Molecular Regulation of Vascular Cambium Identity and Activity

Melis Kucukoglu
Faculty of Forest Sciences
Department of Forest Genetics and Plant Physiology
Umeå

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Cover: Over-expression of the *PtCLE41A* gene in *Populus* results in dwarf plants. A wild type plant (left), transgenic *35S::PtCLE41A* plants (right).
(Photo: Melis Kucukoglu)

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Abstract

In plants, secondary development and wood formation originates from the cell divisions within the vascular meristem, where the vascular stem cells are located. This thesis work presents my results on the molecular regulation of vascular cambium stem cell identity and activity.

I have investigated the role of the receptor-like kinase PXC1 during vascular development in *Arabidopsis thaliana*. Mutant analysis revealed that in the absence of *PXC1*, plants display a pendant phenotype and reduced secondary cell wall thickening and lignification in the inflorescence stems, showing that PXC1 is an important regulator of secondary cell wall formation in *Arabidopsis*.

I also participated in the characterization of members of the TDIF/CLE41/CLE44-TDR/PXY-WOX4 signaling module in hybrid aspen. Functional studies showed that knock down of *PtWOX4* paralogs inhibits vascular cambium activity and secondary xylem formation in transgenic trees. Moreover, over-expression of *PtCLE41A* and related genes induces vascular patterning defects, highly associated with ectopic cambial activity. Results from transcriptional analysis suggested that *PtCLE41A* and related genes positively regulate *PtWOX4* during the regulation of vascular cambium activity. By analysing gene expression patterns in Norway spruce, I provided evidence for the existence of conserved mechanisms in angiosperm and gymnosperm tree species with regard to the regulation of cambium function through *CLE41*, *TDR/PXY* and *WOX4*-like genes.

Finally, I also identified different *PtLCLE* genes as candidate regulators of vascular cambium activity and tree growth. I studied functions of these genes by employing transgenic approaches in hybrid aspen and showed that down regulation or up regulation of these genes affect many different phenotypic properties in hybrid aspen such as cambium activity, internode elongation, leaf size, and adventitious rooting.

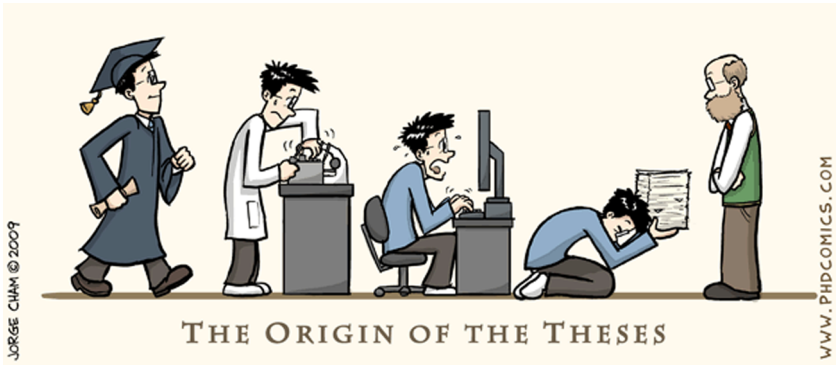
In conclusion, results of these projects provide new insights into the regulation of vascular cambium activity and wood formation.

Keywords: vascular cambium, stem cells, secondary growth, wood formation, *Populus*, *PtWOX4,* and *PtLCLE*

Author's address: Melis Kucukoglu, SLU, Department of Forest Genetics and Plant Physiology, SE901-83, Umeå, Sweden
E-mail: Melis.Kucukoglu@slu.se

Dedication

To the loving memory of my late grandparents, Seviye and Fikret Durmaz
You are missed every day…



"Piled Higher and Deeper" by Jorge Cham, www.phdcomics.com
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Contents

List of Publications

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

I Wang J., **Kucukoglu M.**, Zhang L., Chen P., Decker D., Nilsson O., Jones B., Sandberg G. & Zheng B. (2013). The Arabidopsis LRR-RLK, *PXC1*, is a regulator of secondary wall formation correlated with the TDIF-PXY/TDR-WOX4 signaling pathway. *BMC Plant Biology* 13: 94

II **Kucukoglu M.** *, Nilsson J. *, Zheng B., Sandberg G. & Nilsson O. (2015). *WOX4*-like genes control stem secondary growth in trees. (*Manuscript*).

* These authors contributed equally to this work.

III Nystedt B., Street N.R., Wetterbom A., Zuccolo A., Lin Y.C., Scofield D.G., Vezzi F., Delhomme N., Giacomello S., Alexeyenko A., Vicedomini R., Sahlin K., Sherwood E., Elfstrand M., Gramzow L., Holmberg K., Hällman J., Keech O., Klasson L., Koriabine M., **Kucukoglu M.**, Käller M., Luthman J., Lysholm F., Niittylä T., Olson A., Rilakovic N., Ritland C., Rosselló J.A., Sena J., Svensson T., Talavera-López C., Theißen G., Tuominen H., Vanneste K., Wu Z.Q., Zhang B., Zerbe P., Arvestad L., Bhalerao R., Bohlmann J., Bousquet J., Garcia Gil R., Hvidsten T.R., de Jong P., MacKay J., Morgante M., Ritland K., Sundberg B., Thompson S.L., Van de Peer Y., Andersson B., Nilsson O., Ingvarsson P.K., Lundeberg J. & Jansson S. (2013). The Norway spruce genome sequence and conifer genome evolution. *Nature* 497: 579-584

IV **Kucukoglu M.**, Chaabouni S., Zheng B., & Nilsson O. (2015). Functional characterization of *CLE* genes expressed in the wood-forming zone of *Populus* trees. (*Manuscript*).

V **Kucukoglu M.** & Nilsson O. (2015). CLE peptide signaling in plants – the power of moving around. *Physiologia Plantarum* 155: 74-87.

The contributions of Melis Kucukoglu (M. Kucukoglu) to the papers included in this thesis was as follows:

I M. Kucukoglu generated the transgenic *Arabidopsis* plants expressing the *promoter::GUS* constructs for the candidate *PXC* genes, performed the GUS expression analysis, executed the QPCR expression analysis for main regulators of vascular development, together with D. Decker and L. Zhang characterized the *pxc1* mutants and commented on the final manuscript.

II M. Kucukoglu performed the phylogenetic analyses for the *CLE, LRR-RLK* and *WOX* gene families, executed the QPCR expression analysis for the tissue-specific expression of candidate genes in *Populus*, performed the GUS expression analysis for the *PtPXY* genes, selected and characterized the different *35S::PtCLE41* mutants, performed the QPCR expression analysis for the candidate genes in spruce, performed the auxin study, and together with J. Nilsson, and O. Nilsson co-wrote the paper.

III M. Kucukoglu performed bioinformatics analyses to control the quality of the assembly, analysed the phylogeny of the *phosphatidylethanolamine-binding protein* gene family, and contributed to the writing of the paper.

IV M. Kucukoglu performed the phylogenetic analysis and *in silico* gene expression analysis for the *CLE g*ene family, performed the QPCR expression analysis for the tissue-specific expression of the candidate genes, generated the transgenic *Populus* plants used in the study, together with S. Chaabouni characterized the mutants, and together with O. Nilsson co-wrote the paper.

V M. Kucukoglu together with O. Nilsson co-wrote the paper.

Abbreviations

All abbreviations are explained when they first appear in the text.

1 Introduction

1.1 Why study wood formation?

The influence of wood on human life is astonishing, such that it is one of the most essential natural resources serving mankind. It can be exploited as a raw material for pulp, paper and construction industries or even as a source of energy (Plomion *et al.*, 2001). Moreover, the polymers within the secondary cell walls of the woody biomass, such as cellulose, hemicelluloses and lignin carry a great potential to be utilised as a feedstock for biofuel production as an alternative to fossil fuels or to produce other important chemicals in biorefinery applications (Plomion *et al.*, 2001; Gomez *et al.*, 2008; Karp & Shield, 2008; Nieminen *et al.*, 2012).

Wood conducts water from the root to the shoot and mechanically supports the growing body with its rigid structure within plants, both of which have been instrumental for plant evolution and subsequent colonisation on land. It is generated through the activity of the vascular cambium, a secondary meristem that contains the vascular stem cells (Plomion *et al.*, 2001). The aim of my graduate studies was to understand and deduce how the vascular cambium stem cell identity and activity is controlled on the molecular level. A deep understanding of the vascular cambium regulation is not only significant on a fundamental level but also on a commercial level because transferring this knowledge to forestry practices can assist in engineering and breeding trees with increased biomass production in the future. Throughout my research, I used *Populus* as my main model organism and to some extent *Arabidopsis* and Norway spruce. In this thesis work, I will first introduce how vascular cambium is formed within plants, and which factors regulate the vascular cambium activity. Afterwards I will summarize and discuss my results.

1.2 Plant stem cells

Plants constantly grow and repetitively initiate new structures during the development thanks to the activity of meristems, which harbor and protect the stem cell populations located in the different parts of the plant body (Evert, 2006a). Plant stem cells, or the "meristematic cells" or the "initials", similar to their animal counterparts are reservoirs of cells that can both produce new cells for the generation of new tissues and organs, and self-maintain indeterminately via asymmetric cell division. The asymmetric division of an initial cell generates two daughter cells; one remains as an undifferentiated initial, whereas the other one, or the "derivative of an initial", is targeted for the differentiation pathways. These derivatives can directly differentiate or may divide several rounds before the differentiation (Evert, 2006a).

Even though there is a constant flow of cells from the meristems into the new organs, the number of the stem cells and their dividing derivatives do not change in the meristem (Heidstra & Sabatini, 2014). Therefore, the self-perpetuation of the stem cells and the programmed differentiation of their progeny need to be under a strict control. Then an important question would be "how a stem cell senses to stay as a stem cell and how it knows when to proliferate or differentiate". The answer lies in the concept of the "stem cell niche". First postulated by Ray Schofield in 1978 for the haematopoietic stem cells in mammals, stem cell niche can simply be defined as the local microenvironment that the stem cells are positioned in (Schofield, 1978; Heidstra & Sabatini, 2014). In the niche, stem cells associate with the "niche cells", which act as an organizer to sustain and regulate the activity and identity of the stem cells via local signaling (Gallagher, 2013; Heidstra & Sabatini, 2014). Consequently, the cells residing within the niche stays as a stem cell, whereas the cells pushed outside the niche differentiate. Similarly, if a cell that has not undertaken a terminal differentiation fate is displaced into the stem cell niche, it might regain the identity of a stem cell (Gallagher, 2013).

Plant stem cells are "pluripotent" in their nature, so they can give rise to most but not all types of cells in the plant body (Verdeil *et al.*, 2007). However it is worthy to mention that almost all plant cells can be considered as "developmentally totipotent" due to the fact that they have the "competence" to generate all types of cells in the plant body and even a complete, new plant under certain stimuli. This ability gives a growing plant a certain level of plasticity to adopt to the environmental conditions as sessile organisms (Evert, 2006a).

1.3 Structure and function of the vascular cambium

1.3.1 Primary growth

Plant development commences with the embryogenesis (ten Hove *et al.*, 2015). Following the fertilization, consecutive oriented cell divisions of the zygote generate the primary meristematic regions (protoderm, ground meristem and procambium) as well as the apical meristems, eventually forming a mature sporophyte. With the germination of the sporophyte, primary meristematic regions start to differentiate into functional tissues (epidermis, ground tissue system and vascular tissues), and further development in plants is propagated by the activity of the apical meristems (ten Hove *et al.*, 2015).

Apical meristems, more specifically shoot apical meristem (SAM) and root apical meristem (RAM), contain the stem cell niches to give rise to the shoot and the root system of the plant body (Miyashima *et al.*, 2013). As the name implies, they are found at the tips of the main and lateral shoots and roots. Following the cell divisions in these meristems, newly formed cells elongate, and mature; causing an increase in plant height and root length, as well as a slight expansion in the width of the plant (Evert, 2006c; Beck, 2010). This growth, arising from the activity of the apical meristems is referred to as the "primary growth". For instance, monocotyledons and most of the seedless vascular plants demonstrate only this type of growth during their life cycle (Evert, 2006c; Beck, 2010).

