samach et al., 2000; Menzel et al., 1996). Apart from *AP1*, *LEAFY* (*LFY*) is another well-studied FM identity gene that has been shown to be crucial for the activation of floral identity genes (Weigel et al., 1992; Irish & Sussex, 1990). Mutant studies have revealed some possible overlapping functions among FM identity genes; for example, double mutants of *AP1* and *LFY* drastically increase the phenotypes seen in single mutants (Weigel et al., 1992). *CAULIFLOWER* (*CAL*) and *FRUITFUL* (*FUL*) are two other MADS-box genes, closely related to *AP1*, that display redundancy with *AP1* in specifying the FM (Ferrándiz et al., 2000). Nevertheless, *LFY* might be the central regulator in establishing an FM, since *LFY* overexpression in an *ap1*, *cal*, *ful* triple mutant background at least partially can rescue FM defects (Ferrándiz et al., 2000). *SOC1*, *FUL*, *LFY* and *AP1* are all activated by *SPL* transcription factors in the SAM, involving signals from both FT/FD and miR156 (Jung et al., 2012; J.-W. Wang et al., 2009; A. Yamaguchi et al., 2009). Furthermore, SOC1 directly regulates *SPL* genes in response to photoperiod signals, and the SOC1 mediated SPL regulome also mediate GA signals to promote flowering (Jung et al., 2012). The differentiation of the FM from an IM is also dependent on other phytohormones, and especially the accumulation of auxin, which is dependent on polar auxin transport (Heisler et al., 2005; Benková et al., 2003; Reinhardt et al., 2000). Auxin also regulates the expression of *LFY*, via the auxin-responsive transcription factor MONOPTEROS (MP), and, in turn, LFY promotes auxin signaling in emerging FMs (N. Yamaguchi et al., 2013).

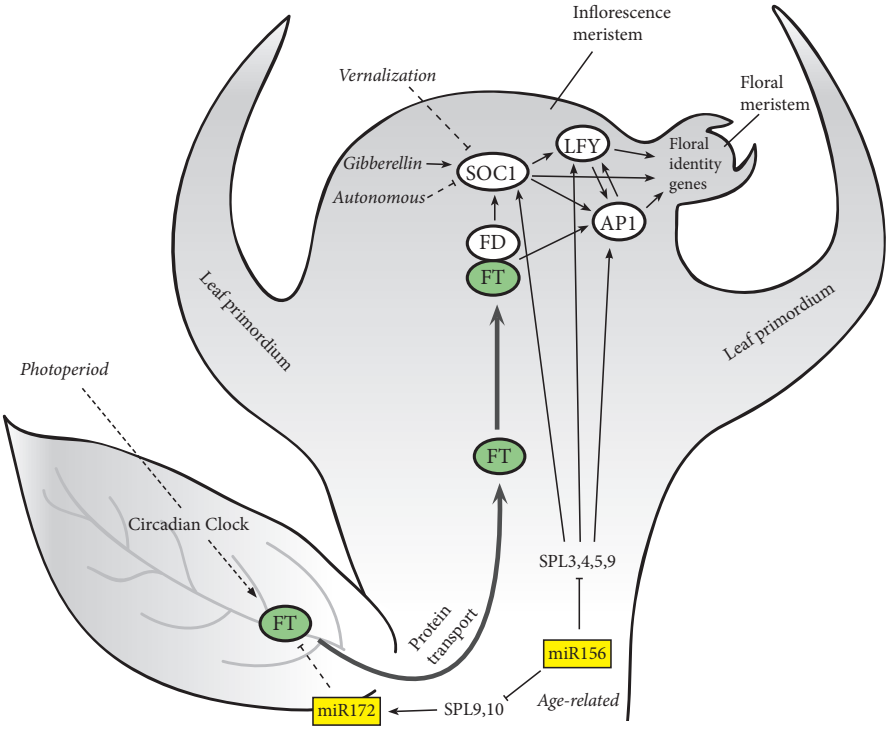

Figure 2. Simplified overview of flowering-time regulation in Arabidopsis. Photoperiodic signals are sensed in the leaves and involve the production of the mobile FT protein, which is then translocated to the apical meristem. FT binds to FD and the FT/FD protein complex triggers the *AP1* and *SOC1* genes, which activate *LFY* gene expression. *SOC1*, *LFY* and *AP1*, in turn, activate the floral identity genes (ABC genes). The autonomous and vernalization pathways, through intermediate steps, negatively regulate *SOC1* in the apical meristem. The gibberellin pathway act to promote *SOC1* expression in the apical meristem. miR156 represses flowering by targeting *SPL* genes, which, without miR156 repression, either promote flowering by activating *SOC1*, *LFY* or *AP1*, or repress flowering by activating mir172-dependent deactivation of *FT* in the leaf. This figure does not discriminate between proteins and genes. Lines with an arrow represent activation and those with a perpendicular bar represent repression. Dashed lines indicate that intermediary steps have been left out. (Image after Amasino (2010), Huijser and Schmidt (2011), Taiz and Zeiger (2010, 5th ed)).

After the specification of the FM, the FM identity genes, especially *LFY* and *AP1,* activate the floral identity genes that act to specify the organs of the flower (see e.g. Irish, 2010 and references within). The specification of organ identity was discovered through mutant analyses in Arabidopsis and snapdragon (*Antirrhinum majus*), which led to the classic ABC model of flower organ identity specification (Coen & Meyerowitz, 1991; Bowman et al.,

