Cellular Aspects of Lignin Biosynthesis in Xylem Vessels of Zinnia and Arabidopsis

Henrik Serk
Front Cover: 6-weeks old greenhouse grown *Arabidopsis thaliana* plant; Lignin UV autofluorescence of an *Arabidopsis* stem cross-section and a tracheary element from *Arabidopsis in vitro* cultures; molecular structure of lignin with monomer radical.
(by Henrik Serk)

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Umeå, Sweden 2015
To my dear family and friends.

“Always do your best. What you plant now, you will harvest later.”
(Og Mandino, American Author, 1923 – 1996).

“It does not matter how slowly you go as long as you do not stop.”
(Confucius, Chinese Philosopher, 551 BC – 479 BC).
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Abstract

Lignin is the second most abundant biopolymer on earth and is found in the wood (xylem) of vascular land plants. To transport the hydro-mineral sap, xylem forms specialized conduit cells, called tracheary elements (TEs), which are hollow dead cylinders reinforced with lateral secondary cell walls (SCW). These SCWs incorporate lignin to gain mechanical strength, water impermeability and resistance against pathogens. The aim of this thesis is to understand the spatio-temporal deposition of lignin during TE differentiation and the relationship with its neighbouring cells. *In vitro* TE differentiating cell cultures of *Zinnia elegans* and *Arabidopsis thaliana* are ideal tools to study this process: cells differentiate simultaneously into 30-50% TEs while the rest remain parenchymatic (non-TEs). Live-cell imaging of such TEs indicated that lignification occurs after programmed cell death (PCD), in a non-cell autonomous manner, in which the non-TEs provide the lignin monomers.

This thesis confirms that lignification occurs and continues long after TE PCD in both *in vitro* TE cultures and whole plants using biochemical, pharmacological and cytological methods. The cooperative supply of lignin monomers by the non-TEs was demonstrated by using *Zinnia* and *Arabidopsis in vitro* TE cultures. Inhibitor experiments revealed further that the non-TEs supply reactive oxygen species (ROS) to TEs and that ROS are required for TE post-mortem lignification. Characterization of the non-TEs showed an enlarged nucleus with increased DNA content, thus indicating that non-TEs are in fact endoreplicated xylem parenchyma cells (XP). The cooperative lignification was confirmed in whole plants by using knock-out mutants in a lignin monomer synthesis gene, which exhibit reduced TE lignification. The XP specific complementation of these mutants led to nearly completely rescuing the TE lignin reduction. Using microscopic techniques, the spatial distribution of lignin was analyzed in TEs from *in vitro* cultures and whole plants, revealing that lignification is restricted to TE SCWs in both protoxylem and metaxylem. These specific deposition domains were established by phenoloxidases, *i.e.* laccases localized to SCWs and peroxidases, present in SCWs and the apoplastic space. Laccases were cell-autonomously produced by developing TEs, indicating that the deposition domains are defined before PCD.

Altogether, these results highlight that the hydro-mineral sap transport through TEs is enabled by the spatially and temporally controlled lignification of the SCW. Lignification occurs post-mortem by the supply of monomers and ROS from neighbouring XP cells and is restricted to specific deposition domains, defined by the pre-mortem sequestration of phenoloxidases.
Sammanfattning


Sammantaget visar resultaten att vätsketransporten genom KE möjliggörs av spatialt och temporalt reglerad SCV lignifiering. Lignifieringen inträffar efter celldöden, genom tillförsel av monomerer och ROS från angränsande XP och begränsas till vissa depositionsdomäner som definieras av pre-mortem fenoloxidas produktion.
Preface

Xylem or wood is the major tissue of higher vascular land plants and exhibits a porous structure which is primarily composed of cellulose, other structural polysaccharides and lignin. The specific structure and composition gives wood its remarkable properties, consequently wood has become a valuable material in human history mostly for the usage as energy source or as building material. Today the utilization of wood as energy source and construction material still persists while it is also used for paper production and increasingly as source for biofuels. In the pulp and paper industry lignin reduces the processability of wood dramatically due to the need of additional extraction and bleaching steps for the elimination of lignin. The conversion of wood into biofuels is based on the saccharification of the cellulose into sugars and the fermentation of the sugars into ethanol. In this process, lignin has also initially been considered as a waste product that restricts the effective conversion of cellulose, while its value is nowadays increasing through the utilization, for example as concrete or asphalt additive, as antioxidant in the food industry or as source for carbon fiber synthesis.

Understanding lignin biosynthesis is of major importance for the efficient conversion of cellulose into biofuels as well as for the optimization of lignin properties for its different applications and lastly to improve the production of pulp and paper. The modulation of lignin formation using genetic or classical breeding tools has shown to improve the lignin polymer properties and thereby the productivity of the industrial processes mentioned above. One example is the improved hydrolytic cleavage through the increased presence of non-condensed bonds in the lignin polymer, resulting in the enhanced lignin extractability. Nevertheless, changes in lignin polymer properties generally lead to the disturbance of the overall tree physiology that causes growth deficiencies, which in turn results in reduced biomass yields. This problem could be solved by the modulation of the spatio-temporal deposition of lignin for example by maintaining natural lignin properties only in specific subcellular compartments and/or specific cell types that are important for the overall development of the plants. Xylem vessels are essential to distribute the hydro-mineral sap to lateral organs of the plant. In this thesis xylem parenchyma cells have been demonstrated to contribute to xylem vessel lignification and appear to be good candidates for the controlled adjustment of the lignin properties.
# List of Abbreviations

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<tr>
<td>ABC</td>
<td>ATP-binding cassette-like</td>
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<tr>
<td>Arabidopsis</td>
<td>Arabidopsis thaliana</td>
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<td>CAT</td>
<td>Catalase</td>
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<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
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<td>CTAB</td>
<td>Cetyltrimethylammonium bromide</td>
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<tr>
<td>DDC</td>
<td>Diethylthiocarbamate</td>
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<tr>
<td>DHP</td>
<td>Dehydrogenative polymer</td>
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<td>DPI</td>
<td>Diphenylene iodonium</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>FT-IR</td>
<td>Furrier-transform infrared</td>
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<tr>
<td>FDA</td>
<td>Fluorescein diacetate</td>
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<td>G</td>
<td>Guaiacyl</td>
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<td>G</td>
<td>Gosslingia</td>
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<td>GUS</td>
<td>β-glucoronidase</td>
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<td>H</td>
<td>p-hydroxyphenyl</td>
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<tr>
<td>HL</td>
<td>Hydroxylamine</td>
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<tr>
<td>IF</td>
<td>Interfascicular fiber</td>
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<tr>
<td>irx</td>
<td>Irregular xylem</td>
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<tr>
<td>LAC</td>
<td>Laccase</td>
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<tr>
<td>NADPH</td>
<td>Nicotineamide adenine dinucleotide phosphate hydrogen</td>
</tr>
<tr>
<td>NBD-CA</td>
<td>γ-nitrobenzofuran-tagged coniferyl alcohol</td>
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<tr>
<td>P</td>
<td>Psilophyton</td>
</tr>
<tr>
<td>PA</td>
<td>Piperonylic acid</td>
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<tr>
<td>PCD</td>
<td>Programmed cell death</td>
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<td>PCW</td>
<td>Primary cell wall</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>PRX</td>
<td>Peroxidase</td>
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<td>S</td>
<td>Syringyl</td>
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<tr>
<td>S</td>
<td>Sennicaulis</td>
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<tr>
<td>SCW</td>
<td>Secondary cell wall</td>
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<td>SHAM</td>
<td>Salicylhydroxamic acid</td>
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<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
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<td>STS</td>
<td>Silver thiosulfate</td>
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<td>SSH</td>
<td>Suppression subtractive hybridization</td>
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<td>TE</td>
<td>Tracheary element</td>
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<td>TEM</td>
<td>Transmission electron microscopy</td>
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<td>XP</td>
<td>Xylem parenchyma</td>
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<td>XF</td>
<td>Xylary fiber</td>
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<td>Zinnia</td>
<td>Zinnia elegans</td>
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*Other abbreviations are explained in the text, when they first appear.*
List of Publications

Paper I

Paper II

Paper III

*These authors contributed equally to the manuscript.

Paper IV

*These authors contributed equally to the manuscript.

In the following text the papers will be referred to by their roman numbers.

Related publications not included in this thesis:

Contributions as an Author

Paper I
In Paper I Henrik Serk contributed by performing promoter::GUS expression analysis of xylem parenchyma specific genes in *Arabidopsis* stems and hypocotyls as well as phloroglucinol-HCl staining of *Zinnia elegans* sections from different internodes of the stem.

Paper II
For Paper II Henrik Serk wrote the first draft, designed the figures and performed promoter::GUS expression analysis of xylem parenchyma specific genes in *Arabidopsis* seedlings.

Paper III
In Paper III Henrik Serk contributed by performing serial sectioning and histological measurements of lignin and cellulose of the shoot apex of *Zinnia elegans* using confocal microscopy and by the characterization of lignin in TE differentiating cultures treated with different phenoloxidase inhibitors using FT-IR spectroscopy. Henrik Serk also contributed to the revision of the initial draft and editing of the figures.

Paper IV
In Paper IV Henrik Serk performed the characterization and analysis of the *Arabidopsis in vitro* TE differentiation time course using biochemical and cytological methods, RT-qPCR expression analysis along the time course, measurements of promoter::GUS expression in seedlings, analysis of promoter::GUS expression in whole plants, complementation of mutants and characterization of the phenotype and lignin by pyrolysis-GC/MS, TGA lignin and confocal microscopy, as well as genotyping of the complemented mutants and confocal imaging of H2B-RFP expressing cell culture lines. Henrik Serk also contributed to the revision of the first draft and editing of the figures.

Related publications not included in this thesis:

For the review article Henrik Serk contributed by making the figures and revising the text.
Introduction

Higher plants (tracheophytes) have developed a vascular system to allow the upright growth for the competition for sunlight against other photosynthetic organisms. The vascular system transports water, hormones and mineral nutrients, i.e. the hydro-mineral sap (Ye, 2002; Pesquet and Ménard, 2015), from the roots to the leaves, where they are required for photosynthesis: the production of energy rich carbohydrates from carbon dioxide (CO₂). These organic nutrients as well as hormones, proteins and mRNA required for cell growth and differentiation are then transported from the leaves to other sink tissues within the plant body (Ye, 2002). The tissue that transports the hydro-mineral sap is called xylem (Greek: xylos, wood), whereas the tissue that transports the photosynthetic products is called phloem (Greek: phloios, bark). In the xylem, transport is maintained by a negative pressure and thus occurs passively through dead hollow cells, called tracheary elements (TEs), which are divided in two morphological classes: tracheids and vessel elements (Carlquist, 2013). In the phloem, transport is associated to a positive pressure resulting from the active transport of living cells, called sieve elements (De Schepper et al., 2013). The structural organization of xylem and phloem depends on the type of species: dicotyledonous angiosperm (flowering) plants that do not undergo secondary/radial growth per se (mostly annual plants) are usually composed of radially arranged vascular bundles with phloem on the outside and xylem on the inside, occasionally interconnected by interfascicular fibers (IFs). Monocotyledonous angiosperms do not have secondary growth, with some exceptions (e.g. Cordyline terminalis), and exhibit a scattered distribution of vascular bundles within the stem (Jura-Morawiec et al., 2015). Gymnosperm (naked seeded) plants and other dicotyledonous angiosperms that experience secondary growth (e.g. woody species) generally form a continuous cylinder of xylem surrounded by phloem on the outside. While in plants without secondary growth, the central stem is filled with parenchyma cells (ground tissue), plants with secondary growth only exhibit arrays of parenchyma cells that interconnect phloem and xylem to permit the exchange of nutrients and other substrates between these two tissues (Esau, 1965). The xylem or wood which makes up the majority of the plant biomass in trees composes almost entirely of cell types with secondary cell walls (SCWs) (i.e. TEs and fibers). Thus the chemical composition of xylem is represented by the SCW composition which contains polysaccharides such as cellulose (45%) and hemicellulose (25%), the polyphenolic polymer lignin (25%) as well as minor proportions of pectin and proteins (Sjöström, 1993). This composition gives wood its unique properties and together with the sustainable production of wood, provides high economic value.
1. Xylem formation