Procambium contains the stem cells to produce the conductive tissues of the primary plant body and the basic developmental pattern of the procambium established in the embryo reflects the future arrangement of the primary vascular system (Beck, 2010). Early plant biologists referred to the primary vascular system in the stems and roots as well as tissues associate with them (such as pith) collectively as "stele", which is a term still in use today (Beck, 2010). In the stems of dicotyledonous plants and gymnosperms, stele arrangement is in the form of discrete vascular bundles or "fascicles" (eustele arrangement) surrounding central pith, and the procambium within the bundle is designated as the "fascicular cambium" (*Figure 1*) (Evert, 2006a; Beck, 2010). When the formation of the procambium is completed, regions between the vascular bundles differentiate into parenchymatic tissues. Hence this region is referred to as the "interfascicular region", meaning "between bundles". As the vascular development proceeds, periclinal divisions (in parallel to the surface plane of an organ) of the procambium cells generate the functional tissues, primary xylem and primary phloem. The fashion of cell divisions

within the procambium generally leads to formation of collateral bundles, in which primary xylem forms towards the pith and primary phloem forms towards the surface of the plant (*Figure 1*). Phloem tissue transports the nutrients such as sugars and amino acids from the source organs to the sink organs, while xylem tissue is mainly responsible for conducting water and providing structural support for the plants (Evert, 2006a; Beck, 2010). In comparison to the stems, most roots display a protostele arrangement where, primary vascular system consists of central primary xylem, enclosed by primary phloem with procambium cells lying in between (Beck, 2010).

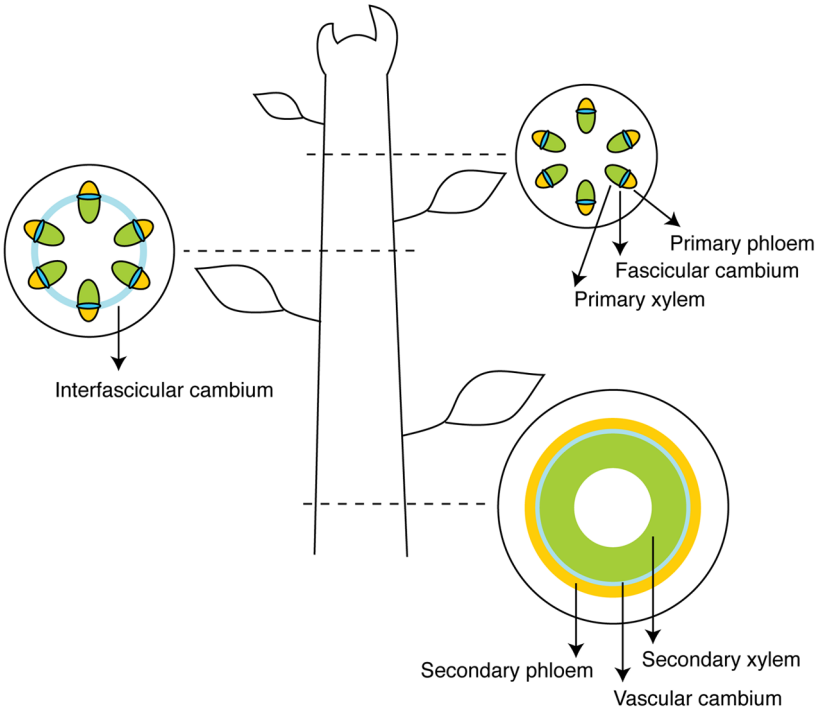

Figure 1: Schematic illustration of vascular cambium formation in a dicot stem. Phloem is depicted in orange, procambium/cambium is depicted in blue and xylem is depicted in green. A stem displaying primary development (top), an interfascicular cambium is formed in the stem (middle), a continuous cylinder of vascular cambium is formed and stem displays secondary growth (bottom). Adopted from (Miyashima *et al.*, 2013; Raven *et al.*, 2013).

With respect to different cell types, primary xylem consists of water conducting tracheary elements (tracheids in gymnosperms vs. vessel members in angiosperms), fibers (only in angiosperms) and parenchyma cells (Beck, 2010). On the other hand, with respect to developmental timing and locations, primary xylem is comprised of protoxylem and metaxylem cells (Beck, 2010;

Bishopp *et al.*, 2013). Protoxylem cells differentiate and mature earlier during the vascular development while surrounding tissues are undergoing extension growth. Moreover, secondary cell wall depositions on these cells show annular or helical structure giving them a certain level of elasticity during the rapid extension growth. As the surrounding tissues mature, these cells are mostly destroyed. Metaxylem cells, on the other hand, differentiate later in comparison to protoxylem cells, and mature when the extension growth has (almost) finished. They possess pitted or reticulate secondary cell wall thickenings (Beck, 2010; Bishopp *et al.*, 2013). With respect to different cell types, primary phloem is composed of conducting cells (sieve cells in gymnosperms vs. sieve tube members in angiosperms), associated companion cells (only in angiosperms), parenchyma cells and fibers (Beck, 2010). Although it is harder to distinguish, much similar to their xylem counterparts, protophloem cells differentiate from procambium cells first and metaphloem cells differentiate later during the vascular development (Beck, 2010).

1.3.2 Establishment of the vascular cambium and secondary growth

As the plants grow larger, the need for water conduction and nutrients increases, and the growing stems or roots require more structural support (Evert, 2006c; Evert, 2006d; Beck, 2010). Then many plant species among the gymnosperms and dicotyledons go through a process of "secondary growth", where girth of the stems and roots thickens. Secondary growth results predominantly from the activity of a lateral meristem called the "vascular cambium" (Evert, 2006c; Evert, 2006d; Beck, 2010).

In the plant species that would go through secondary development, a layer of undifferentiated procambium cells within the vascular bundles of the stem stays active even after the primary development is completed (Evert, 2006c; Evert, 2006d; Beck, 2010). With the onset of the secondary development, these procambium cells start to divide again. In coordination with these cell proliferations, the parenchymatic cells within the interfascicular region dedifferentiate into interfascicular cambium cells and regain the ability to divide. The fascicular cambium and the interfascicular cambium together create a complete cylinder of meristematic cells within the stem, which constitutes the "vascular cambium" (*Figure 1*) (Evert, 2006c; Evert, 2006d; Beck, 2010). During the secondary development of the roots, vascular cambium arises from the activity of the procambium and the pericycle cells (Bishopp *et al.*, 2013). Vascular cambium contains the vascular stem cells to produce the secondary vascular tissues, more precisely secondary phloem

centrifugally and secondary xylem (wood) centripetally (*Figure 1*) (Evert, 2006c; Evert, 2006d; Beck, 2010).

Although it is not in the scope of this thesis work, it is worthy to mention that cork cambium, another lateral meristem, forms a multilayered protective structure called the "periderm" on the surface of the shoots throughout the secondary growth by producing the phellem (cork) and phelloderm (cortex) (Evert, 2006c; Beck, 2010).

1.3.3 Cell divisions in the vascular cambium

Vascular cambium contains two types of morphologically distinct meristematic cells, fusiform initials and ray initials (Evert, 2006d; Beck, 2010). Fusiform initials are vertically oriented, elongated cells with pointed ends. They are the precursors of all cell types of the axial system: secondary xylem cells (tracheary elements (tracheids in gymnosperms vs. vessel members in dicotyledons), associated fibers (only in dicotyledons), and axial parenchyma cells) and secondary phloem cells (conducting cells (sieve cells in gymnosperms vs. sieve tube members in angiosperms), associated companion cells (only in angiosperms), axial parenchyma cells and phloem fibers) (Beck, 2010). The length of the fusiform initials varies depending on the species as well as the developmental age of the vascular cambium, for instance; older fusiform initials are longer than the newly formed fusiform initials (Evert, 2006d; Beck, 2010). Ray initials, on the other hand, are nearly isodiametric cells and relatively small comparing to the fusiform initials. They give rise to the radial system of ray parenchyma cells, which extends from the secondary phloem to the secondary xylem radially inside the trunks of stems or roots and responsible for the translocation of water, nutrients, photosynthetic assimilates as well as storage (Beck, 2010).

Cell division orientation in the vascular cambium is highly important for the correct growth of the vascular tissues (Evert, 2006d; Beck, 2010). Periclinal divisions of the vascular cambium cells can be considered as "additive divisions" since they increase the number of cells within the secondary xylem and the secondary phloem (*Figure 2*) (Evert, 2006d). Moreover, they occur at a greater frequency on the xylem side in comparison to the phloem side; consequently secondary xylem has a higher growth rate than the secondary phloem (Beck, 2010). When the cambium is actively growing, the rate of cell divisions is rather quick comparing to the rate of the cell differentiation; as a result, there is an accumulation of undifferentiated cells arranged in radial files (Evert, 2006d). These radial files contain the initials as well as the immediate

derivatives of the initials or the "mother cells". Mother cells can directly differentiate or may divide several rounds before differentiating into secondary tissues (Evert, 2006d).

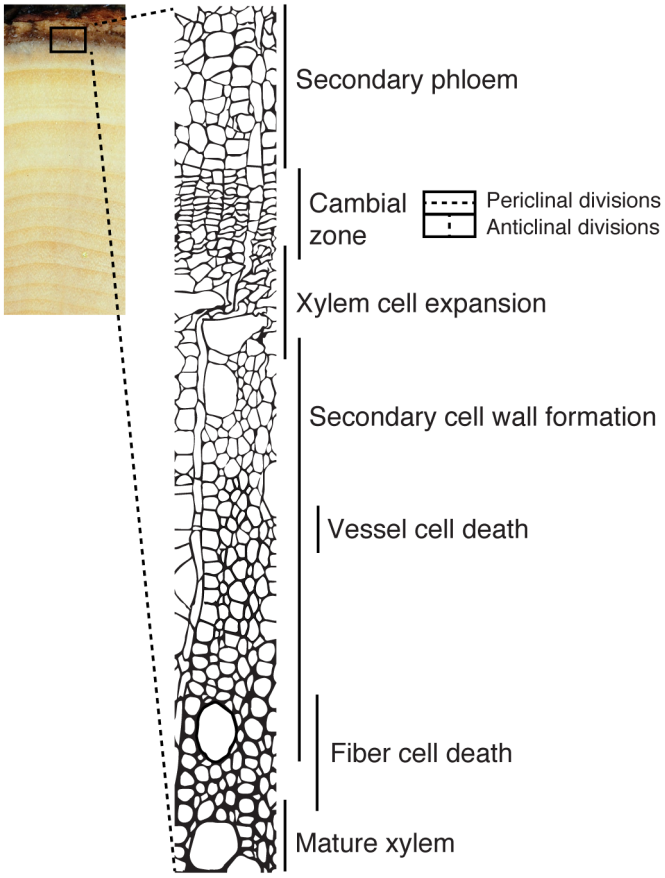

Figure 2: Secondary growth and wood formation in *Populus*. The vascular cambium generates secondary phloem centrifugally and secondary xylem centripetally. Xylem cells pass through different stages of development: cell expansion, secondary cell wall formation, and cell death. Adopted from (Courtois-Moreau *et al.*, 2009; Edvardsson, 2010). *Populus* wood image was kindly donated by Hannele Tuominen and Veronica Bourquin.

Over the years, a number of theories have been suggested regarding to the exact number of the cambial initials and their precise locations; such as the uniseriate (single) initial theory of Sanio or the multiseriate (multiple) initials theory of Raatz (Larson, 1994). This controversy lies in the fact that the initials and the mother cells look cytologically very similar. Therefore, there is a certain level of disagreement about the terminology defining cambium cells.

While some scientists use the term “cambium” just for the initials and the term “cambial zone” for the initials and the mother cells; others refer to them all together as the “cambium” (Larson, 1994). In this thesis work, I used the terms “cambium” and “cambial zone” non-specifically, where both denote initials and the mother cells collectively, unless otherwise stated.

In order to balance with the enlargement of the secondary xylem, the cambial cylinder also needs to increase in its circumference (Evert, 2006d; Beck, 2010). This is accomplished by the anticlinal divisions (in perpendicular to the surface plane of an organ) of the initials (*Figure 2*). Anticlinal divisions can be considered as “multiplicative divisions” since they raise the number of initials within the vascular cambium, which would create new radial cell files (Evert, 2006d). It is assumed that only initials have the ability to divide both anticlinally (to give rise to new cell files) and periclinally (to give rise to new secondary tissues) (Larson, 1994; Evert, 2006d). In stem cross sections of the *Populus* wood-forming zone, most of the anticlinal divisions in cambium cells were observed in close proximity to the secondary phloem during the active growth period (Schrader *et al.*, 2004b). This suggests that initials of the vascular cambium might reside in this region.