1991). The model stipulated that three gene functions (A, B and C) act in a combinatorial manner to specify the different organ identities of the flower. In the first whorl sepals are specified by A activity, while A and B activity together specify petals in the second whorl, B and C together act to specify stamens in the third whorl, and C function determines carpel identity in the fourth whorl. In Arabidopsis, *AP1* and *AP2* are required for A function (Bowman et al., 1993; 1991; Irish & Sussex, 1990), and *AP3* together with *PISTILLATA* (*PI*) is responsible for B function (Bowman et al., 1991), and *AGAMOUS* (*AG*) alone confers C function (Bowman et al., 1991). Although this simple model was proposed over 20 years ago, it still provides a solid framework for understanding the basis of flower development (Bowman et al., 2012). However, today the model is typically extended to include E-function (D function had previously been used for genes specifying ovule identity), which is conferred by the *SEPALLATA* (*SEP1-4*) genes that encode flower-specific co-factors required for organ specification in all whorls (Ditta et al., 2004; Pelaz et al., 2000). According to the so-called floral quartet model, the SEP proteins can form tetrameric complexes together with the ABC genes and, in turn, these 'floral quartets' recognize DNA and elicit transcriptional activation in an organ-specific fashion (Honma & Goto, 2001; Theissen & Saedler, 2001). Another limitation of the original model, which has been highlighted recently, is that an obvious A function seems to be specific to Arabidopsis and its close relatives (Litt & Kramer, 2010). Although already in 1990, Irish and Sussex suggested that the function of *AP1* and *AP2* was to specify the FM identity, and since then more studies have shown that *AP1* and *AP2* play important roles in FM specification (Ferrándiz et al., 2000; Okamuro et al., 1997; Irish & Sussex, 1990). Sepals have more recently been proposed to be the 'ground state' for flower organs (Causier et al., 2010). The fact that sepals are lacking in *ap1* and *ap2* mutants of Arabidopsis, can possibly be explained as an indirect consequence of a failure to properly specify the FMs. Given these facts a refined model has been laid out, the '(A)'BC model, where A and E function ['(A)'] are involved in FM specification and ensuring correct expression domains of the B and C genes, and not floral specification per se (reviewed in Wellmer et al., 2013). An interesting note is that all of the floral identity genes, except for *AP2*, encode proteins belonging to the MIKC-type of MADS-box transcription factors (for reviews see e.g. Dornelas et al., 2011; Gramzow et al., 2010; Kaufmann et al., 2005).

Many of the genes that are involved in reproductive development and flowering in angiosperms were identified through forward genetic screens. However, as previously mentioned, such attempts are difficult in gymnosperms; it time-wise would take almost one human generation to go

from the creation of the mutants until the final assessment of potential reproductive phenotypes. Hence, most of the past and present studies concerning reproductive development in gymnosperms have utilized the methodology of evolutionary developmental biology (evo-devo) (Arthur, 2002). As with angiosperms much of the present work with gymnosperm reproductive development has revolved around transcription factors of the MADS-domain family, in Norway spruce named *DEFICIENS AGAMOUS LIKE* (*DAL*) genes, and the meristem identity gene *LFY*. In gymnosperms the first *LFY* homologs were identified in gnetales (Frohlich & Meyerowitz, 1997) and in conifers two homologs of the *LFY* gene were initially identified in Monterey pine (*Pinus radiata*), *PRFLL* (Mellerowicz et al., 1998) and its paralog *NEEDLY* (*NLY*) (Mouradov et al., 1998). Although the Monterey pine genes at first indicated a sex specific expressional pattern, *LFY* and *NLY* gene homologs from other gymnosperm species have shown that the genes are expressed in both seed and pollen cones (Vazquez-Lobo et al., 2007; Carlsbecker et al., 2004; Shindo et al., 2001). Since gymnosperms lack sepals and petals, organ identity genes homologous to B- and C-class genes have mainly been described. Nevertheless, the recent emerging data sets from conifer genomes suggest that there can be conifer genes that, at least according to phylogeny, relate to angiosperm A function genes (Birol et al., 2013; Nystedt et al., 2013). However, as described earlier: given the evidence that A-class genes do not exert their function as proposed in the original ABC model, species-wide across the angiosperms; such homologous conifer genes connect more plausibly to meristem identity, rather than to organ identity. In Norway spruce a putative C-class ortholog, *DAL2*, has been described and was shown to be expressed mainly in reproductive cones (Sundstrom et al., 1999; Tandre et al., 1998). Putative Norway spruce orthologs to B-class genes comprise *DAL11*, *DAL12* and *DAL13*, which are expressed in pollen cones (Sundstrom & Engström, 2002; Sundstrom et al., 1999). This suggests that the specification of male and female organs involve orthologous genes in both angiosperms and gymnosperms. However, given some 300 million years of evolution, a certain degree of functional divergence between gene orthologs is likely to be expected. Furthermore, apart from B- and C-class genes there are gymnosperm specific genes whose expression is tightly linked to reproductive structures. The expression of the *DAL10* gene of Norway spruce precedes the expression of B- and C-class genes in reproductive organs and potentially the gene aids in the establishment of reproductive identity (Carlsbecker et al., 2003). Carlsbecker et al. (2004) show that expression patterns of the *DAL1* gene are not specific to reproductive organs, and, interestingly, the activity is initiated in shoots of (juvenile) trees and successively increases with age, suggesting a

potential role in an age-dependent pathway of reproductive development Furthermore, the expression also follows a spatial gradient along the stem, paralleling that of the ontogenetic features associated with reproductive competence. In addition, ectopic *DAL1* expression in transgenic Arabidopsis leads to precocious flowering, thus further implicating the role of *DAL1* in reproductive phase transition (Carlsbecker et al., 2004).

1.4.3 *Picea abies* var. *acrocona*

Naturally occurring mutants are attractive model systems to utilize in plant species where classic forward genetic approaches are difficult. One such naturally occurring mutant is the Norway spruce variety *acrocona* (*Picea abies* var. *acrocona*). Reports of this mutant stem from the mid 19th century, from localities in Uppland, Sweden (Fries, 1890). It was first recognized and named for its ability to set seed cones in apical positions on both top shoots and scaffold branches, where wild-type Norway spruce never initiate cones, hence the name *acrocona*, from the Latin for top cone. Apart from setting cones at atypical positions it also produces cones regularly, every growth season – even during years when surrounding natural stands do not produce cones. Both phenotypes, cones at atypical apical positions and frequent cone-setting, results in a short and 'bushy' appearance of an adult *acrocona* tree. Since the original records, *acrocona* plants have been grafted and distributed throughout the temperate world. Owing to limited information of origin it has yet to be determined if the different specimens in gardens worldwide stem from the *acrocona* specimen found in Uppland, or if mutations resulting in the *acrocona* phenotype have arisen in various genetic backgrounds. Previously a German initiative raised inbred crosses of two *acrocona* ramets and observed that around 3% of the population set cones already after 3 growth seasons (Flachowsky et al., 2009). Furthermore, it has been suggested that the early cone-setting phenotype of *acrocona* can be mapped to linkage group 6 in Norway spruce and that the trait follows monogenic inheritance, although the responsible mutation has yet to be found (Achere et al., 2004).