Plant growth is divided into two stages: primary growth and secondary growth. During primary or tip growth, the shoot or root apical meristem, which contains pluripotent stem cells, produces gradually the vascular stem cells, *i.e.* the procambium (Miyashima *et al.*, 2013). The procambium gives further rise to the secondary meristems: the vascular cambium and the cork cambium, which are involved in later stages of plant development and result in secondary or lateral growth. Plants that do not have secondary growth may also develop an interfascicular cambium from the procambium, which interconnects the vascular bundles and produces IFs. Primary xylem and primary phloem differentiate from the procambium, or more specifically from the primordial fusiform initials, which differentiate into xylem and phloem mother cells that are the precursors of the different cell types observed in phloem and xylem (Lachaud *et al.*, 1999). Primary xylem is distinguished into two developmental stages of the plant: the protoxylem which forms during early stages of vascular development and the metaxylem which forms during later stages. Consequently, protoxylem and metaxylem vessels have distinct morphological features. Protoxylem vessels usually die and complete their differentiation before elongation ceases, they develop annular or spiral SCW thickenings that allow the adaptation to the rapid elongation of young tissue by being pulled apart, like a stretching spring (Esau, 1965). Metaxylem on the other hand develops later, when the plant organ is fully elongated and has reticulated or pitted SCW patterns, which provide more strength and less elasticity. Once primary growth is completed, secondary growth may take over, depending on the plant species. Secondary growth, typically observed in perennial species such as trees, is responsible for the formation of xylem or wood. During secondary growth, xylem cells differentiate from the vascular cambium and align with the earlier formed protoxylem and metaxylem vessels. The vascular cambium contains two types of meristematic cells: the fusiform initials and the ray initials (Mauseth, 2014). Fusiform initials are vertically elongated and produce xylem cells towards the center of the stem, the pith, and phloem cells towards the outside. Ray initials on the other hand, are isodiametric cells that form files of ray parenchyma cells, stretching radially through xylem and phloem. The cambial activity defines the proportion of xylem and phloem, hence an accelerated cambial cell division towards the pith of the stem causes that the stem is primarily composed of xylem compared to the smaller phloem layer surrounding the xylem (Nieminen *et al.*, 2012).
2. Xylem cellular composition

Xylem and phloem are composed of different cell types with different morphological and functional characteristics. In general, xylem contains three different cell types: the sap conducting TEs, xylary fibers (XF) for mechanical support, and living xylem parenchyma (XP) cells that provide nutrients and other substrates (Figure 1). These different cell types are described in detail below.

*Figure 1. Anatomy and cell types of Arabidopsis vascular bundle. A, transverse section of 8-weeks old stem stained by phloroglucinol-HCl (lignin stain) indicating different tissues and cell types: ep, epidermis; co, cortex; ph, phloem; ca, cambium; mx, metaxylem; px, protoxylem; XF, xylary fiber; XP, xylem parenchyma; TE, tracheary element; IF interfascicular fiber. B, different xylem cell types obtained by maceration of Arabidopsis stems: px-TE, protoxylem tracheary element; mx, metaxylem tracheary element. Scale bars: 100 µm.*

*Tracheary elements:* TEs are hydro-mineral sap conducting sclerenchyma cells that maintain mechanical integrity and support during sap conduction (Mauseth, 2014). In order to allow the sap to flow, these cells die and lose their cell content during differentiation to form a hollow tube (Groover *et al.*, 1997). TEs further need to reinforce their lateral walls by forming lignified SCWs to withstand the negative pressure associated to the sap conduction. The two types of TEs differ in morphological appearance: tracheids are narrow elongated cells, while vessel elements are shorter and wider in diameter. Both types of TEs can develop spiral, helical, reticulated or pitted SCW patterns (Carlquist, 2013). The presence of tracheids or vessels depends on the plant species. In angiosperms the xylem comprises of both tracheids and vessel elements, whereas gymnosperms are only composed of tracheids, except for plants of the order Gnetales, which also contain vessel elements (Karam, 2005). To generate a continuous conduit that maintains the sap transport, TEs
assemble end-to-end. Tracheids are connected by bordered pits (pit-pairs), which are membrane-like cavities in the lateral walls at both terminal ends that allow the permeation of the sap but prevent the spread of pathogens and cavitation (Choat et al., 2008; Sperry, 2003). The pit membrane of gymnosperm tracheids exhibits further a central thickening, known as torus-margo, dislocation of the torus-margo causes closing of the pit cavity and thus prevents the progression of cavitation (Taiz and Zaiger, 2010). Vessel elements, on the other hand, assemble end-to-end by the formation of terminal perforation plates that permit the passage of the sap. In contrast to pits, perforation plates are openings without membrane barrier and exhibit different shapes, such as simple (one pore), scalariform (ladder-like), foraminate (several pores), and reticulate (net-like), with simple being the most common structure (Esau, 1965). The water transport through TEs is enabled by the cohesion-tension mechanism which relies on the cohesion of water and the tension resulting from the decreasing water potential along the vertical axis of the plant, generated by transpiration and the osmotic potential of the tissue (Tyree and Ewers, 1991; Steudle et al., 2001). In addition, there is a lateral flow of water through the unthickened side walls of TEs to adjacent XP cells, contributing to the negative water potential (Ménard and Pesquet, 2015).

**Xylary fibers:** XFs are another type of sclerenchyma, which function is essentially to provide mechanical resistance against gravity and environmental disturbances to the plant body. They are thin elongated cells with thick lignified SCWs that provide mechanical strength. Contrary to TEs, XF remain alive after differentiation in order to maintain storage capacity of starch, lipids or other nutrients (Esau, 1965). XFs are further divided into two classes: the libriform fibers, which resemble phloem fibers, and the fiber-tracheids, which are intermediates of libriform fibers and tracheids. These two types are morphological very similar but differ in the type and distribution of pits: simple pits dominate in libriform fibers, while pit-pairs are more common in fiber-tracheids (Esau, 1965).

**Xylem parenchyma:** thin-walled XP cells belong to the ground tissue and have the function to store and transport water, minerals, signaling molecules, organic nutrients such as sugars, starch and lipids as well as nitrogen in form of proteins (Ye, 2002; Spicer, 2014). To permit the exchange of such substances, XP have large amounts of pits in their lateral walls through which they are connected to TEs. This connection allows XP to modify the TEs’ sap by loading or unloading minerals and signaling molecules, *i.e.* hormones, as well as to refill embolized TEs with water (Ménard and Pesquet, 2015). In this regard XP are suggested to change the sap flow rate by altering the pH and ionic strength according to environmental changes. In secondary xylem, XP
have been reported to prevent pathogen invasion through the vascular system by excreting phenolic compounds and by filling the vascular conduits with tyloses, gums or resins (Tyree and Ewers, 1991). XP are further connected with each other through plasmodesmata, to allow the symplastic transport of nutrients between XP (Botha et al., 2008). In plants that undergo secondary growth this connection allows the nutrient transport from the phloem into the xylem and vice versa (Spicer, 2014). Anatomically, XP differ from TEs and fibers due to the absence of a lignified SCW, although XP may form some kind of SCW (also known as amorphous layer), which is however much thinner compared to TE and fiber SCWs and contains less lignin (Spicer, 2014). In woody plants, XP cells can form vertical strands (i.e. axial parenchyma), which derive from elongated fusiform initials in the cambium, or horizontal rays (i.e. ray cells) which derive from shorter ray initials of the cambium (Esau, 1965). Together, axial and ray parenchyma cells form a network that allows the exchange of nutrients between phloem and xylem (Ye, 2002). The ray parenchyma cells further differ in the amount of cells: angiosperms usually contain multiseriate rays (several cells wide), while uniseriate rays (only one cell wide) are mostly found in gymnosperms (Esau, 1965).

### 3. Xylem vessel differentiation

Xylem vessel or TE differentiation is initiated in the procambium and vascular cambium, potentially through a polarity gradient of the plant hormone auxin and other signaling molecules, such as cytokinin, brassinosteroids, phytosulfokines and ethylene (Ye, 2002; Fukuda, 2004; Motose et al., 2009; Pesquet and Tuominen, 2011). This gradient is suggested to determine the cell fate, leading to the differentiation of xylem cells towards one side and phloem cells towards the other side. To form hollow sap conducting cylinders, TE differentiation goes through three essential steps: elongation, SCW formation and programmed cell death (PCD) (Fukuda, 1996; Plomion et al., 2001).

#### 3.1. Cell elongation

Xylem vessels as well as fibers and in some cases also XP cells undergo cell elongation before they become fully differentiated. The longitudinal and radial cell elongation is initiated by auxin and brassinolides (Turner et al., 2007). Cell elongation is further facilitated by the rearrangement of the microtubule network and remodeling of the primary cell wall (PCW), which is enabled by expansins, enzymes that loosen up the cellulose-hemicellulose matrix (Ye, 2002). Little is known about the molecular mechanism behind cell elongation. In *Zinnia*, the expansins EXPANSIN1 (*EXP1*) and *EXP2* have been found to be expressed in XP cells next to xylem vessels and were localized to the apical and basal extremities of the cell, thus enabling elongation through tip growth
(Turner et al., 2007). The remodeling of the PCW has further been reported to be controlled by the action of xyloglucan endotransglycosylases/hydrolases (XTHs), which release the hemicellulose xyloglucan from the cell wall to allow cell expansion (Bourquin et al., 2002). The cell elongation process is terminated with the initiation of SCW formation. The termination of cell elongation is accomplished by an increase in acidic pectin (homogalacturonan) and calcium (Ca$^{2+}$) in the PCW and the middle lamella. The acidic pectin forms rigid cross linkages with calcium and thereby prevents further elongation and cell growth. The acidification or de-esterification of pectin has been shown to be mediated by pectin methylesterases (PMEs) (Goldberg et al., 1996; Siedlecka et al., 2008).

### 3.2. Secondary cell wall formation

In TEs, SCW formation is essential to provide rigidity to the lateral walls to withstand the negative pressure during sap conduction. Anatomically, wood cell walls are composed of the middle lamella, the PCW and, depending on the cell type, the SCW. The middle lamella (0.5-1.5 µm) is shared with its neighboring cell and consists principally of pectin and lignin. The PCW thickness (0.1-0.3 µm) depends on the cell type and contains various layers of irregular oriented cellulose microfibrils embedded with pectin, lignin and hemicellulose (Derbyshire et al., 2007). The SCW, which bulges towards the cell interior, contains mainly cellulose, hemicellulose and lignin, and can be divided into three sublayers: S1 (0.1-0.35 µm), S2 (1-10 µm) and S3 (0.5-1.1 µm), that differ in size depending on the species (Ruel et al., 2012). These layers exhibit further different angles of aligned cellulose microfibrils: the S1 layer has a microfibril angle of 60°-80°, the S2 layer of 5°-30° and the S3 layer of 60°-90°, relative to the vertical axis of the plant (Plomion et al., 2001). The microfibril angle influences the strength of the cell wall: an increased angle reduces rigidity and elasticity, whereas a reduced angle has the opposite effect (Plomion et al., 2001). Because the S2 layer makes up the