In species that have a nonstoried cambium (cambium with long fusiform initials), anticlinal divisions happen with the formation of oblique cell walls (Evert, 2006d; Beck, 2010). These oblique anticlinal divisions shorten the length of the fusiform initials. Resulting cells elongate in their apices by intrusive apical growth, so that the length of an initial can be sustained during the growth over the years. Following anticlinal divisions of the fusiform initials, many fates can follow. For instance, fusiform initials might fail to enlarge normally and lost, they might turn into ray initials, or mother cells, which might divide further, periclinally to give rise to secondary tissues. It has been observed that contact with the ray cells increase the survival rate of the fusiform initials (Evert, 2006d; Beck, 2010).

In woody angiosperms, once the xylem mother cells are pushed out of the cambial zone, they enter the radial expansion zone where vessel members no more elongate but expand via intrusive lateral growth and symplastic growth, while fibers continue elongation in height via further intrusive apical growth as well as enlarge via symplastic growth (*Figure 2*) (Siedlecka *et al.*, 2008). Following the cell expansion, both cell types deposit secondary cell walls, lignify and subsequently undergo programmed cell death (*Figure 2*) (Beck, 2010). As the phloem mother cells leave the cambial zone, sieve tube elements

and parenchyma cells behave similar to vessel elements where they mainly enlarge laterally, while phloem fibers might continue to grow via intrusive apical growth (Evert, 2006b).

In temperate zones of the world, the vascular cambium displays a short day induced growth cessation and dormancy period usually in between mid autumn to late winter-early spring (Beck, 2010). With the onset of dormancy, cell walls of cambial cells thickens, large central vacuoles in the cambium cells divide into small vacuoles, cytoplasmic streaming stops and storage products such as lipid drops and starch grains accumulate in the cells to be used in cold hardening and in resumption of cambial cell activity in spring. During this period, formation of new cambial initials and mother cells stops almost entirely and the cambial zone is usually restricted to a single cell layer (Evert, 2006d). In the end of winter to early spring, when the cambial cells start to expand in radial directions via cambial swelling, the aforementioned changes are reversed and the periclinal divisions are reinitiated (Evert, 2006d; Beck, 2010).

1.4 Control of procambium initiation

The initiation and maintenance of the procambium/cambium stem cells is multifaceted, and recent studies reveal some fundamental insights about the regulatory mechanisms in this aspect of the plant growth and development. In section 1.4 and 1.5 of the introduction, I summarize some of the factors controlling the procambium/cambium formation and activity in the context of my PhD studies. For a more comprehensive overview of the factors affecting the primary and secondary vascular development, I invite the readers of this thesis work to check some brilliant reviews published in this research area (Miyashima *et al.*, 2013; Furuta *et al.*, 2014; Guerriero *et al.*, 2014; Nieminen *et al.*, 2015; Rodriguez-Villalon, 2015; Ruzicka *et al.*, 2015; ten Hove *et al.*, 2015).

1.4.1 The role of auxin

Early embryonic development in *Arabidopsis* provides an excellent model system to study the vascular initiation process, because it demonstrates an easy-to-follow developmental pattern in regard to both orientation and number of cell divisions (ten Hove *et al.*, 2015). During embryo formation in *Arabidopsis,* with the transition from octant stage to dermatogen stage, tangential divisions of all cells in the proembryo form an outer cell layer of protoderm (future epidermis) and an inner cell layer of subprotoderm (future ground/vascular tissue). Later, with the transition from dermatogen stage to

early globular stage, periclinal divisions of the subprotoderm cells within the lower tier of the proembryo separates the ground tissue from the first vascular initials. As the embryo development continues, further proliferations of the vascular initials generate the diarch vascular bundle of an early postembryonic root with two phloem poles, a central xylem axis with the intervening procambium (ten Hove *et al.*, 2015). *Arabidopsis* leaf vein patterning provides another easily accessible model system to study the procambium formation and as well as the vascular continuity (Sawchuk *et al.*, 2008). During development of the veins, preprocambial cells are generated in an iterative manner from the cells of the ground meristem and organized into continuous procambium strands (Sawchuk *et al.*, 2008; Miyashima *et al.*, 2013).

One of the major factors contributing to the establishment and continuity of the procambium is the plant hormone auxin (Sawchuk *et al.*, 2008), as the generation of new procambium strands occurs along the narrow paths with an increased polar auxin transport (Mattsson *et al.*, 2003). *PIN-FORMED1 (PIN1)* encodes for an auxin efflux carrier protein which positions asymmetrically on the plasma membrane (Galweiler *et al.*, 1998). The polarity of the auxin transport is mediated mainly by the activity of PIN1, during the vein development (Scarpella *et al.*, 2006) and the procambium formation in embryos (Friml *et al.*, 2003). Restricted and polarized expression of *PIN1* represents one of the earliest events during the vascular initiation process and marks the routes for future procambium strands (Sauer *et al.*, 2006; Scarpella *et al.*, 2006).

During embryogenesis in *Arabidopsis,* auxin response within the ground/vascular tissues stimulates the degradation of BODENLOS (BDL), an AUX/IAA family member protein, which in turn activates the auxin-dependent transcription factor MONOPTEROS (MP)/AUXIN RESPONSE FACTOR5 (ARF5) (Hamann *et al.*, 2002; Dharmasiri *et al.*, 2005; Weijers *et al.*, 2006). In terms of vascular initiation phenotypes, plants with a mutation in the *MP/ARF5* gene display reduced number of vascular initial cells in the lower tier of the embryo proper (Berleth & Jurgens, 1993; Hardtke & Berleth, 1998), which suggests a critical role for this gene during the specification of procambium. *mp/arf5* mutants were shown to have reduced levels of *PIN1* expression, indicating that the mutant phenotypes of *mp/arf5* can be related to a reduced auxin transport and that MP/ARF5 might positively regulate *PIN1* (Wenzel *et al.*, 2007).

During their studies on the *Arabidopsis* leaf vein development, Tyler Donner and his colleagues identified that *ATHB8* is a direct target of MP/ARF5 (Donner *et al.*, 2009). *ATHB8* is a member of the *HOMEODOMAIN-LEUCINE ZIPPER III (HD-ZIP III)* gene family. It appears that the *ATHB8* promoter harbors an auxin-response element, where MP/ARF5 can bind, and positively regulate *ATHB8* expression. Because *athb8* plants show enhanced and broad expression of *PIN1* comparing to wild type plants in the presence of auxin transport inhibitors, it has been suggested that ATHB8 might control the expression domain of *PIN1* and hence stabilizes the auxin flow during the vein development (Donner *et al.*, 2009). Therefore, in agreement with the auxin canalization theory of Sachs (Sachs, 1981), auxin-MP/ARF5-ATHB8-PIN1 seems to form a self-reinforcing mechanism of auxin flow during the formation of procambium.

Other genes that are acting downstream of MP/ARF5 were also identified by transcript profiling (Schlereth *et al.*, 2010). One of these target genes is the basic helix-loop-helix (bHLH) transcription factor *TARGET OF MONOPTEROS* (*TMO5*), which is specifically expressed in the vascular initials of the early *Arabidopsis* embryos. *TMO5* expression is strongly down regulated in the *mp/arf5* plants, suggesting that MP/ARF5 positively regulates *TMO5* expression (Schlereth *et al.*, 2010). Although loss-of-function *tmo5* mutants do not have a visible phenotype, *tmo5 tmo5-like1* (*t5l1*) double mutant embryos show significant reductions in the periclinal divisions of the vascular initials (De Rybel *et al.*, 2013). These mutants also have a smaller vascular bundle size in the postembryonic roots with only one xylem pole and one phloem pole in comparison to the diarch vascular bundle of the wild type roots (De Rybel *et al.*, 2013). A similar morphological phenotype in the postembryonic roots was previously observed in the mutants of *lonesome highway (lhw)* (Ohashi-Ito & Bergmann, 2007). *LHW* also encodes a bHLH transcription factor (Ohashi-Ito & Bergmann, 2007). Further studies revealed that members of the TMO5 and LHW sub-clades interact both *in vitro* and *in vivo*, suggesting that they might function as heterodimers (De Rybel *et al.*, 2013; Ohashi-Ito *et al.*, 2014). Studies showed that *LHW* is expressed broadly both in the embryo and in the postembryonic roots, overlapping with the *TMO5* expression in the vascular initials during the globular stage of the embryogenesis and in the xylem precursor cells of the root meristem during the postembryonic development, which suggests that the TMO5-LHW dimer complex accumulates in these cell types. Moreover, ectopic co-expression under a meristematic promoter of both genes increase the rate of periclinal divisions within the vascular tissues of postembryonic roots, revealing a critical

role for this dimer complex in the control of periclinal divisions and vascular tissue establishment (De Rybel *et al.*, 2013; Ohashi-Ito *et al.*, 2014).

Since the xylem precursor cells, where the TMO5 and LHW interaction occurs, do not divide periclinally in the root meristem, it has been speculated that the TMO5-LHW dimer might control the cell divisions of the procambium non-cell autonomously (De Rybel *et al.*, 2013; Ohashi-Ito *et al.*, 2014). How TMO5-LHW regulate procambium cell divisions non-cell autonomously have been recently elucidated, with the identification of *LONELY GUY3 (LOG3)* and *LOG4* as direct targets of TMO5-LHW (De Rybel *et al.*, 2014; Ohashi-Ito *et al.*, 2014). *LOG* genes encode for enzymes responsible for converting inactive forms of the plant hormone cytokinin to active forms during its biosynthesis (Kurakawa *et al.*, 2007; Kuroha *et al.*, 2009). Expression of *LOG3* and *LOG4* overlap with the sites where TMO5-LHW dimers accumulate both during embryogenesis and post-embryonically (De Rybel *et al.*, 2014; Ohashi-Ito *et al.*, 2014). Higher order *log* mutants suppress the increased periclinal division phenotype of TMO5-LHW over-expressing roots, indicating the involvement of cytokinin as a diffusible factor to control the periclinal divisions of the procambium cells (De Rybel *et al.*, 2014; Ohashi-Ito *et al.*, 2014).

1.4.2 The role of cytokinin

Several reports indicate an important regulatory function for the plant hormone cytokinin during the regulation of the procambium identity and activity (Mahonen *et al.*, 2000; Mahonen *et al.*, 2006a; Mahonen *et al.*, 2006b). Cytokinin signaling commences with the perception of cytokinin by the two-component histidine kinase proteins, which are located on the endoplasmic reticulum and/or the plasma membrane (El-Showk *et al.*, 2013). The interaction between the cytokinin and the receptors initiates a phosphorelay cascade through the activity of histidine phosphotransfer proteins. These proteins are able move between the cytosol and the nucleus, and convey the phosphate groups to the response regulators within the nucleus. Phosphorylation of the type-B response regulators starts the transcription of the cytokinin-responsive genes, as well as the type-A response regulators, which are considered as negative regulators of cytokinin signaling (El-Showk *et al.*, 2013).

In the genome of *Arabidopsis thaliana*, there are three members of the histidine kinase receptor gene family, *WOODENLEG (WOL)/CYTOKININ RESPONSE1 (CRE1)/ARABIDOPSIS HISTIDINE KINASE (AHK4)*, *AHK2* and

AHK3 (Mahonen *et al.*, 2006b). In terms of vascular phenotypes, both the *wol* mutation, in which the cytokinin binding capacity of the receptor is obliterated, and the triple-knockout mutants of the histidine kinase receptors (*cre1 ahk2 ahk3*) show reduced number of periclinal divisions in the procambium cells, leading to a smaller vascular bundle size in the primary roots where all vascular cells in the stele differentiate as protoxylem cells (Scheres *et al.*, 1995; Mahonen *et al.*, 2000; Mahonen *et al.*, 2006b). Moreover, when cytokinin is depleted in the procambium cells by the expression of *CYTOKININ OXIDASE2 (CKX2)* gene under the control of *CRE1* promoter, the defects observed in the *wol* and *cre1 ahk2 ahk3* triple-knockout mutants are phenocopied (Mahonen *et al.*, 2006a), indicating that cytokinin is essential for stimulating the periclinal cell divisions in the procambium, and preventing their differentiation into protoxylem cells. Consistent with this role of cytokinin in the regulation of the procambium, higher order mutants of downstream players such as quintuple mutants of *Arabidopsis thaliana histidine phosphotransfer proteins (ahp1 ahp2 ahp3 ahp4 ahp5)* and triple-knockout mutants of *Arabidopsis thaliana response regulators (arr1 arr10 arr12)* also generate similar vascular phenotypes to *wol* plants (Hutchison *et al.*, 2006; Yokoyama *et al.*, 2007; Argyros *et al.*, 2008).