2 Aims of the study

Put in a general perspective, this thesis has aimed at aiding the forest industry, and especially forest tree breeding, in supplying basic knowledge that could be of direct use in tree improvement programs. Gaining control over developmental transitions holds the potential to significantly shortening breeding cycle intervals and would thus be of great economical importance. Furthermore, to fully understand selected traits, improved knowledge of the underlying genetic components and regulatory mechanisms is essential, not the least, considering the industry's ambitions towards producing novel biomaterials, building materials, and fuels.

More specifically, my work has been focused to identify and study regulatory genetic mechanisms important during conifer embryogenesis and reproductive development, with specific emphasis on the transitions between developmental phases.

Specific aims have been to:

- Pinpoint important processes during early embryo development assessed by global gene expression changes.
- Identify genes potentially involved in the embryo to vegetative transition.
- Characterize the transition from vegetative shoot to female cone and associated regulatory gene expression changes occurring in the *acrocona* mutant.
- Utilize the early cone-setting phenotype of *acrocona* to identify novel regulators implicated in reproductive competence

3 Results and Discussion

3.1 Embryo development (I, II and appendix)

To study early aspects of embryo development in Norway spruce we utilize the unique features of somatic embryo cultures. This allows for more accurate and abundant sampling of material from a well-characterized sequence of developmental stages that can be synchronized by different treatments (Figure 3).

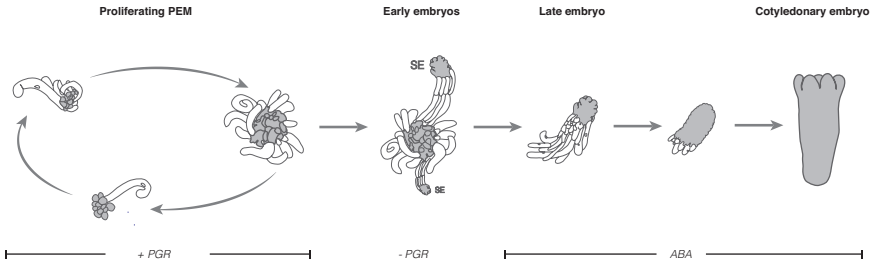

Figure 3. Embryogenic cultures of Norway spruce are usually established from mature zygotic embryos on medium supplemented with the PGRs auxin and cytokinin. The proliferating cultures constitute a combination of densely cytoplasmic cells adjacent to enlarged vacuolated cells referred to as proembryogenic masses (PEMs). Withdrawal of PGRs stimulates the differentiation of early embryos and further development and maturation of embryo requires exogenous supplementation of abscisic acid (ABA). Image after Filonova et al. (2000).

3.1.1 Global gene expression changes during early embryo development (I)

Compared to model angiosperms such as Arabidopsis, knowledge of the molecular regulation of conifer embryology is limited. Although cytological, morphological and temporal differences during embryo development are evident between angiosperm and gymnosperms, there are still striking sequence similarities between corresponding genes of the two groups (Cairney

& Pullman, 2007). It is not until very recently that the first complete conifer genome sequences were published, although the emerging genome and transcriptome data sets are far from fully annotated or complete (Birol et al., 2013; Nystedt et al., 2013). Gene expression changes during conifer development had earlier been studied using microarray techniques, in which complementary DNAs (cDNAs) were spotted onto microarray slides. Early studies of changes in gene expression during Norway spruce embryogenesis include microarrays spotted with 2178 cDNAs from five libraries of loblolly pine (xylem tissues, shoot tip and pollen cone). High hybridization efficiencies between spruce and pine species on microarrays have previously been shown (Stasolla et al., 2003; van Zyl et al., 2002). Different embryogenic cell lines of Norway spruce were assayed and comparative gene expression analysis revealed that global gene expression initially is repressed and subsequently induced during differentiation of early somatic embryos (Stasolla et al., 2004; van Zyl et al., 2003). Microarrays have also been assessed during embryo maturation in white spruce (Stasolla et al., 2003) and Norway spruce (Stasolla et al., 2004).

To gain further knowledge on early events during somatic embryo development in Norway spruce, microarray slides spotted with 12,536 cDNA clones from loblolly pine cDNA libraries originating from zygotic and somatic embryos at various developmental stages, and megagametophytes, were assayed. The focus of the study was to capture gene expression changes during the early aspects of embryo development in Norway spruce, i.e. the stages covering the differentiation of early embryos from PEMs and the beginning of late embryogeny. To study these important early events, we sampled embryogenic cultures at specific developmental stages and assayed global gene expression states. Embryogenic cultures assayed in this study were: (1) one week after transfer to PGR medium (PEM structures), (2) 24 h after withdrawal of PGRs (mostly PEM structures), (3) one week after withdrawal of PGRs (early embryos), and (4) one week after transfer to medium containing ABA (late embryos) (Figure 3).

After hybridization to the loblolly pine array, 720 transcripts with unique differential expression patterns between all developmental stages were identified. Among the different assayed samples 106, 208 and 464 transcripts showed differential expression 24 h after withdrawal of PGRs, during differentiation of early embryos and development of late embryos, respectively. To verify the accuracy of the microarray methodology we tested a set of differentially expressed genes on the same material used in the array by qRT-PCR. We also tested the biological significance of the array by assaying a

set of genes in tissue samples from a different cell line with qRT-PCR. From these verifications we could conclude that the experimental data were reliable.

3.1.2 Important processes (I)

Based on the results of the microarray analysis important processes during early embryo development in Norway spruce could be inferred by comparing the 720 differentially expressed transcripts to their closest putative gene homolog in Arabidopsis. This resulted in 86 transcripts differentially expressed 24 h after withdrawal of PGRs, 152 transcripts differentially expressed during the transition from proliferation to early embryos and 383 transcripts differentially expressed during development of late embryos. Selected important processes and their representation during early embryo development in Norway spruce are presented in Table 1, and include response to biotic and abiotic stress, programmed cell death (PCD), nurse cell function, auxin biosynthesis and response, and cell specification.

Table 1. *Processes occurring during early stages of embryo development based on the microarray analysis. Plus signs (+) indicate differentially expressed genes, and minus signs (-) indicate no differentially expressed genes*.