![Figure 2. Simplified SCW architecture based on the structure observed in tracheary elements. Note that only the S2 layer is shown. Cellulose microfibrils are coated with hemicelluloses. Hemicelluloses form branches between cellulose microfibrils. Microtubules form the boundaries of the SCW. PM, plasma membrane; CML, compound middle lamella.](image-url)
majority of the SCW (~80%), this layer is mostly responsible for the stiffness of the xylem tissue, and consequently displays a low microfibril angle. The subcellular site of SCW formation is defined by the cortical microtubules, forming the boundaries of the SCW by aligning on the plasma membrane (Figure 2) (Turner et al., 2007). Microtubule associated proteins (MAPs) determine the different patterns of the SCW associated to protoxylem and metaxylem, by bundling, stabilizing and/or destabilizing microtubules. For example the enzymes MAP65-1/2 bundle microtubules, while MAP70-5 stabilizes microtubules and MIDD1 destabilizes microtubules (reviewed by Ménard et al., 2015a). MAP70-5 expression has been shown to be correlated with TE SCW formation in Arabidopsis in vitro cultures and specifically localizes to the flanking sites of the SCW boundaries and to microtubular arcs that interconnect different SCW boundaries (Pesquet et al., 2010). Genetic modulation of MAP70-5 by overexpression or silencing confirmed further its role in defining the different SCW patterns. Overexpression of MAP65-1 in Zinnia resulted in the formation of large bundles of the normally randomly oriented microtubules (Mao et al., 2006). On the other hand, overexpression or silencing of MIDD1 in Arabidopsis reduced microtubule density or prevented the depletion of microtubules, respectively (Oda et al., 2010). After the site of SCW deposition has been defined, cellulose synthase proteins (CESAs) form rosette-like complexes that are delivered to the plasma membrane via exocytosis, where they align with the microtubules (Mutwil et al., 2008). In Arabidopsis, essentially three different CESAs were found to be involved in the formation of the CESA-complex: IREGULAR XYLEM1 (IRX1; CESA8), IRX3 (CESA7) and IRX5 (CESA4) (Turner et al., 2007). While the CESA-complex moves parallel to the microtubules, it extrudes cellulose microfibrils composed of β-1,4-linked glucan chains that are arranged in a nearly crystalline structure (Harholdt et al., 2010). The enzyme CESA INTERACTIVE PROTEIN1 (CSI1) was identified to be required for the alignment of the CESA-complex to microtubules (Li et al., 2012a). The mutation of CSI1 caused dissociation of the CESA-complex from microtubules and reduced the cellulose content (Li et al., 2012a; Mei et al., 2012). After deposition of the bulk cellulose, the microfibrils are coated with hemicellulose side branches to provide additional strength (Figure 2). The most common types of hemicelluloses in SCWs are xylans and (gluco)mannans, although the composition is strongly dependent on the plant species: glucuronoxylans dominate in dicotyledon SCWs, whereas glucuronoarabinoxylans are mostly found in monocotyledons and galactoglucomannans in gymnosperms (Scheller and Ulvskov, 2010). Unlike cellulose, hemicelluloses have an amorphous structure with many side branches and generally consist of a backbone of various types of β-1,4-linked sugars. The synthesis of hemicellulose occurs in the Golgi apparatus through membrane bound glycosyltransferases and sugar nucleotides, which are then transported to the
cell wall. Some of the most common glycosyltransferases are IRX9, IRX10 and IRX14, which are involved in xylan backbone synthesis (Rennie and Scheller, 2014). Besides hemicellulose, pectin is integrated in the inter-microfibril spaces mostly in the PCW and the middle lamella, to provide cell wall plasticity and cell adhesion (Siedlecka et al., 2008). Pectin is, like hemicellulose, a heteropolymer which varies greatly in structure and composition. The different types of pectin found in cell walls are homogalacturonan, xylogalacturonan, apiogalacturonan, rhamno-galacturonan I and II, of which homogalacturonan is the most abundant pectin (Harholdt et al., 2010). The pectin structure is usually based on a backbone made of α-1,4-D-galacturonic acid or α-1,4-D-galacturonic acid linked to α-1,2-L-rhamnose units with various side chain modifications. Similar to hemicellulose, pectin is suggested to be synthesized in the Golgi and transported to the cell wall (Harholdt et al., 2010). Lignin is another polymer that is placed between the cellulose microfibrils. Further details on lignin are presented in section 4. Proteins represent a minor fraction in SCWs, the most common cell wall structural proteins are: hydroxyproline-rich glycoproteins (HRGPs), arabinogalactan proteins (AGP), proline-rich proteins (PRP) or glycine-rich proteins (GRP) (Fukuda, 2000).

3.3. Programmed cell death

PCD is an integral part of the development of any organism and generally has beneficial effects through maintenance of tissue homeostasis. During TE development PCD is required for the removal of the protoplast to form a hollow tube that conducts the xylem sap. TE PCD starts about 2-6 hours after formation of the cellulosic SCW and lasts for about 10 minutes, according to live cell imaging of Arabidopsis TE cultures (Pesquet et al., 2010 and 2011). While the SCW is formed, small vacuolar structures can be observed in the cytoplasm, which are suggested to be autophagosomes and/or lysosomes that degrade damaged cytosolic content (Kwon et al., 2010, 2011). Moreover, mitochondria appear to be degraded during SCW formation, as they lose their structural integrity along with the release of cytochrome c, a key enzyme of the electron transport chain (Yu et al., 2002). The gaseous plant hormone ethylene (C2H4) and its related biosynthetic enzymes have been found to be elevated during TE differentiation in Zinnia in vitro TE cultures (Pesquet and Tuominen, 2011). In these cultures, inhibition of ethylene synthesis by silver thiosulfate (STS) prevented TE PCD, suggesting that ethylene triggers PCD. Moreover, ethylene is produced during xylem maturation in whole plants (Andersson-Gunnerås et al., 2003). The initiation of PCD is further accompanied by the inflation of the vacuole caused by an influx of calcium (Ca2+) and the inhibition of the transport of organic anions into the vacuole (Kuriyama, 1999; Roberts and Haigler, 1990, 1992). This results in an increase
of the tonoplast permeability and finally to the implosion of the tonoplast, which takes about 3 minutes in Zinnia TE differentiation cultures (Roberts and McCann, 2000; Groover et al., 1997). The vacuolar collapse causes further a release of its hydrolytic content into the cytoplasm, which subsequently leads to a drop in the pH from 7 to 5 and the degradation of the cellular content (Figure 3). The nucleus has been shown to be degraded first, within 10 minutes after vacuolar rupture (Oba et al., 2001). Other organelles, however, are degraded much slower and remain intact for several hours. Single-membrane organelles, i.e. Golgi apparatus and endoplasmic reticulum (ER) usually inflate and degrade before double-membrane compartments such as mitochondria or chloroplasts (Fukuda, 1996). The ER for example stays intact for 40 minutes after vacuolar implosion (Young et al., 2010). Based on the Zinnia in vitro TE differentiation system chloroplasts remain visible up to 24 hours (Groover et al., 1997). The released hydrolytic enzymes are primarily nucleases and proteases that degrade the cell content (Fukuda, 1996, 2000). The Arabidopsis S1-like nuclease BIFUNCTIONAL NUCLEASE1/ENDONUCLEASE1 (BFN1/ENDO1) and its Zinnia homolog ZINNIA ENDONUCLEASE1 (ZEN1) are capable of degrading both DNA and RNA (Lesniewicz et al., 2013). The corresponding genes are transcriptionally coexpressed with SCW CESAs in Arabidopsis and Zinnia TE differentiating cultures (Kubo et al., 2005; Pesquet et al., 2004). BFN1 has further been shown to be relocalized to the ER in close vicinity to the nucleus specifically during PCD (Figure 3) (Farage-Barhom et al., 2011; Ménard et al., 2015b). Functional evidence has further been provided by silencing ZEN1 in Zinnia TE cultures, which caused reduced nuclear degradation (Ito and Fukuda, 2002). In Arabidopsis several proteolytic enzymes were found to be involved in TE autolysis. The papain-like cysteine protease XYLEM CYSTEINE PROTEASE1 (XCP1) and XCP2 are coexpressed with SCW CESAs and specifically localized to developing TEs (Avci et al., 2008). XCP1 and XCP2 further contain an N-terminal peptide signal, which directs these enzymes to the secretory pathway. Immunolocalization revealed that XCP1 and XCP2 localize to the ER, accumulate in the vacuole during progression of TE PCD and associate to protoplast remnants after vacuolar implosion, indicating their specific role in degrading the cell content (Figure 3) (Avci et al., 2008). Moreover, the double knock-out mutant of XCP1 and XCP2 (xcp1 xcp2) presents a delay in the removal of the protoplast thereby confirming its specific function. The Arabidopsis cysteine protease METACASPASE9 (MC9), is also transcriptionally coexpressed with SCW CESAs, specifically expressed in developing TEs and involved in clearing protoplast remnants (Bollhöner et al., 2013). Further investigations indicated that MC9 is present in the cytosol and has an optimal pH of 5.5, which corresponds to the pH after vacuolar rupture (Figure 3).
4. **Lignin**

Lignin is a polyphenolic polymer that is deposited in several different plant cell types and organs, including TEs, sclerenchyma cells, endodermal cells, seed coat cells and siliquae cells (Barros et al., 2015). In TE SCWs the main function of lignin is to provide mechanical support and hydrophobicity during sap conduction, as well as a protective barrier against pathogens (Bhuiyan et al., 2009; Turner et al., 2007). Lignin is composed of three major subunits: \( p \)-hydroxyphenyl (H), guaiacyl (G) and syringyl (S), that derive from the cross coupling of the respective monomers, also known as monolignols: \( p \)-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. These monomers are phenylpropene alcohols that differ in the degree of methoxylation of the aromatic ring, i.e. non-methoxylated, mono-methoxylated and di-methoxylated, respectively. Recently, the new subunit catechyl (C), has been identified in seed coats and derives from the monomer caffeyl alcohol (Tobimatsu et al., 2013). However, this subunit could not be detected in other plant tissues so far. The cross-coupling of the monolignols is principally initiated by the generation of monolignol radicals through phenoloxidase enzymes. The mechanisms of monomer synthesis, transport, oxidation and polymerization are described in detail below.

**Figure 3.** Schematic representation of the different steps of TE differentiation. A, Initiation of differentiation starts with the formation of microtubule scaffold at the site of SCW formation. B, cellulosic SCW formation is complete, PCD is initiated by \( \text{Ca}^{2+} \) and \( \text{C}_2\text{H}_4 \) uptake, organic anion (OA-) transport is inhibited, and mitochondria release cytochrome C (Cyt). C, Vacuole ruptures and releases proteases (XCPs), BFN1 localizes to the nucleus, cytoplasmic pH changes. D, Nucleus and ER are completely degraded, protoplast shrinks, lignification starts. E, lignified TE with remnant of the protoplast and perforation plate. Adapted from Ménard et al., 2015b.
4.1. Lignin monomer biosynthesis

Lignin monomers are synthesized by the phenylpropanoid pathway (Figure 4). This pathway does not exclusively produce lignin monomers but is also involved in many other processes such as the synthesis of hormones, flavonoids, suberins and lignans (Weng and Chapple, 2010). The phenylpropanoid pathway starts with the precursor phenylalanine (and tyrosine in monocots) which derives from the shikimate pathway in the plastids. This precursor is then converted into the different monolignols by sequential enzymatic steps that involve the hydroxylation and methoxylation of the aromatic ring as well as the removal of the amine group and the conversion of the carboxylic acid moiety to an alcohol. Initially, phenylalanine is converted to the phenolic cinnamic acid by the enzyme PAL (for abbreviations please refer to Figure 4). PAL has four homologs from which PAL1 and PAL2 have been demonstrated to be implicated in lignification: the double mutant (pali pal2) revealed a substantial reduction in lignin (Rohde et al., 2004). The quadruple mutant pali pal2 pal3 pal4 displayed further a dwarf phenotype, sterility and severely reduced lignin content (Huang et al., 2010). Subsequently, the p-hydroxylation of the aromatic ring is introduced by the monogenic enzyme C4H, which mutant (ref3) has severe growth defects and decreased lignin content (Schilmiller et al., 2009). The product of C4H (p-coumaric acid) is then converted to p-coumaryl-CoA, by the enzyme 4CL. 4CL has four gene copies and nine gene-like copies in the Arabidopsis genome, but only 4CL1 and 4CL2 have the highest similarity and are essentially involved in lignin biosynthesis: silencing of both genes resulted in a decrease in lignin and an increase in cellulose content (Yang et al., 2011). The product of 4CL is either directly converted to the H-unit lignin monomer p-coumaryl alcohol by CCR and CAD or meta-hydroxylated by the enzymes HCT and C3H (Figure 4). HCT is monogenic while C3H has three homologs, but only one (C3H1) showed substrate specificity (Raes et al., 2003). Both, HCT silenced plants and the C3H1 mutant (ref8) exhibit a dwarf phenotype with altered lignin content and composition as well as the redirection of the pathway towards flavonoid biosynthesis (Besseau et al., 2007; Li et al., 2010). The product of HCT/C3H has been found to be a good substrate for the enzyme CSE, which has recently been identified to play a role in lignification as the corresponding mutant had a reduced lignin content with an increase in H-units (Vanholme et al., 2013). The enzymes CCoAMT and COMT are able to convert the meta-hydroxylation to a meta-methoxylation. There are seven gene copies of CCoAMT in the Arabidopsis genome and only one COMT homolog but thirteen gene-like copies (Raes et al., 2003). Only CCoAMT1 and COMT1 have been reported to be implicated in lignification: the double mutant (comt1 ccomt1) displays reduced lignification with a composition dominating in H-units (Do et al., 2007). The single mutant of CCoAMT1
caused further the collapse of xylem vessels, called irregular xylem (irx) phenotype, indicating weakening of the SCW as the consequence of the reduction in lignin. CCR converts the CoA-conjugate of the propene residue to an aldehyde and therefore has a central position, redirecting the pathway from the general phenylpropanoid synthesis to the more specific production of lignin monomers (Figure 4). CCR has two homologs (CCR1 and CCR2) and five gene-like copies in the Arabidopsis genome (Raes et al., 2003) of which CCR1 appears to be predominantly involved in developmental lignification, while CCR2 is associated to stress lignin formation during pathogen invasion (Lauvergeat et al., 2001). The knock-out mutants of CCR1 (irx4, ccr1-3 and ccr1-6) have a dwarf phenotype associated to a reduction in lignin and the modification of the lignin structure by the incorporation of ferulic acid (Jones et al., 2001; Mir Derikvand et al., 2008; Ruel et al., 2009). These mutants also exhibit an irx phenotype, possibly caused by the decrease in lignin and/or the modification of the lignin properties (Vanholme et al., 2010). The conversion of the aldehyde moiety to an alcohol (the classical lignin monomer) is achieved through the enzyme CAD, which has nine homologs, whereof CAD4 and CAD5 are the major contributors to lignification. The double null mutant of CAD4 and CAD5 (cad c cad d) causes a reduction in lignin content and affects the lignin structure by incorporating monomer aldehydes (Lapierre et al., 2000; Eudes et al., 2006). The synthesis of the S-unit monomer sinapyl alcohol involves a second methoxylation, which is introduced by an initial meta-hydroxylation by F5H, followed by a methoxylation by COMT. F5H has two homologs (F5H1 and F5H2) with F5H1 being primarily involved in lignification. The knockout mutant of F5H1 (fah1) results in the absence of S-units (Chapple et al., 1992).
The incorporation of intermediates of the monomer synthesis pathway in the respective mutants clearly illustrates the specific role of each enzyme as well as the high plasticity of lignin for its substrates (Grabber et al., 2010). In this regard other types of phenyl-propene compounds or derivatives of monolignols with different substitutions have been identified to be incorporated as well. For example high proportions of G- and S-units γ-acylated with acetate or p-coumarate were detected in lignin of herbaceous plants (Del Rio et al., 2008; Morréel et al., 2004). This is further supported by the discovery of the enzyme PMT, which catalyses the production and incorporation of monolignols acetylated with p-coumarate in grasses (Petrik 2015).