In the vascular cylinder of the primary roots of *Arabidopsis*, high auxin signaling appears in the central xylem axis, where as high cytokinin signaling appears in flanking procambium cell files (Bishopp *et al.*, 2011). In the protoxylem cells, auxin induces the expression of *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6 (AHP6),* which inhibits the cytokinin signaling in this domain (Mahonen *et al.*, 2006a; Bishopp *et al.*, 2011). Plants with a mutation in *AHP6* gene display a loss of protoxylem cell fate and ectopic formation of procambium cells in the protoxylem cell positions, because in these mutants cytokinin-signaling domain expands (Mahonen *et al.*, 2006a; Bishopp *et al.*, 2011). On the other hand, cytokinin modulates the expression and localization of auxin efflux carrier proteins PIN1 and PIN7 in the procambium cell files, which in turn direct auxin transport laterally toward the central xylem axis (Bishopp *et al.*, 2011). This interaction between cytokinin and auxin is very important, which specifies the correct positions of the procambium cells and xylem cells in the vascular cylinder of the roots.

1.5 Control of cambium activity and cell specification

1.5.1 The power of moving around - The TDIF peptide signaling module

The *CLAVATA3* (*CLV3*)/*EMBRYO SURROUNDING REGION* (*ESR*)-*RELATED* (*CLE*) gene family encodes for small proteins that are usually 60-120 amino acids long (Cock & McCormick, 2001; Oelkers *et al.*, 2008). Each CLE protein consists of a signal peptide residing at the N-terminus, a CLE domain(s) residing at the C-terminus and a variable domain between these two sequences (Oelkers *et al.*, 2008). CLE domain is proteolytically processed and post-translationally modified to be biologically active and is thought to be released to the apoplastic space where it acts non-cell autonomously through interactions with plasma membrane-associated LEUCINE-RICH REPEAT RECEPTOR-LIKE KINASEs (LRR-RLKs) (Hirakawa *et al.*, 2008; Ohyama *et al.*, 2008; Ohyama *et al.*, 2009). The most known members of this peptide family include CLV3 and CLE40, regulators of stem cell niches in the SAM and the RAM, respectively (Clark *et al.*, 1995; Stahl *et al.*, 2009). Other members are shown to have diverse roles in plants such as embryo and endosperm development (Fiume & Fletcher, 2012), pollen-pistil interactions (Endo *et al.*, 2013), autoregulation of nodulation (Okamoto *et al.*, 2009; Mortier *et al.*, 2010; Reid *et al.*, 2011), and lateral root expansion (Araya *et al.*, 2014). More information about functional diversification of the CLE peptide family in different plant species can be found in Paper V in this thesis work.

Several reports indicate an important regulatory function for the TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF)/CLE41/CLE44 peptide in the vascular development process (*Figure 3*) (Hirakawa *et al.*, 2008; Etchells & Turner, 2010). Originally identified as a secreted peptide in the *Zinnia* cell culture system, TDIF/CLE41/CLE44 is encoded by two genes in the *Arabidopsis* genome, namely *CLE41* and *CLE44* (Ito *et al.*, 2006). *Arabidopsis* plants over-expressing *CLE41* or *CLE44* under the control of the 35S promoter, as well as plants grown in the presence of a synthetic TDIF/CLE41/CLE44 peptide, display increased vascular cell divisions in the hypocotyls and inflorescence stems, and interrupted vessel strand formation in the leaves (Hirakawa *et al.*, 2008; Whitford *et al.*, 2008; Etchells & Turner, 2010). In addition, ectopic over-expression of *CLE41* or *CLE44* causes defects in the patterning of the vascular tissues with a xylem intermixed with phloem phenotype during both primary and secondary growth (Etchells & Turner, 2010). Taken together, these results indicate that the TDIF/CLE41/CLE44 peptide not only regulates the fate of procambium/cambium cells by promoting vascular stem cell proliferations and

inhibiting their specification into xylem cells, but also by controlling the cell division orientation in the vascular meristems.

CLE41 and *CLE44* genes are expressed mainly in the phloem cells, but the TDIF/CLE41/CLE44 peptide is secreted into the apoplastic space and functions non-cell autonomously (Hirakawa *et al.*, 2008; Ohyama *et al.*, 2008; Etchells & Turner, 2010). In the model plant *Arabidopsis thaliana,* TDIF/CLE41/CLE44 peptide signals through TDIF RECEPTOR (TDR)/PHLOEM INTERCALATED WITH XYLEM (PXY) (*Figure 3*) (Hirakawa *et al.*, 2008; Etchells & Turner, 2010). *TDR/PXY* is expressed in the procambium/cambium cells and encodes an LRR-RLK (Fisher & Turner, 2007; Hirakawa *et al.*, 2008; Etchells & Turner, 2010). Although *tdr/pxy* mutations cause little reductions in vascular cell numbers, when this mutation is combined with the over-expression of *CLE41,* the increased vascular cell division phenotypes of the peptide signaling is almost abolished (Fisher & Turner, 2007; Etchells & Turner, 2010). Moreover, knockout *tdr/pxy* plants also show defects in the organization of vascular tissues, similar to the over-expression phenotypes of *CLE41* and *CLE44* where phloem cells appears to be adjacent or intermixed with the xylem cells (Fisher & Turner, 2007; Etchells & Turner, 2010). These results collectively demonstrate that TDR/PXY both promotes vascular cell divisions and controls cell division planes in the procambium/cambium. Observations regarding to the transcriptional regulations between the components of the TDIF/CLE41/CLE44-TDR/PXY signaling module revealed that *TDR/PXY* expression is up regulated in the *tdr/pxy* mutants, indicating a negative feedback mechanism where *TDR/PXY* controls its own expression (Fisher & Turner, 2007; Etchells & Turner, 2010). In addition, in *35S::CLE41* lines, transcript levels of *TDR/PXY* were reduced, suggesting that also *CLE41* can negatively regulate *TDR/PXY* expression (Fisher & Turner, 2007; Etchells & Turner, 2010).

Previous reports revealed that CLV3 and CLE40 peptides control the expression of the homeodomain transcription factors *WUSCHEL (WUS)* and *WUSCHEL-RELATED HOMEOBOX5 (WOX5),* in the SAM and the RAM respectively (Brand *et al.*, 2000; Schoof *et al.*, 2000; Stahl *et al.*, 2009). By analogy, a peptide screening disclosed that transcript levels of *WOX4* and *WOX14* were increased upon application of the TDIF/CLE41/CLE44 peptide in a TDR-dependent manner in *Arabidopsis* seedlings (*Figure 3*) (Hirakawa *et al.*, 2010; Etchells *et al.*, 2013). Both *WOX4* and *WOX14* are expressed predominantly in the procambium/cambium region of the vascular tissues. While *wox14* mutants do not show any obvious vascular phenotypes, *wox4*

mutants have a reduced procambium/cambium cell proliferation both in the inflorescence stems and in the hypocotyls. Intriguingly, the cambial cell division activity is not completely abolished in the *wox4* mutant plants and over-expression of the *WOX4* gene does not stimulate procambium/cambium formation in *Arabidopsis,* suggesting that *WOX4* mainly controls the cell division activity in the procambium/cambium rather than procambium/cambium cell identity. In addition, mutations in *WOX14* enhance the *wox4* phenotype of reduced cell division activity, indicating that *WOX4* and *WOX14* might function redundantly to control the cell proliferations in the procambium/cambium (*Figure 3*) (Hirakawa *et al.*, 2010; Etchells *et al.*, 2013). On the other hand, *wox4 wox14* double mutants do not show any alterations in terms of vascular organization, therefore it seems unlikely that they are involved in this aspect of the procambium/cambium regulation (Etchells *et al.*, 2013). *wox4* mutants behave as wild type plants in response to TDIF/CLE41/CLE44 peptide application in terms of discontinuous vessel strand formation in leaves, indicating that *WOX4* is not required for the suppression of xylem cell specification from procambium/cambium cells (Hirakawa *et al.*, 2010).

Interestingly, genes similar to *TDR/PXY* and *WOX4* genes in tree species recently started to get characterized. An elegant transcriptional profiling study over the *Populus* wood-forming zone demonstrated that *PttRLK3* and *PttHB3* (orthologous to *Arabidopsis TDR/PXY* and *WOX4*, respectively) transcripts are highly abundant over the cambial zone (Schrader *et al.*, 2004b). While transgenic hybrid aspen trees ectopically over-expressing *PttPXY* or *PttCLE41* (orthologous to *Arabidopsis TDR/PXY* and *CLE41*, respectively) demonstrate reduced plant growth and defects in the patterning of the vascular tissues, in contrast, tissue-specific co-over-expression of these genes under a cambium-specific promoter and a phloem-specific promoter respectively display improved plant growth and wood formation (Etchells *et al.*, 2015). Although more evidence is needed, these outcomes indicate that in *Arabidopsis* and *Populus,* cambium cell proliferations might be regulated via similar pathways.

In terms of xylem cell fate determination, GLYCOGEN SYNTHASE KINASE 3 (GSK3) proteins have been shown to be downstream targets of the TDIF/CLE41/CLE44-TDR/PXY signaling (*Figure 3*) (Kondo *et al.*, 2014). GSK3 proteins, also known as Shaggy Kinases, are important players in the BRASSINOSTEROID (BR) signaling, where they negatively regulate the BR responses. A recent report showed that GSK3s, including BRASSINOSTEROID-INSENSITIVE 2 (BIN2), interact with TDR/PXY at

the plasma membrane, and TDIF/CLE41/CLE44 peptide treatments induce the BIN2 kinase activity in a TDR/PXY dependent manner. Consistent with a role in the inhibition of xylem differentiation, *gsk3* quadruple mutants do not display a discontinuous leaf vessel strand formation in response to TDIF/CLE41/CLE44 peptide treatments, while they still show a procambium cell number increase in the hypocotyls, as do the wild type plants. Anatomical observations of the *gsk3* quadruple mutants showed that they also display an increased xylem ratio and a decreased procambium ratio against all cell types in the hypocotyls, indicating that they normally repress the differentiation of procambium cells into xylem cells. This suppression of xylem differentiation seems to be through the inactivation of BRI1-EMS-SUPPRESSOR 1 (BES1)/BRASSINAZOLE RESISTANT 2 (BZR2) (*Figure 3*). Confirming the involvement of BES1/BZR2, combination of *wox4* mutants with either bikinin (a plant specific GSK3 inhibitor), or the *bes1-D* gain-of-function mutants mimic the xylem-adjacent-to-phloem phenotype of *tdr/pxy* plants in the hypocotyls because of their combinatory effects on cambial cell depletion and enhanced xylem cell differentiation (Kondo *et al.*, 2014).

With regard to regulation of vascular patterning, ERECTA (ER) signaling is suggested as an interacting pathway with TDIF/CLE41/CLE44-TDR/PXY signaling (*Figure 3*) (Etchells *et al.*, 2013; Uchida & Tasaka, 2013). In the *Arabidopsis* genome *ER*, and its paralogs *ERECTA-LIKE1* (*ERL1*) and *ERL2* encodes for LRR-RLKs and ER has been previously reported to play a role in stem elongation (Uchida *et al.*, 2012). Recent studies revealed that the *er* mutation enhances the vascular organization defects observed in *tdr/pxy* both during primary and secondary growth (Etchells *et al.*, 2013; Uchida & Tasaka, 2013). Among the three ER paralogs, only *ER* and *ERL1* show expression in the vascular tissues both in phloem and xylem, however tissue-specific rescue experiments showed that activity of ER in the phloem tissue is essential for the correct procambium development (Uchida *et al.*, 2012; Uchida & Tasaka, 2013). The nature of the interaction between ER signaling and TDR/PXY signaling is currently unclear, since the expression domains of *ER* and *TDR/PXY* are separated (Fisher & Turner, 2007; Hirakawa *et al.*, 2008; Etchells & Turner, 2010; Etchells *et al.*, 2013; Uchida & Tasaka, 2013). ER has been previously shown to interact with EPIDERMAL PATTERNING FACTOR LIKE (EPFL) family peptides, namely EPFL4 and EPFL6/CHALLAH (CHAL), expressed in the endodermal tissues (Uchida *et al.*, 2012; Uchida & Tasaka, 2013). Similar to the vascular organization defects observed in *er tdr/pxy* double mutants, the combination of the *tdr/pxy* mutation

with *epfl4 epfl6* double mutants also enhances the defects observed in the vascular bundles of the inflorescence stems (Uchida & Tasaka, 2013).