Process	PEM to early embryo differentiation	PEM to early embryos	Early embryos to late embryos
Programmed Cell Death (PCD)	+	-	-
Stress related processes	-	+	+
Nurse cell function	-	+	+
Auxin biosynthesis and response	-	+	+
Cell specification	-	+	+
Embryonic to vegetative transition	-	-	+

As expected from previous studies, an over-representation of transcripts involved in the response to stresses (stress-related factors include heat-shock proteins, drought-, salt- and cold-induced proteins and peroxidases) was observed among the differentially expressed transcripts (see e.g. Stasolla et al 2004). Stresses are potent triggers of cell dedifferentiation, which ultimately may lead to somatic embryogenesis (Kikuchi et al., 2013; 2006; Ikeda-Iwai et al., 2003). Oxidative stress is also associated with the initiation of PCD (Swidzinski et al., 2002). Consistent with previous studies indicating that PCD is important for suspensor differentiation (Filonova et al., 2000), PCD-related

genes such as *CATHEPSIN B-LIKE CYSTEINE PROTEASE* and *METACASPASE9* were up-regulated during differentiation of early embryos. Three homologs to the *MEE49*, *MEE66* and *ATHB22/MEE68* genes (*MEE = MATERNAL EFFECT EMBRYO ARREST*) were differentially expressed during early embryo differentiation. In Arabidopsis, several genes expressed from the female gametophyte, including MEE genes, have major effect on embryo development. *MEE49* is crucial for endosperm development and *MEE66* and *ATHB22/MEE68* are involved in early embryo development (Pagnussat et al., 2005). A Norway spruce chitinase gene, *Chia4-Pa*, is expressed in megagametophyte tissues of the seed and in certain cells at the base of the embryonal mass in embryogenic cultures (Wieweger et al., 2003). It is thus tempting to speculate about the presence of so-called 'nurse cells' in our embryogenic cultures and that genes such as *Chia4-Pa* and the *MEE* genes possibly confer some megagametophyte-signaling function. An up-regulation of the auxin-responsive gene *SMALL AUXIN UP RNA* and a down-regulation of *SUPERROOT1* indicate an increased auxin biosynthesis during early embryo development. Moreover, in the beginning of late embryo development genes encoding the auxin receptor TIR1 and the auxin-induced protein IAA11 were up-regulated, suggesting that auxin response processes start to become important concomitantly with the initiation of organogenesis of the embryos. A putative homolog to *LEUNIG* (*LUG*) was up-regulated during early embryo development. *LUG* and its close homolog *LUH* (*LEUNIG HOMOLOG*) are involved in cell identity and SAM establishment and maintenance both in flowers and in embryos (Stahle et al., 2009; Sitaraman et al., 2008). This expression pattern suggests that the conifer homolog to *LUG* may potentially be implicated in cell specification already during early embryo development.

We also found differentially expressed genes involved in the phase transition from embryonic to vegetative development. Putative homologs to the well-characterized master regulators *LEC1* and *ABI3* were found amongst our differentially expressed genes. A Norway spruce homolog to *ABI3*, *PaVP1*, has previously been identified in Norway spruce (Footitt, 2003). From this study we chose to further focus our studies on these differentially expressed master regulatory genes in Norway spruce (for more info on *LEC1* and *ABI3* see also 1.3.2)

3.1.3 Evolution and Expression of Embryonic Genes (II and appendix)

To search for more conifer homologs of *LEC1* and the *AFL* genes, public EST databases were screened. Only two genes highly similar to *LEC1* and *ABI3* were found in databases during the time point for our studies, and recent searches in the transcriptome database from the Norway spruce genome project

(http://congenie.org/) did not generate any additional genes. Furthermore, it has been suggested that the *LEC2* gene is specific to dicotyledonary angiosperms (Sreenivasulu et al 2012). Still we cannot rule out that there in fact exist homologs to *FUS3* and *LEC2* in conifers, considering that samples from specific embryonal stages often are underrepresented in transcriptome libraries.

Two conifer *LEC1* homologs, *PaHAP3A* and *PsHAP3A*, in Norway spruce and Scots pine respectively, were isolated. Both genes encode sequences similar to LEC1-type HAP3 subunits of the CCAAT-box binding factor (CBF, or NF-Y) (Lotan et al., 1998). The encoded amino acids also include the important asp 55, which has been shown to be important for proper binding to the CBF-complex (H. Lee, 2003). The conifer *LEC1*-type genes grouped together as a conifer-specific subclade among the other *LEC1*-type genes. The phylogenetic analysis shows that an ancestral *LEC1*-type gene was probably present in a common ancestor of all seed plants. A homologous gene to *ABI3*, *PaVP1*, has previously been described for Norway spruce (Footitt, 2003). We isolated its Scots pine homolog, *PsVP1*. B3 factors comprise a large gene family of plant specific transcription factors. The conifer homologs group closest to angiosperm *ABI/VP1* genes within the *AFL* clade (*ABI3*, *FUS3* and *LEC2*).

Expression patterns of the Scots pine *PsHAP3A* and *PsVP1* genes were assayed during both zygotic and somatic embryogenesis and expression patterns of the Norway spruce *PaHAP3A* and *PaVP1* genes were assayed during somatic embryogenesis. Our results show that the expression of *PaHAP3A* and *PsHAP3A* is high during early embryo development and that the expression gradually decreases during the maturation phase. Various studies in angiosperms have shown that *LEC1*-type genes are highly expressed during early seed development and continue to play important roles throughout embryo maturation, by regulating *AFL* genes but also directly targeting genes of the fatty acid biosynthesis pathway (Braybrook & Harada, 2008; Santos-Mendoza et al., 2008 and references within). The expression of *PaVP1* and *PsVP1* did not initiate until the maturation stage, and after the initial increase remained at a high expression level. This pattern is similar for angiosperm *ABI3/VP1* genes, which have been shown to initiate their expression upon the onset of maturation and to be tightly correlated with ABA-dependent regulation of seed protein gene expression (Santos-Mendoza et al., 2008; Gutierrez et al., 2007 and references within). The complementary expression profiles of the conifer LEC1-type and *ABI3/VP1* genes indicate direct or indirect regulation, or at least a common regulatory system. Complex interactions, including several feedback loops, of *LEC1* and the *AFL* genes

have been demonstrated by numerous studies in Arabidopsis (see e.g. Sreenivasulu & Wobus, 2013; Kagaya, 2005).