**Figure 4.** General phenylpropanoid pathway. Dotted boxes indicate lignin monomers incorporated into lignin. PAL, phenylalanine ammonia-lyase; TAL, tyrosine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; C3H, p-coumarate 3-hydroxylase; HCT, p-hydroxyphenyllanoyl-CoA:quinone/shikimate p-hydroxycinnamoyltransferase; CSE, caffeoyl shikimate esterase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; COMT, caffeic acid O-methyltransferase; F5H, ferulate 5-hydroxylase; PMT, p-coumaroyl-CoA:monolignol transferase. Adapted from Barros, Serk et al., 2015.
et al., 2013). Furthermore, γ-p-hydroxybenzoate esters have been found in the lignin of various species (poplar, aspen, willow and palm) and are potentially produced by the incorporation of sinapyl p-hydroxybenzoate monomers (Lu et al., 2004). Lan et al., (2015) have recently shown that in grasses the flavonoid tricin is incorporated into lignin and appears to act as lignin monomer. In addition, di- and trilignols have been found to accumulate in lignifying poplar xylem as well as in the extracellular medium of TE differentiating Zinnia cultures (Morreel et al., 2004; Tokunaga et al., 2005). However, it is not clear whether these compounds are: directly incorporated in the lignin polymer, intermediates of lignin biosynthesis or unrelated phenolic compounds.

Regarding the subcellular localization of the lignin biosynthesis proteins, the hydroxylation enzymes C4H, C3H, and F5H are all cytochrome P450 oxidoreductases and localize to the surface of the ER. All other enzymes (PAL, 4CL, CCoAOMT, CCR, CAD and COMT) are located in the cytosol (Ruelland et al., 2003; Takabe et al., 2001). However, several enzymes have been found to form interactions with the ER bound enzymes, such as PAL with C4H (Achnine et al., 2004) as well as HCT and/or 4CL with C3H, although these enzymes showed a rather loose association (Bassard et al., 2012). Furthermore, all the ER bound enzymes (C4H, F5H and C3H) have been reported to form associated protein complexes (Chen et al., 2011). Altogether, this suggests that the lignin monomers are synthesized in the cytosol near the surface of the ER.

4.2. Lignin monomer transport

In order to polymerize in the SCW, the lignin monomers need to be transported across the plasma membrane. To do so, three different transport mechanisms have been hypothesized: i) passive diffusion, ii) vesicle mediated exocytosis, and iii) ATP dependent transport via membrane-bound ATP-binding cassette (ABC) transporters and/or proton coupled antiporters (Figure 5). The passive diffusion was initially suggested based on in vitro experiments, which showed that lignin monomers were able to diffuse into liposomes and lipid bilayer discs (Boija and Johansson, 2006; Boija et al., 2007). However, the possibility of this mechanism lacks currently in vivo evidence. Vesicle mediated secretion of lignin monomers has long been assumed to be true due to feeding experiments with radiolabeled monomers, such as tritium (3H) labeled phenylalanine, tyrosine or cinnamic acid (Pickett-Heaps, 1968; Fujita and Harada, 1979; Takabe et al., 1985). In these studies the label appeared in the rough ER, Golgi apparatus and vesicles associated to the plasma membrane. Nevertheless, Kaneda et al., (2008) have shown that the label incorporation is at least partially caused by proteins and not monolignols. The selective transport of the monolignols coniferyl alcohol and
sinapyl alcohol via ATP dependent transporters was demonstrated by using isolated plasma membrane vesicles in combination with different transport inhibitors (Miao and Liu, 2010). Moreover, loss-of-function mutants of the Arabidopsis ABCG29 transporter showed a reduction in lignin by 23-32%, caused by the equivalent decrease in H-, G- and S-units (Alejandro et al., 2012). Contradictory, the results could not be confirmed by ABC transporter inhibition experiments in xylem tissues of poplar, Japanese cypress and pine (Tsuyama et al., 2013). This study however showed that the monolignol transport was affected when proton coupled antiporters were inhibited, suggesting that they could contribute to the export of monolignols as well.

The unconjugated lignin precursors are considered toxic and exhibit low water solubility which suggests the formation of monomer conjugates such as 4-O-glucosides (Boerjan et al., 2003; Bonawitz and Chapple, 2010; Väisänen et al., 2015). In this regard it was shown that the 4-O-glucosides of coniferyl alcohol and sinapyl alcohol (coniferin and syringin, respectively) were transported across isolated vacuolar membrane vesicles by ABC transporters, while the respective aglycones were not (Miao and Liu, 2010). Monomer glucosides have also been found to accumulate in mutants of phenoloxidase enzymes (e.g. the laccase triple mutant, Zhao et al., 2013), suggesting that monomer glucosides are stored in the vacuole and mobilized only when required. The glycosylation and deglycosylation has further been shown to be facilitated by the enzymes UDP-glycosyltransferase (UGT) and β-glucosidase (BGLU), respectively (Lanot et al., 2006; Escamilla-Trevino et al., 2006). The BGLU was detected in SCWs, which suggests that the monomer glucosides are transported to the cell wall, where they are deglycosylated by BGLUs for lignification de muro (Figure 5) (Liu, 2012). Nevertheless, mutants in UGTs and BGLUs did not show any reduction in lignin (Lanot et al., 2006; Chapelle et al., 2012), indicating that monomer glucosides do not play a major role in cell wall lignification. Dima et al., (2015) further reported that glycosylated lignin oligomers are synthesized
in the cytosol and stored in vacuoles of Arabidopsis leaves (Figure 5). However, such oligomers do not seem to participate in lignin biosynthesis.

4.3. Lignin polymer formation

Once in the wall, the lignin monomers polymerize by the oxidative coupling of generated monomer radicals. These radicals are produced by two different types of phenoloxidases: laccases and peroxidases, which are further dependent on secondary substrates: molecular oxygen (O\textsubscript{2}) and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), respectively. Laccases and peroxidases are crucial for the initiation of the polymerization of lignin monomers. However, the monomer radicals have a relatively long half-life (~45s, Harkin, 1967), which means that they can diffuse through the wall to couple at a position different from where they were activated. The role of laccases and peroxidases during lignification is discussed below.

4.3.1 Lignin monomer oxidizing enzymes

Laccases (EC 1.10.3.2) are multi-copper containing glycoproteins that are able to catalyse the polymerization of various lignin precursors in vitro (Kärkönen et al., 2002; Liang et al., 2006) and are localized to lignifying xylem tissue (Caparrós-Ruiz et al., 2006; Koutaniemi et al., 2015). Because these types of enzymes have many isoforms (17–39 in angiosperms) with redundant functions it is difficult to determine the role of specific isoforms during lignification. However, recently it has been reported that four different laccase isoforms LACCASE4 (LAC4), LAC11, LAC15 and LAC17 are involved in lignin polymer formation in Arabidopsis. All these isoforms are expressed in the inflorescence stems (Berthet et al., 2011), except for LAC15 which has been shown to be involved in seed coat lignification (Liang et al., 2006). LAC4 is expressed in the cambium, in XP and in IF, and is localized to the SCW and middle lamella of fibers as well as the SCW of xylem vessels (Berthet et al., 2011; Schuetz et al., 2014). LAC11 is expressed in the cambium and in XP cells but the exact protein allocation is currently unknown (Turlapati et al., 2011). LAC17 is expressed in the cambium, in IF and in XP, and is localized to SCWs of fibers and xylem vessels (Berthet et al., 2011; Turlapati et al., 2011; Schuetz et al., 2014). Single mutants of LAC4, LAC11 and LAC17 did not show a strong phenotypic change with a slight or no reduction in lignin, whereas double mutants (lac4 lac11 and lac11 lac17) also exhibited a wild type phenotype but a significant reduction in lignin with up to 40%, mostly associated to a decrease in G-units (Berthet et al., 2011; Zhao et al., 2013). The LAC4 mutant also exhibits an irx phenotype indicating the importance of this laccase for vessel SCW lignification. Moreover, the triple mutant lac4 lac11 lac17 revealed severe growth defects with abolished xylem lignification suggesting that all
three isoforms are required for lignification. In poplar, the downregulation of a laccase (LAC3) did not affect the overall lignin content but showed an accumulation of phenolics in XP as well as the deformation of fiber cell walls (Ranocha et al., 2002). Furthermore, the overexpression of the laccase microRNA397a in poplar caused reduced expression of 17 out of 49 laccase and decreased the lignin content by 22% (Lu et al., 2013). Overall, this indicates that in both Arabidopsis and poplar, xylem lignification is dependent on multiple laccase isoforms.

Peroxidases, more specifically heme containing class III peroxidases (EC 1.11.1.7), have been shown to catalyse the polymerization of lignin monomers in vitro (Sterjiades et al., 1993; Guerra et al., 2000) and are expressed in lignifying tissues (Sato et al., 2006; Tokunaga et al., 2009). Like laccases, peroxidases have many isoforms, with 73-138 in angiosperms. Many of these isoforms have however different physiological functions and are not directly involved in lignification. Recent evidence supports that Arabidopsis PEROXIDASE2 (PRX2), PRX4, PRX25, PRX47, PRX52, PRX64, PRX66, PRX71 and PRX72 play important roles in lignification of different cell types. Knock-out mutants of PRX4 and PRX52 have a reduction in lignin, predominantly in fibers associated with a decrease in S-units (Ferández-Pérez et al., 2014, 2015). The peroxidases PRX47, PRX64 and PRX66 are expressed in lignifying xylem, with PRX47 being associated to XP, PRX64 to fibers and PRX66 mostly to xylem vessels (Tokunaga et al., 2009). In root endodermal cells, PRX64 was also found to enable lignification of the Casparian strip, as shown by the reduced Casparian strip lignification in PRX64 silenced plants (Lee et al., 2013). The Zinnia homologous protein of PRX66 (ZPO-C) was also localized to TE SCWs in TE differentiating cell cultures (Sato et al., 2006), supporting its role in vessel lignification. The peroxidases PRX2, PRX25 and PRX71 are all expressed in stems and both single and double mutants (prx2 prx25, prx2 prx71, prx25 prx71) displayed a reduction in lignification, although the phenotype did not reveal any morphological differences (Shigeto et al., 2013, 2015). The double mutants exhibited further a change in the lignin chemistry with an increase in S-units. Another potential candidate for xylem vessel lignification is PRX72, which mutant showed reduced lignification and stem height as well as collapsed xylem vessels (Herrero et al., 2013). In tobacco the peroxidase TP60 was identified by homology search of the French bean peroxidase FBP1 (60% identity to PRX2). Down regulation of this enzyme resulted in a reduction of lignin of nearly 50% (Blee et al., 2003). Similarly, in hybrid aspen the peroxidase PrxA3a (60% identity to PRX52) was found to be expressed in xylem tissue and the suppression resulted in a decrease of lignin with an altered lignin composition dominant in S-units (Li et al., 2003). Generally peroxidase mutants appear to have an increase in S-type lignin, which is supported by in vitro dihydrogenative polymer (DHP) studies showing that the majority of peroxidases use coniferyl alcohol as
substrate and are unable to use sinapyl alcohol (Demont-Caulet et al., 2010; Østergaard et al., 2000).