A recent report identified two other LRR-RLKs involved in the regulation of cambium cell proliferation via transcriptomic analysis during interfascicular cambium initiation (Agusti *et al.*, 2011). In this study, it was shown that MORE LATERAL GROWTH1 (MOL1) is a repressor of cambial activity, whereas REDUCED IN LATERAL GROWTH1 (RUL1) is a promoter of cambial activity in the *Arabidopsis* inflorescence stems. Moreover, it is possible that MOL1 functions upstream of TDR/PXY, since the transcript levels of *TDR/PXY* were up regulated in the *mol1* mutants (*Figure 3*) (Agusti *et al.*, 2011). In the future, it would be intriguing to identify interacting CLE peptide ligands for these LRR-RLKs, their mode of action and crosstalk via the *TDR/PXY* signaling.

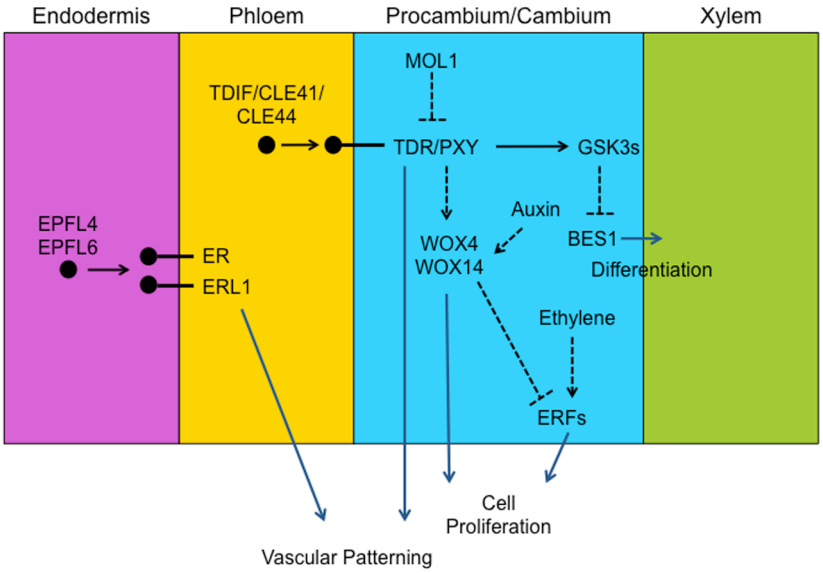

Figure 3: Regulation of procambium/cambium activity by the TDIF peptide signaling module, downstream targets of this pathway and hormonal crosstalk. The secreted TDIF/CLE41/CLE44 peptide interacts with the TDR/PXY receptor in the procambium/cambium. This interaction modulates cell proliferation, cell differentiation towards xylem and vascular patterning. The secreted EPFL4 and EPFL6 peptides interact with the ER and ERL1 receptors in the phloem. This interaction modulates vascular patterning in harmony with the TDIF/CLE41/CLE44 peptide signaling module. Black arrows depict physical interactions; dashed arrows/lines depict transcriptional regulation; dark blue arrows depict control of different vascular development-related processes. Adopted from (Nieminen *et al.*, 2015).

1.5.2 Crosstalk between the TDIF peptide signaling module and the plant hormones

The plant hormone auxin and the basipetal auxin transport plays a major role in the regulation of vascular cambium activity and secondary growth; as evidenced by the observations that decapitation of shoot tips causes decreased cambial activity in plants and the loss of cambial activity can be re-established by the apical application of auxin (Snow, 1935; Ko *et al.*, 2004; Bjorklund *et al.*, 2007). Measurements of auxin in the wood-forming zone of *Populus* and Scots pine revealed that auxin concentration peaks in the cambial zone, and declines towards the secondary tissues during the active growth period (Uggla *et al.*, 1996; Tuominen *et al.*, 1997; Uggla *et al.*, 1998; Schrader *et al.*, 2003). Similarly, the transcript levels of genes responsible for auxin transport correlates with the auxin distribution radially (Schrader *et al.*, 2003), giving rise to the hypothesis that auxin could act as a morphogenetic signal to regulate cell proliferations in the vascular cambium. However auxin concentrations in the vascular cambium do not vary during the annual activity-dormancy cycle (Uggla *et al.*, 1996; Uggla *et al.*, 1998; Schrader *et al.*, 2003; Schrader *et al.*, 2004a). Further studies revealed that cambial growth cessation and dormancy involves the modulation of auxin responsiveness instead of changes in the auxin levels in the vascular cambium (Baba *et al.*, 2011). Consistent with this, transgenic trees with reduced auxin response (by over-expressing a mutant version of a *Populus* Aux/IAA gene) display decreased the frequencies of both periclinal and anticlinal cell divisions in the vascular cambium, indicating that auxin positively regulates cambium cell activity (Nilsson *et al.*, 2008). Interestingly, a recent report indicated that expression of *WOX4* could be induced by auxin (*Figure 3*), and both *TDR/PXY* and *WOX4* genes are necessary for the auxin dependent cambium stimulation in the inflorescence stems of *Arabidopsis*, proposing a role for *WOX4* in translating the auxin signals into cambial activity (Suer *et al.*, 2011).

Another important plant hormone having a function in the regulation of cambium activity and wood formation is ethylene (Love *et al.*, 2009). In *Populus,* application of aminocyclopropane-1-carboxylate (ACC, the precursor of ethylene) into the growth medium of the trees, or feeding the trees with gaseous ethylene, or over-expression of *ACC oxidase (ACO*, the enzyme catalysing the conversion of ACC into ethylene in the final step of its biosynthesis) all stimulates the growth of secondary xylem via increased cambial cell divisions (Love *et al.*, 2009). Interestingly, recent reports show a crosstalk between the ethylene signaling and the TDIF/CLE41/CLE44-TDR/PXY signaling during the regulation of cambial cell proliferations

(Etchells *et al.*, 2012). According to this report, expression levels of the *ETHYLENE RESPONSE FACTORs (ERFs) ERF1*, *ERF018* and *ERF109* are found to up regulated both in the *tdr/pxy* and the *wox4* mutant backgrounds, which might suggest that TDIF/CLE41/CLE44-TDR/PXY signaling normally represses the expression of *ERFs* (*Figure 3*). Consistent with the results from *Populus* trees, *ethylene overproducer1 (eto1)* mutants also display increased numbers of vascular cells in the hypocotyls and inflorescence stems of *Arabidopsis* plants. Instead, when the *tdr/pxy* mutation is combined with either *ethylene insensitive 2 (ein2),* in which the ethylene signal transduction is obliterated, or with the *erf109 erf018* plants, significant reductions in vascular cell numbers are observed in comparison to *tdr/pxy* single mutants proving a genetic interaction. These results collectively suggest that TDR/PXY signaling modulates the levels of *ERF* transcription factors and that ethylene and peptide signaling pathways might work in harmony to regulate the vascular cell divisions (Etchells *et al.*, 2012).

The plant hormone cytokinin also plays an important role in the regulation of vascular cambium activity and secondary growth (Matsumoto-Kitano *et al.*, 2008; Nieminen *et al.*, 2008). Decreasing cytokinin levels in the cambium region of *Populus* trees by the expression of the *Arabidopsis CKX2* gene from a birch *CRE1* promoter leads to reduced number of cambium cells in trees (Nieminen *et al.*, 2008). *ISOPENTENYLTRANSFERASE (IPT)* genes encode for enzymes catalysing the initial step of all isoprenoid cytokinin biosynthesis (Matsumoto-Kitano *et al.*, 2008). Quadruple *Arabidopsis* mutants of *ipt1 ipt3 ipt5 ipt7* cannot generate a cambium and display reduced secondary growth in stems and hypocotyls (Matsumoto-Kitano *et al.*, 2008). These observations collectively signify the importance of cytokinin as a positive regulator of cambium cell proliferation. It will be interesting in the future to identify if cytokinin signaling and TDIF/CLE41/CLE44 signaling interact during the regulation of cambium activity.

1.5.3 Battle of two armies - *HD-ZIP III* vs. *KANADI* genes

The correct polarity of the vasculature in the stems and leaves is coordinated by the antagonistic functions of two gene families: HD-ZIP III transcription factors and KANADI (KAN) transcription factors (Emery *et al.*, 2003). In *Arabidopsis thaliana,* the *HD-ZIP III* gene family contains 5 genes: *PHAVOLUTA* (*PHV*), *PHABULOSA* (*PHB*), *CORONA/ATHB15* (*CNA*), *REVOLUTA* (*REV*)*/INTERFASCICULAR FIBERLESS1* (*IFL1*), and *ATHB8* (Prigge *et al.*, 2005). The *KAN* gene family, on the other hand, belongs to the GARP transcription factors and consists of 4 genes: *KAN1, KAN2, KAN3* and

KAN4 (Ilegems *et al.*, 2010). *HD-ZIP III* transcripts are mainly found in procambium, cambium and developing xylem, while *KAN* genes are mainly expressed in the phloem (Emery *et al.*, 2003; Prigge *et al.*, 2005; Ilegems *et al.*, 2010). The reciprocal expression pattern of the two gene families is important for the correct functioning of these transcription factors. Consistent with their roles, radial organization defects are observed in many of the mutant combinations; loss of function mutations of *phb phv rev* are highly stunted in the overall growth and the vasculature in the cotyledon produces an amphicribal vascular bundle pattern (phloem surrounding xylem). In contrast, triple mutants of *kan1 kan2 kan3* possess an amphivasal vascular bundle pattern (xylem surrounding phloem) in the inflorescence stems of *Arabidopsis* (Emery *et al.*, 2003). On the other hand, gain of function mutations in *REV/IFL1* copies the triple *kan* triple loss of function vascular phenotypes, suggesting their antagonistic functioning (Emery *et al.*, 2003).

Homologs of *REV/IFL1, CNA* and *ATHB8* genes can also be found in the *Populus* genome and they show differential expression patterns in the vascular tissues (Du *et al.*, 2011; Robischon *et al.*, 2011; Zhu *et al.*, 2013). Functional studies indicate that tissue-specific knock down of *PtrHB7* (orthologous to *ATHB8*) inhibits differentiation of cambium cells into xylem but stimulates their differentiation into phloem (Zhu *et al.*, 2013). Conversely, over-expression of *PtrHB7* suppresses the phloem differentiation but stimulates the xylem differentiation, suggesting that *PtrHB7* is a positive regulator of cambium cell proliferation and balances the differentiation process of cambium cells into secondary conductive tissues (Zhu *et al.*, 2013). On the other hand, when a microRNA resistant version of *popREVOLUTA (PRE,* orthologous to *REV/IFL1)* is over-expressed in transgenic *Populus* trees, cambium cells are produced in peculiar places in the stem such as in cortical parenchyma and secondary vascular tissues are generated in reverse polarity, suggesting that *PRE* regulates the cambium initiation and vascular patterning in trees (Robischon *et al.*, 2011). Similar approaches were used to study the functions of *POPCORONA (PCN,* orthologous to *CNA)* (Du *et al.*, 2011). Results show that while down regulation of *PCN* causes irregular lignification in the pith tissue, over-expression of a microRNA resistant version of *PCN* results in delayed lignification both in secondary xylem and phloem fibers, implying, also for this gene, an important role during the formation of secondary vascular tissues in trees (Du *et al.*, 2011).

With regard to the rate of vascular cell divisions, it is observed that in the hypocotyls of higher order *kan* mutants cambial cell divisions are increased,

while ectopic expression of *KAN1* inhibits the procambium formation, suggesting that *KAN* genes also function as negative regulators of procambium/cambium cell proliferations in *Arabidopsis* (Ilegems *et al.*, 2010). This phenotype was also associated with a change in the expression and polar localization of *PIN1*, indicating that *KAN* genes might regulate auxin transport during vascular development (Ilegems *et al.*, 2010).

1.5.4 Regulation of *HD-ZIP III* genes by microRNAs

In the *Arabidopsis* roots, specification of protoxylem and metaxylem cell types is regulated by the HD-ZIP III transcription factors in a dosage dependent fashion; reduced amounts of *HD-ZIP IIIs* promote protoxylem cell identity while increased amounts of *HD ZIP III*s promote metaxylem cell identity (Carlsbecker *et al.*, 2010). This restricted spatial expression pattern of *HD-ZIP IIIs* relies on the activity of SHORT ROOT (SHR) and SCARECROW (SCR) transcription factors. *SHR* transcripts are mainly found in the stele, however SHR proteins translocate from stele to the adjacent cell layer of endodermis, where it activates the *SCR* (Nakajima *et al.*, 2001; Carlsbecker *et al.*, 2010). In the endodermis, they, together, promote the expression of the genes encoding the microRNA165a and microRNA166b. MicroRNAs eventually modulate the levels of the *HD ZIP III* mRNAs non-cell autonomously on the post-transcriptional level. Consistent with this model, in the *shr* or *scr* mutants metaxylem cells are specified at the protoxylem cell positions, since there is no dosage dependent regulation of *HD-ZIP III*s and this phenotype could be recovered by the expression of microRNAs in the ground tissue. MicroRNA resistant gain-of-function *phb-7* mutant plants also show ectopic metaxylem formation. Moreover, in the absence of all 5 *HD-ZIP III* genes, plants lack xylem completely; indicating HD-ZIP IIIs are positive regulators of xylem specification (Carlsbecker *et al.*, 2010).