To further investigate the role of *PaHAP3A* during embryo maturation and germination, transgenic cultures harboring a ß-estradiol inducible *PaHAP3A* transgene were established. Embryogenic cultures overexpressing *PaHAP3A* during embryo maturation displayed a mild delay of maturation progression, although the total yield of mature cotyledonary embryos was similar to untreated controls. However, some of the embryos from the transgenic cultures developed ectopic embryos, a phenotype not detected in any non-transformed control culture. During embryo germination no visible delay in germination progression could be observed, nor did induced germinating embryos display any deviating phenotype. In Arabidopsis, studies have shown that exogenous supplementation of ABA strengthens embryonic phenotypes of ectopic *LEC1* action during germination (Junker et al., 2012; Kagaya, 2005). Exogenous supplementation of ABA during germination did not affect induced transgenic *PaHAP3A* embryos. No ectopic phenotype was observed on induced germinated embryos, nor did embryos display ectopic embryonal storage compound accumulation, such as lipids or starch.

The phylogenetic analysis of *LEC1*-type genes revealed that the conifer genes formed a distinct conifer-specific subclade separated from their angiosperm counterparts. Furthermore, the expression analyses indicated low expression levels of the conifer genes throughout embryo maturation, in contrast to the increase in angiosperm *LEC1* expression levels during later maturation stages. In addition, ectopic expression of *PaHAP3A* did not display embryonal and postembryonic phenotypes similar to those observed for *LEC1* overexpression in angiosperms. Taken together, this suggests a divergent evolutionary history of the conifer and angiosperm *LEC1*-type genes, indicative of either neo- or subfunctionalization. In contrast, the conifer *ABI3/VP1* homologs display similarities closer to their angiosperm homologs, both considering gene expression patterns and phylogeny.

3.1.4 Embryogenic potential is retained by histone deacetylase inhibition (II and appendix)

In many plants, including the conifers, embryogenic cultures are routinely established from immature or mature zygotic embryos. When the plants start their vegetative growth period, upon the onset of germination, the capacity to initiate embryogenic cultures is rapidly decreased – i.e. they loose their embryogenic potential (Bonga et al., 2009). However, rare exceptions are found in the literature, e.g. Klimamaszewska and colleagues found that individual genotypes of 10 year old trees derived from somatic embryos still

harbored the capacity to initiate embryogenic tissues (Klimaszewska et al., 2010). There is a great interest to be able to propagate trees with valuable traits via somatic embryogenesis. To be able to do this, more knowledge on the molecular regulation of totipotency and embryogenic potential in plants is required.

The gradual loss of embryogenic potential together with the rapid genetic switchover during germination can, at least partly, be attributable to epigenetic changes. Histone acetylation, together with certain specific histone methylation marks, are essential for proper transcription of embryonic genes (Bemer & Grossniklaus, 2012; Holec & F. Berger, 2012). The action of histone deacetylases (HDACs), and demethylases, are thus crucial for proper repression of the embryonic gene program, prior to the transition to the vegetative phase of growth (Bouyer et al., 2011; Tai et al., 2005).

Genome-wide inhibition of histone deacetylases (HDACs) using the chemical inhibitor trichostatin A (TSA) was investigated during maturation and germination of somatic embryos of Norway spruce. During embryo maturation, cultures treated with TSA continued to proliferate, and maturation progression was arrested. In addition, when embryos were treated with TSA during the first 10 days of germination, vegetative development was partially inhibited by the HDAC inhibitor simultaneously as the competence to differentiate embryogenic tissue was maintained at a level corresponding to that of mature embryos (80%). However, once embryogenic competence in germinating embryos already had decreased, HDAC inhibition was not enough to regain totipotency, but only enough to retain the level of competence to that of the level before treatment. The expression of embryonic genes was altered by HDAC inhibition. HDAC inhibition during maturation led to a maintained expression of *PaHAP3A* and a repression of *PaVP1*, contrary to their expression in untreated cultures. During germination, altered embryonic gene expression was not detected; in fact, we could not detect any expression of the genes either in treated or untreated embryos.

Reports in angiosperms have earlier suggested an important role of cytosine methylation patterns and embryogenic competence (Elhiti et al., 2010 and refrences within). The effect of DNA hypomethylation on embryogenic potential and embryo maturation was also studied in somatic embryos of Norway spruce using the methyltransferase inhibitor zebularine (1-(ß-D-ribofuranosyl)-1,2-dihydropyrimidine-one). Zebularine is a more stable cytidine analogue, but with an analogous mode of action, than the commonly used 5-azacytidine (Baubec et al., 2009). Supplementation of zebularine during embryo maturation led to a rapid degeneration and death of treated cultures (unpublished data). Thus, proper DNA methylation patterns seem to be crucial

during embryo maturation. However, to further study the effects of repressed methylase activities on maturation, more thorough dose-dependent methylation inhibitor assays are needed. Treatment of embryos during germination was not lethal, but only partially inhibited germination progression, similar to the effects of TSA to germinating embryos. However, unlike TSA-treated embryos, inhibition of DNA methylation did not increase embryogenic potential (unpublished data).

Taken together, it is tempting to speculate that only some cells remain totipotent during germination in the presence of TSA, and thus retain their capacity to differentiate embryogenic tissue, and if it would be possible to isolate these cells it would then be possible to see an altered embryonic gene expression. Reporter gene studies are now being assayed to see if embryos germinated on TSA in fact retain an ectopic expression of *PaHAP3A*.

3.2 Reproductive Development (III and IV)

The presence of a gradient of developmental stages over the tree makes conifers an attractive model for studying developmental transitions in plants. However, conifers do not set cones every year, thus complicating studies of the reproductive identity initiation and reproductive organ development. The interval between cone-producing years varies, but is usually 3-5 years once the tree reaches general reproductive maturity (Tirén, 1935). To gain knowledge of the events that determine vegetative or reproductive fates in a shoot, we took advantage of the early cone setting properties of the naturally occurring *acrocona* mutant.

3.2.1 Characterization of the *acrocona* mutant (III and IV)

The mutant phenotype of an *acrocona* tree was first recognized as a naturally occurring variety that produced cones regularly and at positions not normally observed for Norway spruce (Fries, 1890). An *acrocona* cone is typically characterized by a conversion from vegetative to reproductive identity (compare Figure 4A to 4B-F). The conversion follows a gradient from the base of a vegetative leading shoot to the top where sometimes a full transition to reproductive structures has taken place (Figure 4B-D). The transition is represented by an alteration in needle morphology, as the needles are broader and more resembles bracts the further along the base-top gradient they reside (Figure 4C-D). The conversion to reproductive character also brings an altered phyllotactic pattern and a decrease in ergastic substances, normally present in vegetative shoots. Along the base-top gradient ovuliferous scale-like structures emerge in the axil of bract-like needles (Figure 4E). The conversion is more

pronounced at apical positions of the tree, and at more basal regions, where the phenotype is less penetrant, the meristem does not terminate and a vegetative shoot can emerge the next growth season. A wild type ovuliferous scale consists of a single structure with two ovules forming with their integuments facing the cone axis. Ovuliferous-scale-like structures of the *acrocona* mutant display bifurcated or fused scales that at their most basal position on the shoot can consist of three scales that all can carry an ovule-like structure with integuments containing ovules that are inverted (Figure 4E- F). Based on our observations it is tempting to draw parallels to inflorescence mutants found in angiosperms (Ungerer et al., 2002).