Most laccases and peroxidases show expression and/or localization in different tissues/cell types, which suggests that laccases and peroxidases have distinct functions. This is further demonstrated by the laccase triple mutant, which has severely reduced xylem lignification but no effect on Casparian strip lignification (Zhao et al., 2013). Though, some laccases and peroxidases indicate overlapping functions, e.g. LAC4 and PRX72 mutants exhibit both collapsed xylem vessels suggesting that they are both involved in xylem vessel lignification. As both mutants do show any growth defects, this supports the hypothesis that laccases and peroxidases may act in a sequential order, with laccases being active during early stages and peroxidases during later stages of development (Sterjiades et al., 1993).

**Figure 6.** Lignin monomer radical activation mechanism in TEs. Superoxide is produced by NADPH oxidases (NOX) and converted to O$_2$ and H$_2$O$_2$ by SODs. O$_2$ may further be provided through the atmosphere (atm). Alternatively, lignin is activated by a redox shuttle systems involving the oxidation (Ox.) and reduction (Red.) of radical carriers. Lignin monomers are provided by neighboring XP (see results section). Black dotted lines indicate alternative route for the production of O$_2$ and H$_2$O$_2$ by SODs in SCWs. Blue dotted lines indicate alternative route for the generation of monomer radicals by peroxidases in the apoplast (see results section).
4.3.2 Secondary substrates

Laccases and peroxidases require the secondary substrates O₂ and H₂O₂ respectively in order to produce lignin monomer radicals. Generally there is no limit in oxygen as it diffuses freely from the air and is available through the xylem sap (Gansert, 2003). H₂O₂ has an extremely short half-life (10⁻⁹ – 10⁻³ s, D’Autréaux and Toledano, 2007), which suggests that it is constantly produced by the lignifying tissue. The requirement of H₂O₂ for lignification has been addressed by using H₂O₂ scavengers such as potassium iodide (KI) and catalase (CAT), which caused a reduction in extracellular lignin of Picea Abies cell suspension cultures and reduced lignification of the Casparian strip, respectively (Kärkönen et al., 2002; Lee et al., 2013). Reactive oxygen species (ROS), such as H₂O₂, are by-products of photosynthetic or respiratory processes, and are produced by chloroplasts, mitochondria or peroxisomes. Under normal (non-stress) conditions the level of ROS is balanced by ROS scavengers in order to avoid tissue damage. In contrast, stress conditions may lead to an imbalance and an oxidative burst, which has been associated to PCD (Vianello et al., 2007). However, Zinnia TE differentiating cultures did not indicate the presence of an oxidative burst, but rather a continuous presence of ROS/H₂O₂ during the differentiation period and beyond (Gómez Ros et al., 2006). Gómez Ros et al., (2006) further showed that H₂O₂ was produced by both TEs and non-TEs with non-TEs being the major source. This was confirmed by the localization of H₂O₂ near the outer side of the plasma membrane and in the apoplast of XP cells in contact with TEs in whole Zinnia plants (Ros Barceló et al., 2005). The supply of H₂O₂ is believed to be the result of the combined action of two enzymes: nicotinamide adenine dinucleotide phosphate hydrogen oxidase (NADPH oxidase) and superoxide dismutase (SOD) (Ogawa et al., 1997). NADPH oxidases or respiratory burst oxidase homolog (RBOH) proteins produce superoxide (O₂⁻) from molecular oxygen, whereas SODs catalyse the reaction of superoxide to H₂O₂. Superoxide has been found to be localized near the plasma membrane, the site where NADPH oxidases have been detected as well (Ros Barceló et al., 1998). SODs were confined to SCWs of xylem vessels and fibers as well as the apoplastic space of XP, neighboring xylem vessels (Karpinska et al., 2001). NADPH oxidases are involved in Casparian strip lignification, as demonstrated by using the NADPH oxidase inhibitor diphenylene iodonium (DPI) and loss-of-function mutants of RBOH-F (Lee et al., 2013). These mutants exhibited reduced Casparian strip lignification, while xylem vessel lignification was unaffected, indicating the specific function of RBOH-F. The role of SODs during lignification was confirmed by using the SOD inhibitor diethyldithiocarbamate (DDC) which caused a reduction of TE lignification in Zinnia TE differentiating cultures (Karlsson et al., 2005) and abolished lignification of the Casparian strip (Lee et al., 2013). Poplar plants
downregulated in a high-isoelectric-point (hipl) SOD showed further a slight reduction of lignin and an accumulation of phenolics (Srivastava et al., 2006). Altogether, these studies support that H$_2$O$_2$ is delivered by XP through the NADPH oxidase mediated production of superoxide, which diffuses to the apoplast and/or the TE SCW where SODs convert it to H$_2$O$_2$ and O$_2$, required for phenoloxidases to activate lignin monomers (Figure 6).

4.3.3 Monomer oxidation by radical carriers

Monolignols need to be activated to radicals by phenoloxidases prior to cross-coupling. According to Terashima et al., (2012) radicals need to be generated at the growing ends of the lignin polymer as well and suggest that this could be accomplished by radical carriers such as ions and/or enzymes that diffuse through the wall to the reaction site. However, it is quite unlikely that phenoloxidase enzymes diffuse through the dense polysaccharide matrix of the SCW leaving only the possibility of ion based radical carriers. Manganese (Mn) has been identified to be such a carrier, acting as a redox shuttle system in which Mn$^{2+}$ is oxidized to Mn$^{3+}$ by phenoloxidases and diffuses to the growing lignin polymer where it is reduced back to Mn$^{2+}$, hence closing the loop (Figure 6) (Önnerud et al., 2002). This study is based on in vitro DHP formation in a two-phase redox shuttle system, composed of coniferyl alcohol, peroxidases, Mn$^{2+}$ oxalate/acetate, dioxane and water. In this system peroxidases are never in direct contact with the lignin monomers and the resulting lignin polymer was found to be similar to natural spruce wood lignin. Similar results were also obtained by Landucci (2000) using Mn$^{3+}$ acetate in acetic acid or pyridine solution. Further support for this mechanism arises from Berglund et al., (1999) who found that manganese is one of the most homogenously distributed elements in spruce wood. Westermark (1982) proposed that superoxide anions form a complex with calcium and possible act as redox shuttle system. It is further suggested that a superoxide producing enzyme, which could be NADPH oxidase located near the PCW (Ros Barceló et al., 1998), supplies the superoxide to the site of lignification. Calcium has also been found to be homogenously distributed in wood and forms calcium pectate specifically in the middle lamella and the cell corners (Berglund et al., 1999; Boerjan et al., 2003), the site where lignification is initiated. It is therefore tempting to hypothesize that calcium superoxide complexes are involved in radical activation at the growing lignin polymer. The participation of diffusible redox shuttles would further explain the structural differences observed between DHPs and natural lignins. DHPs generally constitute a higher proportion of condensed (C-C) linkages compared to natural lignins which dominate in non-condensed (C-O-C) linkages. The formation of DHPs appears to follow the classical lignin radical coupling model proposed by Freudenberg (1968) which is based on the dimerization of lignin monomers.
On the other hand, the end-wise addition of monomers to the growing lignin polymer would result in higher proportions of non-condensed bonds due to the steric accessibility (Önnerud et al., 1999). The redox shuttle system promotes monomer-polymer coupling reactions and would therefore explain the lignin structures observed in nature.

### 4.4 Lignin structure and deposition

The lignin structure relies on the proportion of each of the three different subunits in the lignin polymer and varies depending on species and cell type. Gymnosperm lignins comprise mainly of G-units and a small proportion of H-units, whereas angiosperm lignins exhibit a combination of G- and S-units, and an H-unit composition that is higher in monocots compared to dicots (Boerjan et al. 2003). Concerning the xylem cell types, lignin of TEs is composed almost entirely of G-units, while fibers exhibit both G- and S-units. The subunit composition appears to be further controlled by the initiation of lignification and follows a sequential order. It is important to mention first that lignification of wood cells starts in the cell corner and the middle lamella, and extends then to the different layers of the SCW (S1, S2 and S3) (Donaldson, 2001). H-units are deposited first during the beginning of lignification, in the middle lamella and the cell corners, while G-units are deposited subsequently in the middle lamella and in the SCW layers. S-units have been shown to be deposited during later stages of lignification. The time-dependent lignification is further related to the different cell types: vessels were observed to be lignified first, in the cell corner, middle lamella and the SCW followed by the deposition of lignin in fiber cell walls during later stages (Donaldson, 2001). This observation is in agreement with the dominance of S-units in fiber, which are deposited at later stages. Moreover, the deposition of lignin has been associated to the formation of the different SCW layers. Cell corner and middle lamella start to get lignified after formation of the S1 layer, which then gets slowly lignified during the formation of the other SCW layers, while the majority of lignin is actually deposited after completion of SCW formation (Terashima and Fukushima, 1988). This is coherent with the speed of lignification, the middle lamella is considered to lignify very rapidly, whereas SCW lignification occurs slowly (Terashima et al., 2012).

Furthermore, lignin deposition is influenced by the intermolecular spaces in the different cell wall compartments. The middle lamella and PCW represent a highly porous carbohydrate network, which allows the deposition of larger spherical lignin structures (Figure 7) (Donaldson, 2001). The SCW on the other hand is densely packed due to the parallel arrangement of cellulose microfibrils, leading to the formation of laminar lignin structures that align with the microfibril threads. Nakashima et al., (1997) mention further that lignin forms spherical structures around the microfibrils at the
onset of SCW lignification and completely fills the inter-microfibrilar spaces during later stages. The progression of lignification further effects the organization of the microfibrils from being slightly irregular at earlier stages of lignification, to straight during later stages (Nakashima et al., 1997). The structural relationship of lignin with other cell wall polymers leads to the varying quantities observed in the different cell wall compartments. The highest amount of lignin is generally found in the cell corner and the middle lamella, whereas the lignin quantity in the SCW is lower but increases from the S1 to the S3 layer (Figure 7) (Fromm et al., 2003; Gierlinger, 2014). The amount of lignin in the middle lamella and cell corner has been estimated with about 60-70% of the total lignin, while the SCW comprises of 22-32%, based on studies in gingko and spruce (Terashima et al., 2012). Taken together, the spatial and temporal deposition of lignin in xylem vessel and fiber appears to be a strictly controlled process.

**Figure 7.** Lignin structure and deposition in tracheids of secondary xylem. Note that only the S2 layer is shown. Lignin has a globular structure in the compound middle lamella (CML), and a laminar structure in the SCW. Lignification progresses gradually from CML to SCW. The lignin quantity is highest in CML and increases along the SCW.

### 4.4.1 Lignin intermonomeric linkages

The activated monolignol radicals are polymerized by cross-coupling with other monomer/oligomer radicals. Monolignol radicals can displace their electric charge by resonance stabilisation which results in the generation of five possible resonance structures with three being most favourable: radical charge at position O-4, β or 5 (Morreel et al., 2010). The polymerization is then initiated by the formation of a dimer, which needs to be reactivated for the end-wise addition of another monomer radical to form a trimer (Vanholme et al., 2010). The cross-coupling of these oligomers is suggested to
lead to the formation of the irregular highly branched lignin structure. The types of linkages observed in lignin are divided into condensed (C-C) linkages: 5-5' (biphenyl), β-β' (resinol), β-5' (phenylcoumaran), β-1' (diarylpropane), and non-condensed (C-O-C) ether linkage: β-O-4' (β-aryl-ether), 5-O-4' (biphenyl ether) (Figure 8A,B). Because monomers preferably couple at the β position, β-O-4', β-5' and β-β' are the most abundant linkages, with β-O-4' being most common (Vanholme et al., 2010). The linkages 5-5' and 5-O-4' are present at lower proportions and potentially form by the coupling of oligomers (Boerjan et al., 2003). Nevertheless, differences in the abundance of the various linkages mentioned above can be observed between different species. Gymnosperms contain generally more condensed linkages due to the dominance of G-units compared to angiosperms which also incorporate S-units (Boerjan et al., 2003). Higher levels in β-5' linkages, which may occur in G-unit rich lignins, are usually associated to an increase in rigidity and hydrophobicity due to the carbon-carbon bond. Genetic modulation of lignin monomer synthesis genes can lead to substantial changes in the formation of subunit linkages. The mutant of COMT for example has increased levels of benzodioxane (β-O-4'/α-O-5') linkages due to the incorporation of 5-hydroxyconiferyl alcohol, which are normally present at very low proportions (Lu et al., 2010). The mutation of F5H on the other has shown elevated β-5' and dibenzodioxocin (5-5'/β-O-4'[α-O-5']) linkages, which are also rare under wild type conditions (Figure 8C) (Marita et al., 1999).