1.5.5 Master regulators of phloem and xylem cell fate

A MYB coiled-coil-type transcription factor ALTERED PHLOEM DEVELOPMENT (APL) is considered as a key regulator of phloem identity in *Arabidopsis* (Bonke *et al.*, 2003). Expression studies showed that *APL* transcripts are abundant in both sieve elements and companion cells. Although ectopic expression of *APL* under the control of the *WOL/CRE1/AHK4* promoter does not induce ectopic phloem formation, it suppresses the protoxylem cell differentiation in primary roots. Moreover, a recessive *apl* mutation causes formation of xylem cells where phloem normally develops. These results indicate that APL acts as a promoter of phloem differentiation and a repressor of xylem differentiation (Bonke *et al.*, 2003).

The NAC domain transcription factors VASCULAR-RELATED NAC-DOMAIN6 (VND6) and VND7 are shown to be key regulators of xylem vessel differentiation (Kubo *et al.*, 2005). Studies showed that *VND6* and *VND7* are expressed specifically in immature metaxylem and protoxylem vessels of *Arabidopsis* roots, respectively. While single *vnd6* and *vnd7* mutants do not reveal any visible altered phenotypes, *VND6* and *VND7* over-expression caused trans-differentiation of numerous cell types in the hypocotyls, roots and leaves into xylem vessel elements. In particular, ectopic expression of *VND7* causes protoxylem formation, whereas ectopic expression of *VND6* causes metaxylem formation (Kubo *et al.*, 2005). On the other hand, SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1 (SND1)/NAC SECONDARY WALL THICKENING PROMOTING FACTOR1 (NST3) jointly with NST1 are identified as regulators of xylem fiber cell differentiation (Zhong *et al.*, 2006; Mitsuda *et al.*, 2007). GUS analyses revealed that *SND1/NST3* and *NST1* are expressed in the interfascicular and xylary fibers of the stems and the secondary xylem of the hypocotyls of *Arabidopsis*. Functional studies indicated that ectopic over-expression *SND1/NST3* also induce ectopic secondary cell wall formation in various cell types of different organs. In comparison, loss-of-function mutants of *snd1/nst3 nst1* show complete loss of secondary wall thickenings in fiber cells (Zhong *et al.*, 2006; Mitsuda *et al.*, 2007). The above-mentioned NAC master regulators (VND6, VND7, SND1/NST3 and NST1) stimulate the expression of *MYB46* and *MYB83* transcription factors, which activates genes involved in the secondary cell wall biosynthesis (Zhong *et al.*, 2007; Zhong *et al.*, 2008; Ko *et al.*, 2009; McCarthy *et al.*, 2009; Zhong & Ye, 2012; Hussey *et al.*, 2013).

1.5.6 CLE45 signaling and protophloem formation

Previous studies revealed five protophloem marker genes, namely *PHLOEM DIFFERENTIATION 1 (PD1)* to *PD5* (Bauby *et al.*, 2007). Among these *PD1, PD2* and *PD3* show developmental stage specific expression in differentiating protophloem cells following their specification, whereas *PD4* and *PD4* are expressed in the provascular cells and later in the phloem initial cells, preceding the phloem differentiation (Bauby *et al.*, 2007). Successively, *PD4* and *PD5* were described as *BREVIS RADIX* (*BRX*), and *OCTOPUS* (*OPS*), respectively (Mouchel *et al.*, 2004; Truernit *et al.*, 2012). They both encode plasma membrane-associated proteins with a polar localization on the membrane and loss of function mutations in both *BRX* and *OPS* genes causes mutant phenotypes of small root meristem and short primary roots (Scacchi *et al.*, 2010; Truernit *et al.*, 2012). Anatomically, gap cells can be observed in the

protophloem cell files of mutant roots where individual protophloem cells cannot differentiate due to the interruption of nuclear degradation and cell wall thickening. These findings indicate that BRX and OPS are positive regulators of protophloem differentiation (Scacchi *et al.*, 2010; Truernit *et al.*, 2012). A subsequent genetic screen revealed that *barely any meristem (bam3)* could suppress the phenotypes of *brx* and *ops* (Depuydt *et al.*, 2013; Rodriguez-Villalon *et al.*, 2014). Since *BAM3* encodes for an LRR-RLK and these receptors mainly interact with CLE peptides, authors of this study tested *bam3* mutants for the insensitivity to the various CLE peptides with regard to the root growth inhibition phenotypes, and identified CLE45 as a putative ligand for this receptor (Depuydt *et al.*, 2013). CLE45 peptide treatments to growing *Arabidopsis* seedlings, or over-expression of a variant of CLE45, result in similar defects to *brx* and *ops*, such as short roots and inhibition of protophloem formation, suggesting that CLE45 is a negative regulator of protophloem cell fate (Depuydt *et al.*, 2013; Rodriguez-Villalon *et al.*, 2014). The mode of action of the CLE45-BAM3 ligand-receptor pair seems to be through inhibiting the sieve element precursor cells to gain the sieve element cell fate and locking them in their current developmental state (Rodriguez-Villalon *et al.*, 2014). Expression analysis revealed that while *BRX*, *BAM3* and *CLE45* are expressed in the developing protophloem, *OPS* is expressed both in the developing protophloem and metaphloem (Depuydt *et al.*, 2013; Rodriguez-Villalon *et al.*, 2014). *BAM3* expression is found to be increased in peptide treated roots, indicating that CLE45 might positively regulate *BAM3* (Depuydt *et al.*, 2013). Furthermore, enhancement of the OPS activity could decrease the effects of CLE45 peptide application in a BRX-dependent manner (Rodriguez-Villalon *et al.*, 2014). *CLE26* encodes a similar peptide to CLE45 (Rodriguez-Villalon *et al.*, 2015). Interestingly, *CLE26* also shows high expression in the protophloem and addition of this peptide to the growth medium of *Arabidopsis* plants causes similar defects as CLE45 peptide applications (Rodriguez-Villalon *et al.*, 2015). It will be exciting to see if these genes have similar roles during the development of the secondary phloem.

1.5.7 CLE10 signaling and protoxylem formation

The CLE 10 peptide is shown to be involved in the regulation of protoxylem development, by regulating the cytokinin response (Kondo *et al.*, 2011). In the *Arabidopsis* genome, the CLE10 peptide is encoded by *CLE9* and *CLE10.* Both genes are highly expressed in the stele of the primary roots. Exogenous application of CLE10 peptide to the growing *Arabidopsis* seedlings as well as the over-expression of *CLE10* in *Arabidopsis* plants hindered the root growth and inhibited the protoxylem formation, suggesting that CLE10 is a negative

regulator of protoxylem cell fate. The CLE10 peptide is involved in the repression of the type-A response regulators *ARR5* and *ARR6,* and as a result enhancement of the cytokinin signaling. Consistent with this, *arr10 arr12* double mutant plants are insensitive to CLE10 peptide treatment in terms of the protoxylem inhibition phenotype. Moreover, the CLV2-CORYNE (CRN) receptor pair is suggested to be the putative receptor for the CLE10 peptide by the genetic evidence (Kondo *et al.*, 2011).

2 Objectives

The main objective of my PhD work was to understand how the vascular cambium identity and activity is regulated on the molecular level. More comprehensively, I wanted to identify which genes regulate the vascular cell divisions and/or cell specification events in the vascular cambium. My special focus was on three gene families: the *CLE* gene family, the *LRR-RLK* gene family and the *WOX* gene family. Throughout my experiments, I used three model plant species, the herb model *Arabidopsis thaliana,* the angiosperm woody model *Populus tremula L.* × *P. tremuloides* Michx (Hybrid aspen), and the gymnosperm woody model *Picea abies* (Norway spruce). Specific goals of my project were:

- To identify potential new LRR-RLKs functioning in vascular development (Paper I)

- To characterize the homologs of *Arabidopsis CLE41*, *CLE44, TDR/PXY*, and *WOX4* genes in tree species and determine their mode of action in the vascular cambium (Paper II and Paper III)

- To identify potential new *CLE* genes functioning in the regulation of vascular cambium identity and activity in trees (Paper IV)

- To summarize and review the current knowledge with regard to the CLE peptide ligands and their signaling modules in plants (Paper V)

3 Results and discussion

3.1 *PXC1* is a regulator of secondary cell wall formation in *Arabidopsis* (Paper I)

As mentioned earlier, recent reports suggest that in the model plant *Arabidopsis thaliana,* LRR-RLKs such as TDR/PXY, MOL1 and RUL1 are key regulators of procambium/vascular cambium activity (Fisher & Turner, 2007; Hirakawa *et al.*, 2008; Etchells & Turner, 2010; Agusti *et al.*, 2011). However, the *Arabidopsis* genome contains over 200 genes that encode LRR-RLK proteins (Shiu & Bleecker, 2001), many of which are currently uncharacterized. In paper I, we addressed this issue and attempted to identify potential new LRR-RLKs, which might be involved in the regulation of procambium/vascular cambium activity and/or the vascular development process. Our strategy in this paper relied on the general assumption that co-expressed genes might be functionally related. Therefore, we first collected the publicly available transcriptional data sets from the GENEVESTIGATOR database (Zimmermann *et al.*, 2004; Hruz *et al.*, 2008), and performed an *in-silico* co-expression analysis for all the *LRR-RLKs* in the *Arabidopsis thaliana* genome. Results showed that the expression of 7 possible candidate genes cluster together with the *TDR/PXY* gene (Paper I, Figure 1, Additional file 1). Interestingly, three of these 7 genes were *MOL1*, *RUL1* and *VASCULAR HIGHWAY1 (VH1)*, all with functions in the regulation of vascular development (Clay & Nelson, 2002; Agusti *et al.*, 2011). While one gene did not display high expression in the vascular tissues, the remaining three, which we designated as *PXY/TDR-correlated* (*PXC*) genes, showed high expression in the vasculature and were mainly uncharacterized.

Next, in order to validate and examine the expression patterns of *PXC* genes further, we generated transgenic *Arabidopsis* plants carrying the *GUS* gene

under the control of the *PXC* promoter. During this experimental part, *TDR/PXY::GUS* lines were also produced as a reference. Results indicated that all *PXC* genes display strong promoter activity in the vascular tissues (Paper I, Figure 2, Figure 3). Although the *GUS* expression patterns of the *PXCs* and *TDR/PXY* in the one-week-old primary roots differed (Paper I, Figure 2D), all genes showed almost overlapping expression patterns in the vascular strands of the cotyledons, shoot apex, hypocotyls and first true leaves during early stages of development (Paper I, Figure 2A-2C). We also checked expression patterns of these genes in the base of 5-week-old inflorescence stems (Paper I, Figure 2E). In the *TDR/PXY::GUS* lines, GUS staining was seen in the procambium cells, in the interfascicular cambial region, and in the protoxylem region of the vascular bundles. This is consistent with previous observations, with the exception of the protoxylem region (Fisher & Turner, 2007; Hirakawa *et al.*, 2008; Etchells & Turner, 2010). For the *PXC1* gene, GUS lines displayed an expression pattern coinciding with the *TDR/PXY* expression, but the protoxylem expression was very faint, and a certain amount of GUS signal was observed in the newly forming xylem cells. For the *PXC2* gene, GUS expression was restricted to the newly arising vessels members in the vascular bundles, while *PXC3* was expressed in the procambium cells and the protoxylem region of the vascular bundles. This GUS analyses showed that our candidate LRR-RLKs might indeed perform functions during the progression of vascular development.