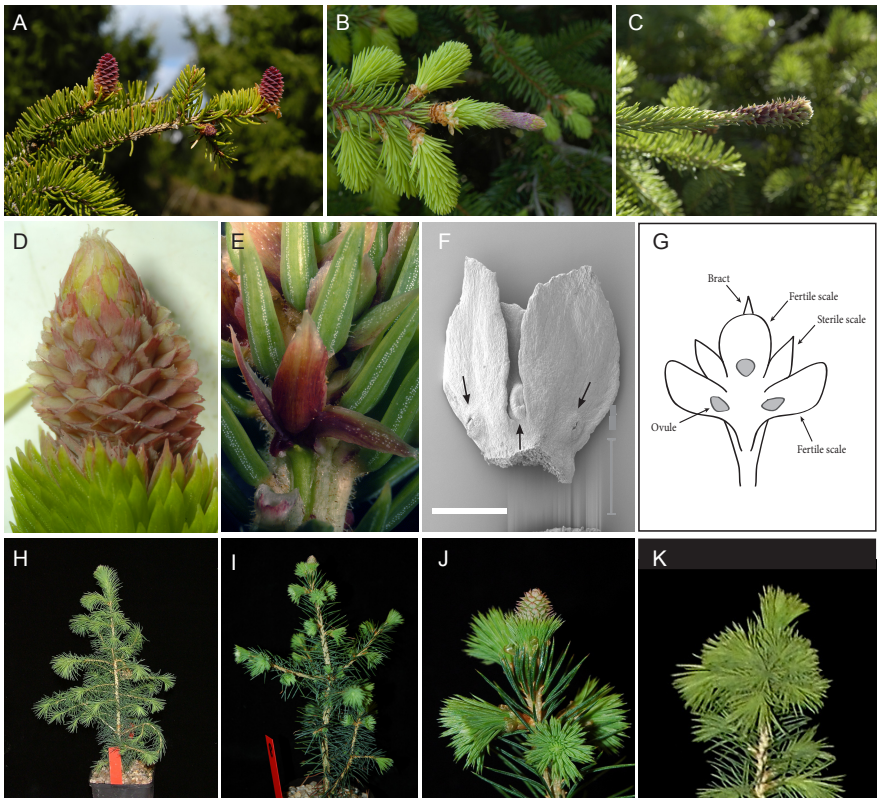

Figure 4. Homeotic conversion of vegetative shoots to shoots with female identity in *acrocona*. (A) Wild-type Norway spruce female cone at pollination. (B-D) transition shoot structure of *acrocona:* after bud burst in spring (B), after shoot elongation in late spring (C), and a close-up of a transition cone after bud burst (D). Needles are formed at basal position of the shoot and a gradual transition towards broader, 'bract-like' structures with red apices appears along a gradient towards the apex of the shoot. (E) Part of an *acrocona* shoot displaying a three-lobed ovuliferous-scale-like structure axillary to needles/bracts (which have been removed for clarity). (F) Scanning electron micrograph image of an ovuliferous scale-like structure from *acrocona* with three scales

united at their base and each carrying ovule-like structures (arrows). Bar = 500 μM. (G) Adaxial view of a reconstructed flower structure of the Permian conifer *Pseudovoltzia liebeana*, after Clement-Westerhof (1988). (H-K) Inbred *acrocona* plants grown under accelerated growth conditions displaying wild-type (H) and transition cone (I) phenotype after three growth cycles. (J) Close-up of the transition cone in (I). (K) Inbred plant with an intermediate *acrocona* phenotype with numerous lateral shoots after five accelerated growth cycles. Photos: 4A-4F Annelie Carlsbecker.

We generated a population of inbred siblings from two *acrocona* trees and raised the offspring under accelerated growth conditions in a phytotron unit (one calendar year corresponded to three growth seasons for the plants). Out of 75 inbred *acrocona* plants 19 set cones already during the third growth cycle (approximately 1/4). Inbred siblings from a second crossing showed a similar segregation pattern (three out of sixteen produced cones after three growth cycles). Together, our segregation patterns further strengthen the hypothesis suggested by Achere et al (2004), that the *acrocona* phenotype is a single monogenic trait; we assume that the parents are heterozygous for the mutation and that the early cone-setting phenotype was manifested only in homozygous plants. After additional growth cycles more individuals displayed cone-related phenotypes; however, control plants did not produce cones and grew normally during the duration of the experiment (five growth cycles). Inbred *acrocona* plants raised under accelerated growth conditions displayed a remarkable phenotype in that they produced a cone on the top apical shoot (Figure 4I-J). The number of cones set by each cone-setting inbred plant varied from one to eleven and every cone-setting plant terminated a cone on the apical shoot. The cones resembled the intermediate cone phenotype found on older *acrocona* trees (Figure 4D and J). Similarly to older trees, needles were formed in the base of a cone-like shoot and the more apically along the axis they appeared the more broad and bract-like they became. Phenotypes emerging the fourth growth cycle included a spirally arranged congregation of lateral shoots within the boundaries of a single shoot, in the position normally occupied by ovuliferous scales, suggesting that this multiple shoot aggregation could be the effect of an indeterminate meristem (Figure 4K).

The intermediate transition cone phenotype of *acrocona* display similarities to the reproductive shoot of extinct Voltziales species (compare Figure 4F to 4G). Thus, at least morphologically, our observations of the *acrocona* phenotype seem to support Florin's theory that the origin of the modern seemingly simple structure of an ovuliferous scale has an origin in more elaborate short shoots of extinct conifer genera (possibly via a planation process) (Florin 1951; Clement-Westerhoff 1988). The cause of this proposed reduction, whether the process was sudden or if it required many intermediate stages, is presently not known. However, modern molecular genetics have

shown that complex morphological and physiological traits do not have to be explained by intricate series of condensations and intermediates, but can simply be explained by simple shifts in regulatory gene expression (reviewed in Mathews & Kramer, 2012).