Figure 8. Lignin intermonomeric linkages, showing (A) condensed linkages, (B) non-condensed linkages and (C) linkages elevated in COMT mutants (benzodioxane) or F5H mutants (dibenzodioxoxcin) that are rarely found under wild type conditions.
The coupling of monomer radicals, specifically coniferyl alcohol radicals, has been suggested to be controlled by dirigent proteins. These proteins stereospecifically restrict the dimerization of two coniferyl alcohol radicals to one type of stereoisomer, such as β-β' linked (+)-pinoresinol, the major product of a dirigent protein in *Forsythia intermedia* (Davin and Lewis, 2000). Although dirigent proteins have been mostly associated with lignan biosynthesis, it is proposed that they are also involved in lignin polymer formation by guiding the stereospecific cross-coupling in the presence of phenoloxidases. Immunolocalization of dirigent proteins revealed the presence in actively dividing cambial cells of the stem as well as in SCWs of lignifying xylem vessels (Burlat *et al.*, 2001). The role of dirigent proteins during lignification has further been confirmed by the loss-of-function mutant of the Casparian strip localized dirigent protein ENHANCED SUBERIN1 (*ESB1*), which exhibits disordered Casparian strip formation and an increase of lignin in the Casparian strip (Hosmani *et al.*, 2013).

### 4.4.2 Lignin nucleation sites

The remote distance of the onset of lignification (middle lamella) from the site of monomer production (ER/cytosol interface) suggests the presence of specific nucleation sites from which lignification progresses. The nature of such nucleation sites has however not been fully understood yet. One hypothesis is that three dilignols of coniferyl alcohol (β-O-4’, β-5’ and β-β’ linked) together with ferulic acid bind to polysaccharides in the wall and thus form the nucleation sites for new monomers/oligomers in grasses and gymnosperms (Ralph *et al.*, 2004; Terashima *et al.*, 2012). This is further supported by the presence of mono- and di-ferulate esters in grasses, which form linkages with xylan and provide nucleation sites for monomers/oligomers through their para-hydroxylation (Ralph *et al.*, 1995). Recently, the flavonoid tricin has been found to be present in lignins from monocots (Lan *et al.*, 2015). Because this molecule can only initiate the lignin polymer chain and is not incorporated by end-wise addition, tricin is suggested to be the nucleation site, at least in grasses. Dirigent proteins have further been postulated to guide the initiation of lignification due to their specific function and localization as described previously (Davin and Lewis, 2000). Another hypothesis is that GRP proteins act as nucleation sites as the result of their increased proportion of aromatic tyrosine residues. This is supported by the specific localization of GRP proteins in secondary thickenings of protoxylem vessels (Keller *et al.*, 1989). The protein could however not be detected in metaxylem vessels. Alternatively, peroxidases that bind pectin in the presence of calcium have been described to form nucleation sites (Boerjan *et al.*, 2003). As previously mentioned calcium pectate terminates cell elongation and is abundant in the middle lamella and in the cell corners, the sites where
lignification is initiated. This suggests that such peroxidases are specifically localized to these sites and initiate lignification by radical activation of exported lignin precursors.

4.5 Transcriptional regulation of lignin biosynthesis

The SCW formation is controlled by a hierarchical organisation of several transcription factors. Most of these transcription factors are either members of the NAC (NAM, ATAF1/2, CUC2) domain or the MYB (Myeloblastosis) transcription factor family. NAC domain transcription factors are the master regulators of the transcriptional network and control SCW formation in specific cell types (Figure 9). The master regulator SECONDARY WALL-ASSOCIATED NAC DOMAIN1 (SND1) also known as NAC SECONDARY WALL THICKENING PROMOTING FACTOR3 (NST3) has been found to regulate SCW formation in fibers. This transcription factor is specifically expressed in IFs and XFs of Arabidopsis stems and the dominant repression of SND1/NST3 reduces the thickness of fiber SCWs (Zhong et al., 2006). Besides SND1/NST3, the two other NAC transcription factors NST1 and NST2 have been identified to play a role in SCW formation in the endothecium of anthers (Mitsuda et al., 2005) and in fibers of stems (Zhong and Ye, 2015), respectively. In contrast, the SCW formation of xylem vessels is controlled by the master switches VASCULAR-RELATED NAC DOMAIN6 (VND6) and VND7. VND6 and VND7 are specifically expressed in metaxylem and protoxylem vessels respectively and the dominant repression of VND6 or VND7 prevents the formation of either metaxylem or protoxylem vessels in roots (Kubo et al., 2005). VND7 further interacts with the NAC domain protein VND-INTERACTING2 (VNI2) in order to repress VND7 regulated genes that are implicated in protoxylem vessel development (Yamaguchi et al., 2010). Driving VNI2 by the VND7 promoter resulted in the depletion of protoxylem vessels in leaves and roots of seedlings. Therefore, VNI2 possibly functions upstream of VND7 to control the protoxylem vessel formation. VNI2 is expressed in XP cells surrounding mature vessel elements in vascular bundles of stems, which suggest that VNI2 suppresses VND7 in these cells (Yamaguchi et al., 2010). Similar to VND6 and VND7, the down-regulation of the nuclear proteins ASYMMETRIC LEAVES2-LIKE19 (ASL19) and ASL20 showed abnormal vessel development, while overexpression induced ectopic TE formation in non-vascular tissues (Soyano et al., 2008). Soyano et al., (2008) further showed that ASL19 and ASL20 expression is dependent on VND6 and VND7, indicating that ASL19 and ASL20 are downstream targets of VND6 and VND7, and control protoxylem and/or metaxylem vessel differentiation.

The direct targets of SND1/NST3, NST1, NST2, as well as VND6 and VND7 are, among others, the transcription factors MYB46, MYB83, and the
KNOTTED1-LIKE HOMEO DOMAIN (KNOX) gene KNOTTED ARABIDOPSIS THALIANA7 (KNAT7) (Figure 9) (Zhong et al., 2008; McCarthy et al., 2009; Ohashi-Ito et al., 2010). These master regulators bind to their targets through a cis-acting element, called secondary wall NAC-binding element (SNBE), conserved in all target promoters (McCarthy et al., 2011). VND6 and VND7 have also been reported to bind to a cis-element, called tracheary element regulating cis-element (TERE, CTTNAAAGCNA) located in the promoter of their targets, which are mostly genes involved in SCW formation and PCD (Figure 9) (Pyo et al., 2007). The transcription factors MYB46 and MYB83 are secondary transcriptional regulators with many downstream targets and directly activate the transcription of KNAT7, MYB58 and MYB63 (Zhong and Ye, 2012). MYB58 and MYB63 have been shown to activate the transcription of all lignin biosynthesis genes (except for F5H) as well as the phenoloxidase LAC4 (Figure 9) (Zhou et al., 2009). The transcriptional activation was further shown to occur through binding to AC cis-regulatory elements (ACCTACC, ACCAACC or ACCTAAC) present in the promoters of all lignin biosynthesis genes, except for C4H and COMT which presumably contain deteriorated forms of AC elements (Zhou et al., 2009). The S-lignin specific gene F5H does not contain the AC elements and is controlled directly by the master regulator SND1 (Zhong et al., 2006). Concerning MYB58 and MYB63, the overexpression of these genes has led to an ectopic deposition of lignin, whereas dominant repression caused a reduction in lignin along with an irx phenotype, thereby confirming their association to lignin biosynthesis (Zhou et al., 2009). Promoter::GUS expression analysis showed further that MYB58 and MYB63 are expressed in TEs, XFs and IFs. Another MYB transcription factor that was found to be implicated in lignification is MYB85, which is directly regulated by SND1. MYB85 was also shown to control lignin monomer synthesis gene expression through binding to the AC element (Zhou et al., 2009). Because dominant repression of either MYB58, MYB63 or both does not completely abolish lignification, MYB58, MYB63 and MYB85 are potentially redundant activators of lignin biosynthesis. KNAT7 is another transcription factor that is involved in SCW formation, particularly in lignification of vessels and fibers. In contrast to the previous mentioned transcription factors KNAT7 represses SCW formation. Loss-of-function mutants showed an increase in SCW thickness and lignin content along with an upregulation of SCW related genes, whereas overexpression resulted in the reduction of the SCW thickness (Li et al., 2012b). However, the mutant also exhibited an irx phenotype, which suggests that the function of KNAT7 depends on the cell type. MYB75, also known as PRODUCTION OF ANTHOCYANIN PIGMENT1 (PAP1) due to its association to anthocyanin biosynthesis, was further identified to act as a repressor of lignin biosynthesis genes (Bhargava et al., 2010). The loss-of-function mutant showed increased SCW thickness, higher lignin content and
an upregulation of lignin synthesis genes, while the overexpressor displayed an increase in anthocyanin content without affecting the overall lignin composition. This suggests that MYB75 is involved in directing the carbon flux between lignin and flavonoid biosynthesis. A yeast-two-hybrid screen revealed further that MYB75 interacts with KNAT7, indicating that these proteins may form a complex to negatively regulate the deposition of lignin (Bhargava et al., 2010).

**Figure 9.** Simplified transcriptional network regulating TE differentiation in *Arabidopsis*. Colored underlying boxes indicate expression in specific cell types: TEs (blue), XP (red), IFs/XFs (purple) and TEs/IFs and XFs (green). Arrows indicate activation whereas horizontal strokes indicate repression. Colored arrows indicate direct transcriptional activation.
5. Evolution of tracheary elements and lignin