Based on the co-regulation network analysis (Paper I, Figure 1C) and the expression patterns of our candidate genes (Paper I, Figure 1, Figure 2), the strongest predicted association between *TDR/PXY* was with *PXC1*. Hence, in the next step of this work, we turned our attention to the *PXC1* gene and analyzed three T-DNA insertion lines (Paper I, Figure 5A). We monitored the *PXC1* expression in these lines and found that in *pxc1-1* and *pxc1-2* plants the level of *PXC1* expression was only reduced to ≈40% and ≈25% of wild type levels, whereas in *pxc1-3* plants the level of *PXC1* expression was nearly abolished (Paper I, Figure 2B). This indicated that while *pxc1-1,* and *pxc1-2* were weak alleles, *pxc1-3* allele was a null mutant. With respect to overall growth, we identified that the inflorescence stems of the *pxc1* mutants were taller than those of wild type plants (Paper I, Additional file 5), opposed to *tdr/pxy* plants (Fisher & Turner, 2007). When the plants were grown under long day conditions, the anatomy of the inflorescence stems in all *pxc1* lines were indistinguishable from those of wild type plants (Figure 6A, 6B). However when the growth conditions of *pxc1* mutants were altered from long-day conditions to short-day conditions just after bolting to promote secondary

growth, the stems of *pxc1-1* and *pxc1-3* mutants could not maintain a continued upright growth and displayed a pendent phenotype (Paper I, Figure 5C). A more detailed anatomical comparison of the inflorescence stems of the mutants and wild type revealed that two mutant lines showed reduced secondary cell wall thickness and lignification of the xylem cells both in the fascicular and interfascicular regions (Paper I, Figure 5D-5L, Figure 6C, 6D). Consistent with this phenotype, a previous study also showed that a transposon-tagging mutant for the *pxc1* display aberrant leaf morphology associated with defective cell elongation and reduced lignification in the vascular bundles of the leaves (Kim *et al.*, 2009). Organization of the vascular tissues in the mutants was normal, in contrast to the disruption of vascular organization induced by mutations in the *TDR/PXY* gene (Fisher & Turner, 2007; Hirakawa *et al.*, 2008; Etchells & Turner, 2010).

Interestingly, a previous study determined that *SND2* controls the expression of *PXC1* (Hussey *et al.*, 2011). *SND2* expression, in turn, is modulated by *SND1/NST3* (Zhong *et al.*, 2008). As mentioned earlier, SND1/NST3 and NST1 are regulators of xylem fiber cell differentiation (Zhong *et al.*, 2006; Mitsuda *et al.*, 2007). *SND2* expression seems to be preceding the secondary cell wall thickenings both in vessel and fiber cells (Zhong *et al.*, 2008). When SND2 expression is dominantly repressed, plants exhibit reduced secondary cell wall formation in both interfascicular fibers and xylary fibers of the inflorescence stems, in contrast over-expression of *SND2* induces an increase in the secondary wall thickness of these cells (Zhong *et al.*, 2008).

Following the shift from the long-day conditions to short-day conditions, *pxc1* mutants displayed reduced levels of *TDR/PXY* and *WOX4* transcript levels in the inflorescence stems (Paper I, Figure 7B). This might suggest that *PXC1* regulates the expression of *TDR/PXY* and *WOX4* positively. Therefore, in order to understand the functional relationship between *TDR/PXY* and *PXC1,* reciprocal crosses were performed between homozygous *pxc1* and *tdr/pxy* knockout lines, however we were unable to obtain double homozygotes for *pxc1 tdr/pxy* (data not shown), which indicated that a simultaneous loss of function for these genes in plants is lethal. Thus, collectively our results indicated that PXC1 plays a role during secondary cell wall formation and that it controls the maturation of xylem fiber cells, particularly interfascicular fibers. Moreover, PXC1 mediate a pathway that cross talk with the TDIF/CLE41/CLE44-TDR/PXY-WOX4 signaling module.

3.2 *PtWOX4* genes regulate the vascular cambium activity in hybrid aspen (Paper II, Paper III)

Recent studies suggest that in the model plant *Arabidopsis thaliana,* the procambium/vascular cambium activity is mediated by WOX4, a WOX transcription factor and the downstream target of the TDIF/CLE41/CLE44 peptide signaling pathway (Hirakawa *et al.*, 2010). Moreover, evidence suggests that WOX4 and WOX14 might act together whilst regulating the cell divisions of the vascular stem cells (Etchells *et al.*, 2013). However, it is not known whether *WOX* genes also control stem secondary growth in trees, a life strategy, which is absolutely dependent on the formation of a thick trunk in order to outcompete all other plants for access to sunlight. Therefore, in paper II, we isolated and examined the homologs of these genes in *Populus* using functional genomics tools and transgenic approaches.

We first identified the *Populus* homologs of the *Arabidopsis WOX4* gene via genome mining and phylogenetic analysis. Our analysis in this part of the study included protein sequences from several different plant species such as *Arabidopsis thaliana*, *Populus trichocarpa*, *Vitis vinifera*, *Oryza sativa*, *Physcomitrella patens*, *Picea abies, Pinus taeda* and *Amborella trichopoda* (Paper II, Supplemental Figure 1). As WOX proteins are characterized by the presence of conserved homeodomains, these parts of the protein sequences were used to create the phylogenetic tree. Results revealed that the *Populus* genome contains two *WOX4* paralogs, *PtWOX4A* and *PtWOX4B* (Paper II, Supplemental Figure 1). These two paralogs share 97% similarity to each other, reflecting a late duplication event for this gene in the *Populus* lineage.

Next, we monitored the expression pattern of the *PtWOX4* genes in trees, by using diverse experimental methods including the QPCR, *in situ* hybridization, microarray and the use of transgenic *Populus* trees expressing the GUS reporter from the *PtWOX4* promoter (Paper II, Figure 1). Results showed that *PtWOX4* expression was mainly associated with the vascular cambium and the developing xylem in the stems (Paper II, Figure 1A, 1B, 1I-1K). Moreover, this expression followed the establishment of a closed vascular cambium ring, such that the GUS activity was detected in the provascular strands, procambium of the vascular bundles, in the interfascicular cambium and finally in the vascular cambium throughout the rest of development (Paper II, Figure 1E-1J).

In order to study the function of *PtWOX4* genes in hybrid aspen, knockdown plants were generated, in which the expressions of *PtWOX4* paralogs were down regulated using the RNAi approach (Paper II, Figure 2,

Supplemental Figure 2). With respect to primary growth, *PtWOX4 RNAi* plants grew to similar heights as wild type plants (Paper II, Figure 2A, 2C). However with respect to secondary growth, these plants showed a reduced stem thickening such that the plants could not sustain an upright growth without support (Paper II, Figure 2B, 2C). A detailed anatomical analysis revealed that the reduction in the stem thickening was associated mainly with a reduction of secondary xylem rather than of secondary phloem (Paper II, Figure 2D, 2H, 2I). Moreover, the secondary xylem in the stems of the RNAi plants often displayed large gaps. The vascular cambium of the *PtWOX4 RNAi* plants displayed fewer cells in comparison to wild type plants and this was related to the reduced frequency of both periclinal divisions and anticlinal divisions of the vascular cambium cells (Paper II, Figure 2E-2G). As formation of the new xylem and phloem cells is related to the periclinal divisions of the vascular cambium mother cells, our results collectively argues that *PtWOX4* is a positive regulator of cambial activity, and possibly that of the vascular cambium xylem mother cells, in hybrid aspen. Moreover, in contrast to *Arabidopsis* (Hirakawa *et al.*, 2010), *PtWOX4* might also act as a promoter of vascular cambium identity.

During the course of this project, the *Populus* homologs of the *Arabidopsis CLE41* and *TDR/PXY* genes were also identified (Paper II, Supplemental Figure 3, Supplemental Figure 4). Phylogenetic analysis revealed that the *Populus* genome contains two gene models similar to *TDR/PXY*: *PtPXYA* and *PtPXYB;* and 6 gene models similar to *CLE41* and *CLE44: PtCLE41A, PtCLE41B, PtCLE41C, PtCLE41D, Potri.001G075200.1* and *Potri.003G156000.1* (Paper II, Supplemental Figure 3, Supplemental Figure 4). These genes were expressed similarly to *Arabidopsis CLE41*, *CLE44* and *TDR/PXY* (Hirakawa *et al.*, 2008; Etchells & Turner, 2010), such that the transcripts of *PtCLE41* genes were more abundant in the phloem-cambium region of the stems, while *PtPXY* genes were highly expressed in the vascular cambium and the developing xylem (Paper II, Figure 3).

To elucidate the functions of the *PtCLE41A, PtCLE41C* and *PtCLE41D* genes in hybrid aspen, we initially suppressed the expression of the putative paralogs (*PtCLE41A/PtCLE41B* and *PtCLE41C/PtCLE41D*) via RNAi. However although the expressions of these genes were down regulated, transgenic trees grew as wild type trees (data not shown), most probably due to redundancy between the gene family members. Therefore, we constitutively over-expressed them under the control of the 35S promoter (Paper II, Figure 4, Supplemental Figure 5, Supplemental Figure 6). Results showed that over-

expression *PtCLE41A* and the related *PtCLE41C,* and *PtCLE41D* genes caused dwarf phenotypes in *Populus* (Paper II, Figure 4A, Supplemental Figure 6A, 6B). On the anatomical level, these plants displayed defects in the organizations of the vascular tissues where often a xylem intermixed with phloem phenotype was observed in the stems, accompanied with the formation of ectopic xylem islands (Paper II, Figure 4C, 4D, Supplemental Figure 6C-6E). Therefore, we concluded that *PtCLE41* genes regulate woody tissue organization in hybrid aspen. However, due to the severity of the observed phenotypes, it was not possible to quantify the number of cambial cells to determine if *PtCLE41*-like genes also stimulate the cambial cell division activity. These results are in agreement with a recently published report, where over-expression of *PtCLE41A* (previously reported as *PttCLE41*) caused similar phenotypes in hybrid aspen (Etchells *et al.*, 2015). In addition, we also identified that *PtCLE41A*, *PtCLE41C* and *PtCLE41D* genes might positively regulate the expression of *PtWOX4* and *PtPXYA* in hybrid aspen since the transcript of these genes were more abundant in the stems of the over-expression lines (Paper II, Figure 4B, Supplemental Figure 6H, 6I). Thus, we concluded that a signaling module involving similar components as in *Arabidopsis*, functions during the regulation of vascular cambium identity and activity in *Populus*. However, although most aspects of this regulation appear to be similar, there are also differences. For instance, the positive regulation of the receptor gene *PtPXYA* by PtCLE41A and related peptides seems to be opposite to what has been described in *Arabidopsis* (Hirakawa *et al.*, 2008; Etchells & Turner, 2010; Hirakawa *et al.*, 2010).

We also studied the expression of *PtWOX4* genes under conditions mimicking the growth cessation, dormancy and reactivation cycle of trees. We could show that *PtWOX4* expression was correlated with active growth of the vascular cambium (Paper II, Figure 1M-1P). Moreover, this could be related to the modulation of auxin responses affecting the upstream genes of *PtWOX4* such as *PtPXYA* and *PtCLE41B* during the annual growth cycle (Paper II, Figure 6C-6E).

Taking advantage of the Norway spruce draft genome (Paper III), in paper II we also tried to understand the evolution of the function of these gene families. Results indicated that in the genome of *Picea abies PaCLE41A, PaCLE41B, PaPXY* and *PaWOX4* are the genes likely to encode for the CLE41, TDR/PXY and WOX4-like proteins, respectively (Paper II, Supplemental Figure 1, Supplemental Figure 3, Supplemental Figure 4). QPCR analysis for these genes in different tissues of Norway spruce implied that the

TDIF/CLE41/CLE44-TDR/PXY-WOX4 pathway is an evolutionarily conserved program for the regulation of vascular cambium activity between herbaceous plants, angiosperm and gymnosperm tree species (Paper II, Figure 5).

Finally, as the evidence argues for redundant functions of the WOX4 and WOX14 transcription factors in the control of vascular cell divisions in *Arabidopsis* (Hirakawa *et al.*, 2010; Etchells *et al.*, 2013), I also checked the *Populus* genome for the existence of similar genes (Paper II, Supplemental Figure 1). Results showed that the *Populus* genome harbour similar genes to *WOX13* instead of *WOX14,* which resides in the same phylogenetic clade as *WOX14* (Paper II, Supplemental Figure 1). I inspected the expression patterns of these genes *in silico* using the ASPWOOD database (http://aspwood.popgenie.org) (Figure 4). This database presents RNA-seq transcriptional profiling of the genes expressed in the wood-forming zone of *Populus tremula* trees. Interestingly, *Populus WOX13*-like genes are expressed differentially in the wood-forming zone of *Populus* (Figure 4), which might implicate a role also for these genes during the regulation of cambial activity or later stages of the wood formation.