3.2.2 Gene expression during reproductive development (IV)

Studies on plant reproductive identity and development in angiosperms have generated an intricate gene regulatory network that is dominated by proteins encoding MADS-domain transcription factors (Dornelas et al., 2011). Norway spruce homologs to MADS-box genes and the FM identity gene *LFY* have previously been identified (See also 1.4.2). In Table 2 previously characterized genes together with five novel genes (*DAL4*, *DAL9*, *DAL14*, *DAL19* and *DAL21*) paralogous to characterized MADS-box genes are presented.

Table 2. *Expression pattern of previously described MADS-box and LEAFY genes (black text) as well as novel MADS-box genes (blue text) in seed and pollen cones of Norway spruce. Plus signs (+) indicate that the gene is active in tested tissues, minus signs (-) indicate no gene activity, and (n.a.) signifies that the genes have not been tested.*

Gene name	Expression in seed cones	Expression in pollen cones	References
LFY	+	+	Carlsbecker et al 2004, Vazquez-Lobo et al 2007
NLY	+	+	Carlsbecker et al 2004, Vazquez-Lobo et al 2007
DAL1	+	+	Tandre et al 1995, Carlsbecker et al 2004
DAL2	+	+	Tandre et al 1995, Tandre et al 1998
DAL3	+	+	Tandre et al 1995
DAL4	n.a.	n.a.	Carlsbecker et al 2013
DAL9	n.a.	n.a.	Carlsbecker et al 2013
DAL10	+	+	Carlsbecker et al 2003
DAL11	-	+	Sundström et al 1999, Sundström & Engström 2002
DAL12	-	+	Sundström et al 1999, Sundström & Engström 2002
DAL13	-	+	Sundström et al 1999, Sundström & Engström 2002
DAL14	+	+	Carlsbecker et al 2013
DAL19	+	+	Carlsbecker et al 2013, Uddenberg et al 2013
DAL21	+	-	Carlsbecker et al 2013

A comprehensive set of gymnosperm and angiosperm genes was used to generate a consensus phylogeny of previously characterized and novel MADS-box genes. Phylogenetic inferences suggest relationships of: *DAL14* to *DAL1*;

DAL4, *DAL9* and *DAL19* to *DAL3*; and *DAL21* to *DAL10*. Furthermore, the analysis revealed that most conifer genes form clades with characterized angiosperm genes: *DAL1* and *DAL14* group together with the angiosperm *AGL6/SEP* clade; *DAL2* with the *AG* clade; and *DAL3*, *DAL4 DAL9* and *DAL19* with the *TM3/AGL14* clade. *DAL10* and *DAL21* group with a previously proposed gymnosperm-specific clade described as the *GGM7 GNETUM GNEMON MADS7*) clade (Carlsbecker et al., 2003).

To study *MADS* and *LFY* genes during Norway spruce reproductive development, end-point RT-PCR and extensive in situ hybridization assays were performed at distinct developmental stages. Furthermore, the homeotic conversion of intermediate *acrocona* cones was utilized to discern regulatory gene expression differences in the mutant. Among the novel genes, *DAL4* and *DAL9* were not assayed. *DAL14* was expressed in both female and male reproductive organs, *DAL19* was expressed in most tissues and *DAL21* was specific to female reproductive organs. Most novel genes were often found to be expressed in patterns that deviated from those of their previously described paralogs (Carlsbecker et al., 2004; 2003; Tandre et al., 1998) but with some overlapping, implying functional divergence. One interesting notion is the expression pattern of *DAL14*, which during early development was expressed in ovuliferous scales and displayed pronounced activity in the medial apical domain and the lateral parts in which the ovules later develop.

The transition phenotype of *acrocona* cones can be interpreted as an indeterminacy of the meristem to terminate, whereby axillary meristem function remains to produce the shoot-like phenotype seen in the base of the cone. Interestingly, *DAL14* was the only analyzed gene that was differentially expressed between normal cones and *acrocona* cones. The expression was absent in transition cones, or expressed only in the most apical position of the cone, in ovuliferous scales with a functional appearance. It is tempting to speculate that this differential expression pattern can be related to the aberrant morphology of the *acrocona* cone. The pattern suggests that the normal function of *DAL14* is to either suppress meristem activity or to restrict multiple scale formation during ovuliferous scale development. The *DAL14* group together with the AGL6/SEP clade, a group in which angiosperm genes are involved in both floral meristem and organ identity determination (reviewed in Melzer et al., 2010). Furthermore a *Gnetum gnemon* homolog to AGL6-proteins has been shown to form multimeric complexes (floral quartets) with B- and C-class proteins, similar to SEP-proteins in angiosperms (Y.-Q. Wang et al., 2010). This, in turn might suggest that a putative 'floral quartet' complex formation specifying meristem identity in modern cones is lacking in *acrocona* cones, as a result from the absence of DAL14 activity. Put in an evolutionary

perspective, the reduction from a more ancient shoot-like ovuliferous scale to the modern simple structure might have occurred simply by changes in expression patterns of an ancestral *DAL14* gene, from a central position to lateral/apical areas, which might have arrested the more shoot-like scales. These are tempting speculations, although functional evidence is needed to verify evolutionary theories and to draw conclusions about the role of MADS-box genes in conifer reproductive development.

3.2.3 Differential Expression During Initiation of the Cone (IV)

To predict if a shoot will take on vegetative or reproductive identity is difficult, since by the time distinguishable features emerge, the initiation signal is probably already gone. Inbred *acrocona* plants, raised under accelerated growth conditions, results in cone-setting plants that initiates a cone at the position of the top apical shoot during the second growth cycle, and develop cones during the third growth cycle. In this unique plant material we can therefore sample material that will enable us to capture the tissue in which the earliest signals for reproductive competence can be captured.

To find a potential cone-inducing factor, samples were collected during the second growth cycle, when the shoots started to elongate after bud flush. Samples included 5-6 needles from the top apical shoot and needles from a more basal shoot, in which a vegetative identity was expected. The plants were then left to progress into the third cycle and, as predicted, cone phenotypes emerged and could be recorded. This provided a sample population where possible cone-inducing factors could be reliably separated from non-inducing factors. mRNA from samples collected during the second growth cycle (including the putative cone-inducing factor) and third growth cycle were extracted and a massively parallel sequencing approach was chosen to identity differential expression profiles.