The evolution of TEs has been a major step that allowed the colonization and development of vascular land plants. The first land plants are estimated to have evolved during the late Ordovician period, about 450 mya (Weng and Chapple, 2010). The atmospheric CO$_2$ concentration is suggested to be related to the appearance of vascular land plants, along with the water transport ability for photosynthetic processes (Sperry, 2003). During the late Ordovician and early Silurian CO$_2$ concentrations were very high and therefore vascular systems with low hydraulic conductance were more energy efficient. Later during the Devonian and Carboniferous period atmospheric CO$_2$ concentrations declined, resulting in reduced water demand for photosynthesis, making the investment of energy into vascular systems with higher hydraulic capacity possible (Sperry, 2003). The colonization of land by plants possibly began by charophytes (green algae) that were flushed onto marine shores (Weng and Chapple, 2010). The first land plants are presumably similar to liverworts, a division of bryophytes, which do not have a vascular system and are relatively small in size to avoid water stress. These species have developed a cuticle (waxy surface layer) to prevent excessive transpiration, limited by their low hydraulic conductance (Sperry, 2003). Subsequently, more advanced bryophytes such as hornworts developed stomata (epidermal pores) that allow to regulate the transpiration rate more efficiently. In liverworts and hornworts, which lack conducting cells, the water transport is facilitated by a symplastic mechanism involving plasmodesmata (Mauseth, 2014). Mosses on the other hand developed hydroids around 410 mya (Figure 10), which are considered to be the earliest vascular structures (Friedman and Cook, 2000). The hallmark that permits the capillary water transport in contrast to non-vascular plants is the establishment of PCD, thus creating a hollow conduit. The water transport through dead hollow cellular remnants has been reported to be six times more efficient than non-vascular transport (Sperry, 2003). Because some species contain cells that undergo PCD but do not form SCWs, SCW biosynthesis is suggested to have appeared later during evolution (Escamez and Tuominen, 2013). While hydroids are limited in mechanical support and can collapse due to hydraulic tension (Ligrone et al., 2000), an improved transport element was needed. Consequently, microperforate tubes have evolved in protracheaophytes (e.g. Aglaophyton, Hornephyton), which have smooth thin perforated walls similar to hydroids but also incorporate lignin-like polyphenolics. Sennicaulis (S) – type banded tracheids, named after the fossil in which they were discovered, are believed to have evolved from microperforate tubes (Figure 10) (Edwards, 2003). S-type tracheids appeared in rhyniopsids, the first tracheophytes, and resemble modern protoxylem tracheids with annular/spiral secondary thickenings (Friedman and Cook, 2000). However,
contrary to protoxylem vessels these tracheids deposit lignin-like phenolics also between the SCW thickenings, which reduces structural elasticity and lateral water permeability. *Gosslingia* (*G*)-type banded tracheids can be found in lycophytes and are thought to be an improvement of the *S*-type ones, based on the presence of larger pores between the SCW thickenings (Kenrick and Crane, 1991). The typical lignin structure as observed in “modern” plants is proposed to have evolved in *S*- or *G*-type tracheids of lycophytes. Later, during the early Devonian, euphyllophytes developed pitted, *Psilophyton* (*P*)-type tracheids (Figure 10), which probably origin from *G*-type banded tracheids. These tracheids resemble modern metaxylem vessels, being characteristic for the presence of bordered pits with an un lignified pit membrane that improves the lateral water transport (Edwards, 2003). By having the initial building block, *i.e.* the tracheid, it may have taken just a couple of million years to develop a complete woody tissue as nowadays observed in gymnosperms (Figure 10). The evolution of vessel elements from tracheids, on the other hand, has taken nearly 140 million years through the sequential increase in tracheid diameter (Sperry, 2003). The appearance of vessels is suggested to be related to the evolution of leaves together with large amount of stomatas leading to high transpiration rates. At the same time, atmospheric CO$_2$ was lower than ever, thus plants could not afford to produce more woody sap conducting biomass to compensate the increase in transpiration (Sperry, 2003). Therefore, plants developed vessel elements with much greater hydraulic capacity compared to tracheids. The first vessels were observed in *Gigantopteridales*, about 260 mya (Li *et al.*, 1996). However, in angiosperms which appeared about 245 mya, vessel elements evolved very much later, during the mid-Cretaceous (Figure 10).
Once charyophytes were flushed onto marine shores, they had to develop a mechanism to protect themselves from pathogens and UV radiation. This protective mechanism was most likely based on the synthesis of simple phenolic compounds. The green algae *Chlamydomonas reinhardtii* contains three of the lignin biosynthesis genes (*CCoAMT*, *CAD*, and *CCR*) and a related species (*C. nivalis*) has been found to produce phenolics upon UV exposure (Duval et al., 2000; Xu et al., 2009). The moss *Physcomitrella patens* accumulates phenolic compounds such as flavonoids and lignans, and exhibits orthologs of the nine core lignin biosynthesis genes (*PAL*, *C4H*, *4CL*, *C3H*, *HCT*, *CCoAMT*, *COMT*, *CAD* and *CCR*) indicating the beginning of the evolution of lignin biosynthesis (Xu et al., 2009). Although lignin-like compounds may have appeared already in some bryophytes (Edelmann et al., 1998), contemporary lignin structures, composed of H- and G-units, appeared in S- or G-type tracheids of lycophytes. Lignin composed of S-units is suggested to have evolved later, along with the appearance of angiosperms and is associated with improved mechanical properties and pathogen resistance (Weng and Chapple, 2010). However, S-lignin has also co-evolved several times in other species, such as lycophytes, ferns and gymnosperms (Weng and Chapple, 2010). In terms of the evolution of phenoloxidases,

Figure 10. Evolution of TEs (A) and their SCW structure (B) in relation to the geological time periods in million years ago (mya). A: O, Ordovician; S, Silurian; D, Devonian; C, Carboniferous; P, Permian; Tr, Triassic; J, Jurassic; K, Cretaceous; T, Tertiary. B: CML, compound middle lamella; grey, polysaccharides; yellow, PCW; red, lignin/lignin-like compounds.
Laccases were detected in green algae and are suggested to have evolved due to the increase in atmospheric O\textsubscript{2} during the Ordovician period (Lowry \textit{et al.}, 1980). The first lignin associated class III peroxidases occurred later, in mosses, during the late Ordovician to further reduce the oxidative stress (Weng and Chapple, 2010). Both laccase and peroxidase isoform numbers increased dramatically along the evolution of higher plants. Overall, lignin monomer synthesis seems to have evolved before lignification indicating that these phenolics were originally intended as defence compounds.

**Aim and Objectives**

The overall aim of this thesis is to understand how lignin biosynthesis in TEs is controlled spatially and temporally, particularly by testing the function of novel genes/enzymes that are implicated in different aspects of cell wall lignification, such as lignin monomer synthesis, monomer oxidation/polymerization and the production of ROS, required for monomer oxidation.

1. **Hypothesis**

Live cell imaging of TE differentiation indicated that TE lignification is temporally controlled and occurs directly after PCD (Pesquet \textit{et al.}, 2010). This observation suggests a novel mechanism in which monomers are supplied by the adjacent living parenchymatic cells to the dead TEs for their \textit{post-mortem} lignification. The \textit{post-mortem} lignification further leads to the question of the degree of cell autonomy for other substrates and enzymes that are required for the synthesis of lignin, such as ROS and phenoloxidases. Live cell imaging further revealed that lignification is spatially restricted to TE SCWs, which further suggests the existence of a mechanism that controls the specific deposition of lignin. Phenoloxidases appear to be good candidates for controlling the restricted lignification of SCWs (Schuetz \textit{et al.}, 2014).

Based on the hypotheses explained above three main research questions have been formulated, that define the objectives of this thesis:

1. **When does TE lignification occur?** Does lignification occur before or after PCD? How does TE lignification progress?

2. **How does TE lignification operate on the cellular level?** Are the substrates cell-autonomously produced or supplied by XP?

3. **What is the spatial restriction of TE lignification?** Which enzymes and/or substrates controls the spatial restriction?
2. Strategy

The general research strategy was to study the lignification process by using simplified *in vitro* TE differentiation systems of *Zinnia elegans* and *Arabidopsis thaliana* as well as whole plants, to confirm the results obtained with the *in vitro* systems. In the *in vitro* systems, cells are hormonally induced to differentiate into 30-50% lignified TEs, while the rest of the cells remain parenchymatic (non-TEs). Because all cells simultaneous progress towards TE differentiation these systems allow studying the TE differentiation process at different stages of development, including: SCW synthesis, PCD and cell wall lignification. This makes the *in vitro* systems ideal tools to study lignification on the cellular level along with the avoidance of problems that arise when studying lignification in whole plants. Such problems are: i) xylem cells are an integral part of the wood tissue and are only accessible by destructive means, ii) xylem tissue is mostly composed of dead already lignified cells (only a few cells are actively differentiating), and iii) inhibition of lignification in whole plants leads to physiological and developmental defects. The *Zinnia* TE differentiation system is based on the induction of isolated mesophyll cells by auxin and cytokinin, leading to the de-differentiation to cambial cells that can further differentiate to TEs. The TE differentiation process of the *Zinnia in vitro* system has been extensively studied. Genetic and developmental processes are considered very similar to those in whole plants (Fukuda and Komamine, 1982; Ye, 2002; Pesquet *et al.*, 2006; Turner *et al.*, 2007). In addition, genetic studies using transient transformation were made possible in the *Zinnia in vitro* culture system as well (Endo *et al.*, 2008). Nevertheless, the *Arabidopsis* TE differentiation system offers several advantages over the *Zinnia* system. Those advantages are for instance the simplicity of using genetic tools (e.g. stable transformation), the availability of genomic databases as well as the maintenance of cultures and transgenic lines over a long period of time. The initiation of TE differentiation in the *Arabidopsis* system requires, besides auxin and cytokinin, also brassinosteroids but, unlike the *Zinnia* system, the culture is based on cambial-like cells which means that the cells do not de-differentiate prior to TE differentiation. The *Arabidopsis* system has already been shown to be a useful tool to study processes related to TE differentiation (Pesquet *et al.*, 2010; Ohashi-Ito *et al.*, 2010).

In order to confirm the results obtained from the *in vitro* TE differentiation system whole plants of *Zinnia* and *Arabidopsis* were investigated using biochemical and genetic methods. *Zinnia* plants are suitable to study lignification because of the short generation time and the relatively simple arrangement of xylem cells in stems and hypocotyls (Novo-Uzal *et al.*, 2013). However, the *Zinnia* model lacks the availability of genetic tools and information because the genome has not been sequenced yet. The *Arabidopsis* plant model system, on the other hand, is widely used in various
fields of plant science due to its benefits, specifically for genetic studies (Koornneef and Meinke, 2010). The *Arabidopsis* genome has been sequenced, the genome has a relatively small size (157 Mbps) and is composed of five diploid chromosomes. Other benefits are the availability of T-DNA insertion mutants (TAIR, www.arabidopsis.org), the short rotation time, the small size, the simple transformation procedure and the high seed yield through self-pollination (Koornneef and Meinke, 2010). In terms of xylem formation, the hypocotyl of *Arabidopsis* develops a continuous vascular cambium that leads to secondary growth, promoted specifically during short days (8h light, 16h dark) (Chaffey et al., 2002). Moreover, secondary growth has been reported in roots and in inflorescence stems, which makes *Arabidopsis* a suitable model to study primary as well as secondary xylem development (Nieminen et al., 2004).

3. Methodology

**FT-IR Spectroscopy**

In **Paper I, II and III** lignin was characterized by Fourier Transform Infrared (FT-IR) spectroscopy and microspectroscopy. FT-IR microspectroscopy is based on the combination of FT-IR spectroscopy and microscopy, and has been used widely for the compositional analysis of wood tissue (Gorzsás et al., 2011). Resolution and speed of this method has recently been improved by equipping the FT-IR with a focal plane array (FPA) detector, which leads to the simultaneous detection of large amount of spectra over a defined area. The chemical fingerprint obtained from the FT-IR spectra is normally subjected to multivariate data analysis such principle component analysis (PCA) or orthogonal projections to latent structures-discriminant analysis (OPLS-DA), which allows not only the separation of the variables based on a correlated (predictive) response variable but also based on a non-correlated (orthogonal) component (Gorzsás et al., 2011). Changes in lignin structure and composition can be detected by the lignin characteristic peaks at 1510 cm\(^{-1}\) and 1595 cm\(^{-1}\) resulting from aromatic -C=C- vibrations (Gorzsás et al., 2011). The 1510 cm\(^{-1}\) peak is further related to more condensed and cross-linked lignin (Zhong et al., 2000). Moreover, changes in the intensity ratio of the 1510 cm\(^{-1}\) and 1595 cm\(^{-1}\) peaks are related to a shift in the S/G-ratio, hence the 1510 cm\(^{-1}\) peak is associated to G-lignin, and the 1595 cm\(^{-1}\) peaks to S-lignin. One drawback of this method is that proteins could mask the lignin peaks, specifically when working with small amounts of lignin.

**Pyrolysis - GC/MS**

For the characterization and quantification of lignin in transgenic plants, pyrolysis - gas chromatography (GC)/mass spectrometry (MS) was applied in **Paper I, II and IV**. Samples were dried, ball-milled and pyrolyzed at about
450 °C. The pyrolysis degradation products were subsequently separated on a capillary GC column and detected by MS. The drawback of this method is the time consuming data analysis due to the sequential manual processing of chromatograms. However, data processing has recently been improved by multivariate curve resolution by alternate regression (MCR-AR) and automated peak assignment, which permits processing of multiple chromatograms in parallel (Gerber et al., 2012). Pyrolysis-GC/MS provides a chemical fingerprint and has been extensively applied for the compositional analysis of wood. The pyrolysis degradation products are grouped into different classes according to detected compounds with aromatic ring structures that resemble either H-, G-, S-units or generic phenolic residues as well as compounds that derive from carbohydrates. The total amount of lignin is then calculated based on the sum of detected H-, G-, S-unit and generic phenolic related compounds. A particular disadvantage of this methods is that soluble phenolics might be detected and falsely interpreted as lignin subunits. Data analysis is either performed by multivariate data analysis such as PCA and OPLS-DA, or by calculating the relative proportion compared to a reference sample, i.e. wild type.