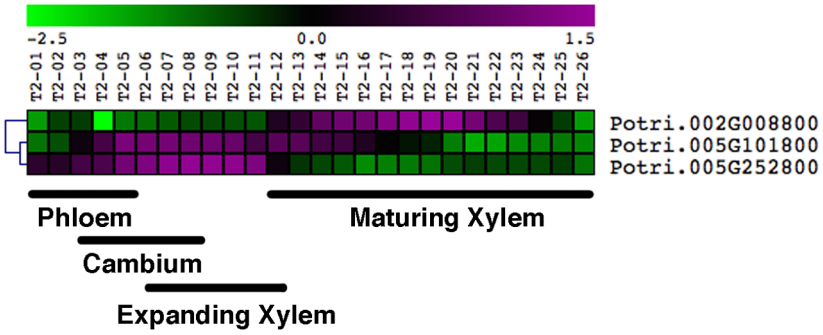

Figure 4: Heat-map of the normalized and scaled RNA-seq data for the *Populus WOX13*-like genes obtained from the ASPWOOD database (http://aspwood.popgenie.org). Hierarchical clustering analysis was performed by the TIGR Multiexperiment Viewer (MeV) suite (http://www.tm4.org/mev.htmls) suite using the Euclidian distance metric (Bailey *et al.*, 2009). T2-NN signifies each tangential section along the wood-forming zone coming from the second tree as a representative of all five trees. Corresponding vascular regions are marked at the bottom of the heat-map.

3.3 The Christmas tree as a model organism in plant biology (Paper III)

Paper III describes the sequencing strategy of the Norway spruce (*Picea abies*) genome and its draft assembly. The sequenced individual was a 43-year-old copy of the *P. abies* clone Z4006, which originally came from central Sweden. The sequencing strategy involved a whole haploid and diploid genome shotgun sequencing in combination with shotgun sequencing of fosmid libraries using next generation sequencing platforms. For the assembly, a hierarchical genome assembly strategy was employed for all the whole genome shotgun data together with RNA-seq data. This was followed by *ab initio* prediction of protein-coding genes. The main new insights that were gained from the analysis of the genome organization and architecture of Norway spruce include a big genome size (20Gbp), 28354 well-supported gene models, several long (>10000 base pairs) introns and the existence of numerous pseudo genes and copious repetitive DNA sequences.

Coniferous trees are evolutionarily ancient and ecologically important members of boreal forests. They have a wide distribution over the northern hemisphere and the Norway spruce together with the Scots pine (*Pinus sylvestris*) represents the two most ecologically and economically important species in Sweden. They also display unique growth and development habits in comparison to angiosperm species in terms of i.e. reproduction, seed production, evolution, adaptation and cold tolerance, wood formation, and composition of the polymers within secondary cell walls. Therefore, the availability of genome sequences from gymnosperm species including the Norway spruce (Paper III), loblolly pine (*Pinus taeda*) (Neale *et al.*, 2014), and white spruce (*Picea glauca*) (Birol *et al.*, 2013) now opens up new possibilities to study these key traits and facilitate the comparative analyses of plant genomes, evolution and annual and perennial life strategies. Moreover, it will allow the forestry industry and tree breeders to develop new tools and strategies for improved productivity, health and quality of trees.

3.4 *PtLCLE* genes regulate growth and development in hybrid aspen (Paper IV)

Numerous studies report that CLE peptide-encoding genes exist in the genomes of plants from different lineages such as monocots (*Oryza sativa* and *Zea mays*), dicots (*Arabidopsis thaliana, Glycine max, Populus trichocarpa* and *Medicago truncatula*), green algae (*Chlamydomonas reinhardtii*), moss (*Physcomitrella patens*), lycopods (*Selaginella moellendorffii*) and conifers (*Picea abies and Picea glauca*) (Oelkers *et al.*, 2008; Miwa *et al.*, 2009; Strabala *et al.*, 2014; Hastwell *et al.*, 2015). However, in plant species other than *Arabidopsis* information with respect to the roles of these CLEs is limited. Thus, in paper IV, we attempted to identify and functionally characterize the members of the CLE peptide family in *Populus* (*Populus trichocarpa* Like-CLE, PtLCLE), which might be involved in the regulation of vascular cambium activity and/or the vascular development process. For this reason, we first identified the *Populus* homologs of *Arabidopsis CLE* genes including the closest homologs of the most known members such as *CLV3*, *CLE40*, and *CLE41* via phylogenetic analysis (Paper IV, Figure 1, Supplemental Figure 1).

In order to determine candidate *PtLCLEs* that might function in the regulation of vascular cambium activity or in different aspects of the wood formation process, their respective expression patterns were assessed via an *in silico* analysis approach (Paper IV, Figure 2). Results indicated that a number of *PtLCLE* genes were expressed in the wood-forming zone of aspen with different tissue specificities such as secondary phloem, vascular cambium, expanding xylem and mature xylem tissues (Paper IV, Figure 2). By using this approach, we chose two *PtLCLE* genes, *PtLCLE1* and *PtLCLE2A* that display preferential expression in the wood-forming zone of *Populus,* and studied their functions using transgenic approaches in hybrid aspen.

The first selected candidate gene that we selected was *PtLCLE1,* which was expressed preferentially in the cambial zone (Paper IV, Figure 2). We further examined the *PtLCLE1* expression in different tissues of hybrid aspen and found that *PtLCLE1* was highly expressed in the phloem-cambium region of the stem, young leaves and roots (Paper IV, Supplemental Figure 2A). In order to dissect the role of the *PtLCLE1* gene during secondary development, we suppressed its expression in transgenic *Populus* trees using the RNAi method (Paper IV, Figure 3). As there is no paralog of this gene in the *Populus* genome (Paper IV, Supplemental Figure 1), the RNAi construct was designed to specifically suppress the expression of the *PtLCLE1* gene. With regard to overall growth, *PtLCLE1 RNAi* plants displayed reductions in height-growth

and stem thickness (Paper IV, Figure 3B-3D), as well as smaller leaf sizes comparing to wild type plants (Paper IV, Figure 5). However in terms of internode number no significant differences were observed (Paper IV, Figure 3E), thus, the decrease in stem height was associated with changes in the internode lengths. Anatomical observations revealed that *RNAi* plants have reduced secondary xylem production in their stems (Paper IV, Figure 4), indicating that *PtLCLE1* might positively regulate the vascular cambium activity, and possibly the periclinal divisions of the xylem mother cells. Moreover, it is also involved in the control of internode elongation and leaf size. The observed phenotypes are opposite to those seen in transgenic *Populus* trees over-expressing a *gibberellin 20-oxidase* gene such as longer plants with elongated internode lengths and broader leaves (Eriksson *et al.*, 2000). This suggests a possible crosstalk or interaction with gibberellin signaling or metabolism, which needs to be investigated further.

The second candidate gene that we selected was *PtLCLE2A,* which is expressed specifically in the maturing secondary xylem (Paper IV, Figure 2). We examined the expression of the *PtLCLE2A* gene and its paralog *PtLCLE2A* in more detail and found that *PttLCLE2* genes are mainly expressed in the roots and young leaves of hybrid aspen trees (Paper IV, Supplemental Figure 2B, 2C). In order to investigate the roles of the *PtLCLE2* paralogs in hybrid aspen, we employed both gene silencing and over-expression approaches (Paper IV, Figure 6, Figure 7). The most significant phenotypes we observed in *RNAi* plants and the over-expresser plants were in terms of height growth, where *PtLCLE2 RNAi* plants were longer than wild type plants while *35S::PtLCLE2A* plants were shorter than wild type plants (Paper IV, Figure 6B, 6C, Figure 7B, 7C). This phenotype proved to be associated partially with changes in internode lengths and partially with changes in the leaf initiation rate at the shoot apex (Paper IV, Figure 6E, Figure 7E). Therefore, we concluded that *PtLCLE2* genes might negatively regulate primary growth in hybrid aspen. Moreover, as the *PtLCLE2* genes were highly expressed in the roots (Paper IV, Supplemental Figure 2B, 2C), we also examined the formation of adventitious roots in our transgenic lines (Paper IV, Figure 10). Results suggested that the *PtLCLE2* genes might also inhibit the formation and growth of adventitious roots (Paper IV, Figure 8).

4 Conclusions and future perspectives

The accumulation of biomass in forest trees mainly relies on the activity of the vascular cambium. Therefore, understanding the principles of stem cell maintenance and activity in this meristematic tissue is fundamentally important. My thesis and its findings contribute to the knowledge on the regulation of the vascular cambium identity and activity by examining the functions of different candidate genes identified by bioinformatics analyses. During the course of this work, I mainly concentrated on three different gene families, specifically the *CLE*, *LRR-RLK* and *WOX* genes:

I described a new potential *Arabidopsis* LRR-RLK, PXC1, which functions, partially in connection with the TDR/PXY signaling module, during secondary cell wall formation, possibly in xylem fiber cells. As LRR-RLKs interact with CLE peptide ligands to regulate diverse biological and developmental processes (Hirakawa *et al.*, 2008; Ohyama *et al.*, 2008; Ohyama *et al.*, 2009), it would be interesting to identify the possible peptide ligands for this receptor and the origin of these ligands. Characterization of upstream regulators of *PXC1* and its downstream targets would be other avenues for research. Moreover, to isolate and characterize the homologs of these genes in other plants, such as trees, would also be informative. This study also suggests other so far uncharacterized genes, such as *PXC2* and *PXC3* (Paper I, Figure 1, Additional file 1), which are co-expressed with the cambium regulator *TDR/PXY* (Hirakawa *et al.*, 2008; Etchells & Turner, 2010) and therefore might have roles in vascular development.

I also identified and characterized the components of the TDIF/CLE41/CLE44-TDR/PXY-WOX4 signaling pathway in hybrid aspen. I demonstrated that *PtCLE41*-like genes, *PtPXY* genes and *PtWOX4* genes are regulators of cambium activity in *Populus*. My results also provided evidence

that the seasonal regulation of vascular cambium activity might be through the regulation of these genes in connection to auxin signaling. Moreover, my results also suggested that the role of these genes in the regulation of secondary growth is evolutionary conserved and probably predates the angiosperm/gymnosperm split, since the expression pattern of the regulatory modules are essentially the same in *Arabidopsis, Populus* and Norway spruce. As a next step, it would be interesting to observe and characterize if *WOX13*-like genes also participate in the regulation of vascular cambium identity or activity in trees. Moreover, cytokinins are important regulators of vascular cambium activity (Nieminen *et al.*, 2008), but the connection between the TDIF/CLE41/CLE44-TDR/PXY-WOX4 signaling module and cytokinin signaling has, to my knowledge, not been studied and might provide interesting insights.

I also identified a group of *Populus CLE (PtLCLE)* genes that are specifically expressed over the wood-forming zone of hybrid aspen. I showed that *PtLCLE1* might be involved in the regulation of cambium cell proliferation, more specifically vascular cambium xylem mother cells, internode elongation and leaf size regulation. I also disclosed that the *PtLCLE2* genes might participate in the regulation of primary growth and adventitious rooting in trees. A deep understanding of how these genes function together with their interacting receptors is necessary in the future. Moreover, there are a number of other *PtLCLE* genes that are expressed highly and differentially in the wood-forming zone (Paper IV, Figure 2), and these genes provide interesting new candidates for future studies, since they could play roles at different stages of the vascular development and wood formation. In this respect, it would be interesting to identify the genes that are regulating the phloem mother cells. By analogy to *PtCLE41A* and *PtPXYA*, it would not be illogical to speculate about the existence of a similar mechanism where phloem mother cells could be regulated via one of these *PtLCLE* genes, maybe expressed in the developing xylem. Moreover, due to the high number of *PtLCLE* genes expressed over the wood-forming zone in trees, one could also expect a highly complex regulatory system for the vascular development and the wood formation process, where different PtLCLEs may interact with different receptor complexes and cross talk with different hormones.

In the future, an important and interesting approach would also be the translation of the knowledge identified here to biotechnology applications. For instance, a recent study revealed that by co-expression of *PtCLE41A* and its receptor *PtPXYA* under the control of tissue-specific promoters, one could

dramatically increase both primary and secondary growth in *Populus* trees (Etchells *et al.*, 2015). This indicates that manipulating genes involved in the regulation of vascular cambium could be a viable approach to increase biomass production in trees. However, it will be important to test these genetically modified trees in the field, and observe how well they perform against biotic and abiotic stresses. If successful, these types of approaches might help to increase the productivity of forests, allowing the growth of more woody biomass on less land.

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