The sequencing generated 136 Gb of RNA sequence, including between 58 and 270 Mb per sample with an estimated coverage of 100X for exonic regions. At the time point for the study no gymnosperm genome sequence was available. Hence, both a *de novo* and an *ab initio* (using a wide-ranging set of 27,720 white spruce transcripts as reference, Rigault et al., 2011) assembly approach were conducted. The *ab initio* method detected an 83% overlap to the white spruce sequences and the *de novo* approach generated a total of 83,650 ORFs. Putative orthologous groups of the translated transcripts were detected together with the white spruce sequences protein sets from Arabidopsis. The *acrocona* ORFs were present in 19,865 orthologous groups and 71% of these also contained white spruce and Arabidopsis proteins, indicating that at least a corresponding set of 19,439 (35%) reconstructed *acrocona* ORFs are valid. To

further detect differentially expressed genes between our samples Bowtie2, Cufflinks, Cuffmerge and Cuffdiff (Trapnell et al., 2012) were used to totally yield expression estimates for 33,383 transcripts. Subsequently, transcript abundance for samples from apical cone-setting shoots with basal non-cone-setting shoots of the same *acrocona* plants were discriminated, which generated 132 significantly differentially expressed transcripts. A comparison of apical shoots from cone-setting and non-cone-setting *acrocona* individuals identified 219 differentially expressed genes. When combined, these two data sets identified a total set of 8 differentially expressed transcripts. Seven of the differentially expressed genes were down-regulated in cone-setting plants and functional annotation against known plant species implied that these transcripts have potential roles in cell wall composition during meristem and organ development, cell signaling and plant stress response. Interestingly, the ORF of the single transcript that was identified as being significantly up-regulated in shoots that the next growth cycle set a cone, was identical to the MADS-box gene *DAL19*. Phylogenetic reconstruction, similarly to the comprehensive MADS-box phylogeny, grouped the *DAL19* sequence to the characterized angiosperm *TM3/AGL14* clade. This clade includes the well-described Arabidopsis floral integrator *SOC1*. The *DAL19* gene specifically groups together with other gymnosperm and Norway spruce genes such as *DAL3*, *DAL4* and *DAL9*. Thus clear orthology to a single angiosperm gene could not be found.

We specifically searched for differential expression of *SPL* genes in our mRNA data set, but did not find any significant up- or down-regulations. We do not rule out the possibility that *SPL* genes and miRNAs are important for reproductive competence and cone-setting in conifers. Samples collected over a broader spatio-temporal gradient would probably be key in resolving possible conservation and/or divergence patterns of miRNAs and *SPL*-genes between angiosperms and gymnosperms.

In addition to the mRNA sequencing, directed qRT-PCR analyses using *DAL19* and eight additional selected potential candidate genes (*DAL1*, *DAL2*, *DAL3*, *DAL9* and *DAL10*, *LFY*, *NLY* and the Norway spruce *FT/TFL1* homolog *PaFTL2* (*PaFTL2*: Karlgren et al., 2013; 2011) were assayed to provide independent *acrocona* gene expression data for the cone initiation event. The analysis showed that only *DAL19* up-regulation was significantly associated with the early cone-setting. This further reinforced the assumption that *DAL19* might be a potentially important factor for the early cone-setting phenotype in *acrocona*. Another interesting observation from the targeted gene expression experiments was that *DAL1*, previously potentially linked to the vegetative to reproductive transition in Norway spruce (Carlsbecker et al., 2004), showed a

dramatic expressional increase in an individual that set a total of eight cones during the growth cycle after sample collection. Yet another trend visible from the experiments, was that the *DAL10* gene showed an up-regulation in samples from cone-producing plants, collected during the growth cycle where cones were already present. This is also in correspondence to previous observations made by Carlsbecker and colleagues, where they hypothesized a role of *DAL10* in specifying reproductive shoot identity (Carlsbecker et al., 2003).

Taken together, expression profiles and localization studies, as well as phylogenetic relationship, suggest a central role for *DAL19* in the vegetative to reproductive transition in conifers. An attractive hypothesis is that the ancestral function of *DAL19/SOC1* genes might have involved the regulation of phase transitions in common ancestral plants. However, functional studies are needed to further assess the role of *DAL19* in reproductive development.

4 Conclusion

Important processes during early somatic embryo development include response to biotic and abiotic stress, programmed cell death, auxin biosynthesis and response, cell specification, potential nurse cell signaling and preparation for the embryonic to vegetative phase transition.

PaHAP3A encodes a putative homolog to angiosperm LEC1-type HAP3 factors. Data presented suggest functional divergence between the gymnosperm and angiosperm lineages.

Proper histone deacetylase (HDAC) activity is crucial for embryo maturation progression and normal embryonic gene expression. The rapid loss of embryogenic potential typically observed following germination can be blocked by inhibition of HDAC activity.

The *acrocona* mutant is characterized by early cone-setting and the occasional gradual transformation of vegetative shoots to female cones. The absence in expression of an AGAMOUS-LIKE6/SEPALLATA (AGL6/SEP) homolog may be functionally associated with the non-determinate development of the ovule-bearing scale of *acrocona*.

Inbred *acrocona* plants grown under accelerated growth conditions produced cones within one calendar year. The initiation of the early cones is associated with increased transcriptional activity of the MADS-box gene *DAL19*.

5 Future perspectives

The possibility to initiate embryogenic cultures from older trees would greatly aid forest tree breeding. To accomplish this, further basic research is needed in order to comprehend dedifferentiation processes and plant totipotency.

The *acrocona* mutant holds the potential to be incorporated into existing breeding programs as e.g. a rootstock with early cone production properties. Furthermore, the establishment of embryogenic cell cultures of *acrocona* presents a possible rapid cycle model system, in which transgenic studies will be possible.

Future research topics include:

- Investigate the epigenetic regulation of totipotency and dedifferentiation in conifers. The recent availability of genome sequences enables e.g. characterization of global patterns of histone methylation (H3K27me3 and Polycomb) changes during the embryonic to vegetative phase transition.
- Functionally characterize potential master regulators of conifer embryogenesis. Transgenic lines utilizing reporter genes and constitutive overexpression are currently being assessed, to further elucidate the role of *PaHAP3A* during embryogenesis and its involvement in the embryogenic potential.
- Functional testing of previously identified conifer MADS-box genes homologous to angiosperm ABC genes, using transgenic embryogenic cell-lines of the *acrocona* mutant.
- Investigate the role of *DAL19* on reproductive development in conifers, using transgenic studies.

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