**Extraction and Quantification of Lignin**

In Paper IV lignin was further quantified by using the thioglycolic acid (TGA) lignin method according to Antonova et al., (2007). This method was optimized to specifically extract the lignin polymer and to prevent disturbances from other cellular components. Initially non-structural proteins were extracted, followed by the degradation and extraction of cell wall components such as cellulose, hemicellulose and pectin, using the respective hydrolyzing enzymes (cellulase, hemicellulase, xylanase and pectinase). Subsequently, structural proteins were digested and extracted to prevent interference of aromatic residues from proteins. The remaining lignin was then derivatized by TGA, solubilized in NaOH and precipitated with HCl to obtain a pure lignin extract. Quantification was done by re-solubilization in DMSO and spectrophotometrical detection at 280 nm.

**Confocal Laser Scanning Microscopy**

In Paper I, II, III and IV, UV confocal laser scanning microscopy (CLSM) was applied to quantify lignin and to analyze its distribution. UV-CLSM is a reliable method for the detection of lignin in wood samples due to the UV autofluorescence of lignin (Donaldson, 2013). Lignin exhibits a distinct UV emission spectrum, with its maximum between 450 and 520 nm when excited with a 405 nm laser. This emission spectrum allows the identification and distinction of lignin from other interfering fluorescent components such as chlorophyll and permits the simultaneous application of fluorescent staining techniques. In Paper III for example, Zinnia sections were stained with the
cellulose stain Congo Red and excited with a 488 nm laser, leading to a non-overlapping emission spectrum with its maximum between 560 nm and 620 nm. The lignin UV emission spectrum further depends on the chemical composition of lignin, thus UV-CLSM can be used to detect difference in lignin composition (Donaldson, 2013). This was further observed in mutants of the lignin biosynthesis gene CCR1, which exhibits a shift of the lignin fluorescence spectrum in xylem vessels (Paper I and VI). A problem of this method is that the lignin UV fluorescence interferes with the emission spectra of proteins fluorescently labeled with CFP, YFP or GFP, which makes it difficult to detect such proteins in lignified tissues.

Transmission Electron Microscopy
In Paper III, transmission electron microscopy (TEM) was applied in combination with potassium permanganate (KMnO₄) staining in order to confirm the presence of lignin and to analyze the detailed structure of lignin. TEM allows the visualization of lignin with a high resolution and has been used for the detection of lignin in various wood samples for many years (Fromm et al., 2003). Usually, the sample is embedded in epoxy resin and subjected to ultrathin sectioning. These sections are then stained with KMnO₄, which reacts with lignin as it oxidizes the alcohol end-groups to carbonic acid and thus results in the precipitation of manganese dioxide (MnO₂) (Koch and Schmitt, 2013). During image acquisition, a beam of electrons passes through the specimen, MnO₂ absorbs electrons and allows the visualization of lignin. In order to visualize the cellulose deposition in TEs by TEM, cells were stained with periodic acid-thiocarbohydrazide silver proteinate (PATAg). This method relies on the oxidation of cellulose by periodic acid, resulting in the rupture of the carbon chain and the formation of aldehyde groups (Rutherford et al., 1942). Thiocarbohydrazide then reacts with the aldehyde groups to thiocarbohydrazones, which reduce silver proteinate, leading to the absorption of electrons (Lo et al., 1987). PATAg staining has been widely used for the detection of periodic acid-labile glycoconjugates and polysaccharides. To confirm the SCW specific deposition of phenolics, caffeine precipitation was used, which was first discovered by Mueller and Greenwood (1977). Phenolic precipitation was induced by fixation with glutaraldehyde and caffeine. Samples were embedded, sectioned and stained by PATAg or KMnO₄.

Methods for Gene Expression Analysis
In all papers the gene expression has been studied by using methods that either allow the cell/tissue specific localization of gene expression: in situ RT-PCR and promoter::GUS (β-glucoronidase) expression, or the quantification of the relative expression: RT-PCR/RT-qPCR and microarrays. In Paper I and III, in situ RT-PCR was used to analyze the gene expression, while in
Paper I, II and IV the gene expression was also studied by using the promoter::GUS reporter system. Compared to promoter::GUS expression, in situ RT-PCR is a very rapid approach that allows the detection of even lowly expressed genes (Pesquet et al., 2004). On the other hand the promoter::GUS system provides more information about the gene function and is not affected by the mRNA turnover. In situ RT-PCR is based on the incorporation of fluorescent primers during PCR amplification directly within the tissue and the subsequent visualization of the fluorescent signal by CLSM. Promoter::GUS expression is more time consuming and requires cloning and transformation of plants or cell cultures with the promoter::GUS construct. Transgenic GUS expressing plants/cells are then supplied with the substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) which is converted to the insoluble blue colored substance 5,5′-dibromo-4,4′-dichloro-indigo, formed by oxidation and dimerization of the enzyme product (Karcher, 2002).

In Paper I, III and IV semiquantitative RT-PCR and quantitative RT-qPCR was used to determine the relative gene expression. For RT-PCR, cDNA is synthesized from extracted total RNA and amplified by PCR thus providing qualitative information about the transcript abundance. RT-qPCR on the other hand provides information about the relative quantity of the transcript abundance and relies on the PCR amplification of the cDNA in the presence of a nucleic acid stain (e.g. SYBR green) that binds only to double stranded DNA. RT-PCR/RT-qPCR is however very time consuming when studying the genes expression of large amounts of genes. Microarrays offer the possibility to obtain the relative expression for many genes simultaneously. In Paper IV the differential expression between control (uninduced) and induced cultures along the Arabidopsis time course was determined by microarray expression data obtained from Derbyshire et al., (2015). Furthermore, a microarray experiment was conducted for the 14d time point of the Arabidopsis time course (at which all TEs are dead), to define genes specifically expressed in non-TEs. Moreover, microarray expression data of another Arabidopsis time course as well as a Zinnia time course are publically available (Kubo et al., 2005; Demura et al., 2002). These data have been studied in Paper I to identify non-TE expressed genes. The Affymetrix microarrays used in all these studies, provide information on the differential expression based on differently labeled mRNA probes of induced and uninduced samples. A mixture of these samples is hybridized to oligonucleotide probes immobilized on the microarray chip, thus enabling the detection of the differential expression. The Affymetrix microarray is considered as being the most widely used method to determine genome-wide expression analysis in various fields of biological science (Jiang et al., 2008; Izarry et al., 2003). Alternatively to microarrays, suppression subtractive hybridization (SSH) libraries were constructed in Paper I to assess the differential expression between induced Zinnia cultures inhibited to perform PCD using STS and normally induced
cultures. In this method suppression PCR is applied on pooled cDNA from control samples and STS treated samples, which results in the hybridization and elimination of equally abundant mRNA/cDNA (Diatchenko et al., 1996). The remaining cDNA represents the differential expressed genes and is analyzed by amplification, cloning and sequencing (Laitinen et al, 2005). SSH libraries are an alternative method to microarrays for the detection of differential gene expression and may be especially useful for the identification of novel genes and genes with very low transcript abundance (Cao et al., 2004).

**Genetic Methods**

In **Paper I and IV** reverse genetic methods were applied to study the role of XP expressed genes during lignification. In **Paper I** the implication of XP expressed genes in lignification was assessed by pyrolysis-GC/MS analysis of their respective T-DNA insertion mutants. T-DNA mutants of nearly all Arabidopsis genes are publically available through the SALK Institute Genomic Analysis Laboratory (http://signal.salk.edu/). Moreover, T-DNA insertion mutants have been widely used in plant research and effectively lead to the loss of gene function through disruption of the gene expression (Gilchrist and Haughn, 2010; Krysan et al., 1999). Such mutants can be screened simply by using T-DNA specific primers. Drawbacks of this method are that the mutation can be lethal due to the complete loss of function and phenotypes may not be visible because of gene redundancy (Gilchrist and Haughn, 2010). This, however is not the case for the mutants studied in this thesis. In **Paper IV** mutants of the CCR1 gene, which display severe growth defects, were complemented with the full-genomic clone, driven by its native promoter or by a promoter that is specifically expressed in XP cells, to demonstrate the cooperative supply of lignin monomers. Besides using multiple mutant alleles, mutant complementation has been reported to definitely prove that the observed mutant phenotype is caused by the mutation of the particular gene (Krysan et al., 1999).

**Pharmacological Treatments**

In all papers different enzyme inhibitors were used to investigate the lignification process, or to block specific features of cells. In **Paper I** the inhibitor STS was applied to inhibit PCD during TE differentiation. STS inhibits the perception of ethylene due to the silver (Ag+) component of STS (Nukui et al., 2000). Ethylene is specifically produced during TE maturation and was found to regulate PCD in a positive manner (Pesquet and Tuominen, 2011; Bouchez et al., 2007). Another inhibitor, used in **Paper I, II, III and IV** was piperonylic acid (PA), which is an inhibitor of C4H and results in the inhibition of lignin monomer synthesis and lignification (Schalk et al., 1998; Schoch et al., 2002; Salvador et al., 2013). Several inhibitors were further
applied in Paper III to inhibit either laccases, peroxidases or both, to identify the role of these enzymes during post-mortem TE lignification. Sodium azide (NaN₃) and hydroxylamine (HL) are both general phenoloxidase inhibitors and inhibit peroxidases as well as laccases. NaN₃ was found to inhibit the activity of horseradish peroxidase (Ortiz de Montello et al., 1988), lignin peroxidase H2 from Phanerochaete chrysosporium (Tuisel et al., 1991) and laccases from Trametes versicolor (Johannes and Majcherczyk, 2000). HL was shown to inhibit peroxidases from Arthromyces ramosus (Wariishi et al., 2000) and, together with NaN₃, laccases from Coriolus versicolor (Mishra and Kumar, 2009). Laccases from peach, spruce and Rhus vernicifera were further inhibited by cetyltrimethylammonium bromide (CTAB) (Walker and McCallion, 1980). Moreover, laccases from Chaetomium thermophilium and Sclerotium rolfsii were inhibited by TGA (Chefetz et al., 1998; Ryan et al., 2003). CTAB and TGA appear to be very specific to laccases as no other phenoloxidases could be found to be inhibited by these compounds. In contrast the compound salicylhydroxamic acid (SHAM) was demonstrated to be a specific inhibitor of peroxidases in maize and Arabidopsis (Hemetsberger et al., 2012; Lee et al., 2013). Although several of the inhibitor studies used phenoloxidases from fungi or algae, these inhibitors potentially act upon plant phenoloxidases as well, due to the universal function of these compounds: all inhibitors are chelating agents that bind metal ions such as copper or iron, which are essential co-factors of laccases and peroxidases, respectively. To investigate the role of ROS during lignification in Paper I and III, the inhibitor DPI was applied to TE differentiating cell cultures. DPI has been reported to specifically inhibit NADPH oxidases through covalent binding to the enzyme and thus prevents the production of superoxide (Jones et al., 2000; O’Donnell et al., 1993). To further define the role of other types of ROS, the general ROS scavenger N-acetylcysteine (NAC) was supplied to TE differentiating cultures. NAC is a precursor of cysteine and/or glutathione compounds, which have well known antioxidant properties (Sun, 2010).

Besides these inhibitors, lignin monomers were applied to TE differentiating cultures in order to induce lignification on demand. In Paper I, II, III and IV coniferyl alcohol was used, while sinapyl alcohol was also supplied in Paper I. Both compounds have been shown to form DHPs in vitro in the presence of phenoloxidases (Sterjiades et al., 1992; 1993) and accumulate in the extracellular medium of lignifying Zinnia TE differentiating cultures (Hosokawa et al., 2001). In Paper III the fluorescently labeled lignin monomer: γ-nitrobenzofuran-tagged coniferyl alcohol (NBD-CA) was applied in combination with laccase and/or peroxidase inhibitors, to define the preference of NBD-CA for either type of enzyme. The successful incorporation of NBD-CA into lignin has recently been described, both in vitro and in whole plants of Arabidopsis and pine (Tobimatsu et al., 2011, 2013; Schuetz et al., 2015).
Results and Discussion

In this section the results of this thesis project are presented and discussed. The results are based on four main papers/manuscripts to which I will refer to. These papers/manuscripts aim to answer the research questions stated in the aim and objectives section.

1. **When does TE lignification occur?**

**Paper I and II** have shown that TE lignification occurs *post-mortem* using the *Zinnia* TE differentiation system as well as whole *Zinnia* plants. In **Paper III** the *post-mortem* lignification was confirmed in *Zinnia* TE differentiating cultures and the progression of lignification was investigated in whole plants. **Paper IV** revealed further that TE lignification continues long after TE PCD using *Arabidopsis* TE differentiating cultures.

2. **How does lignification operate at the cellular level?**

**Paper I** describes that the *post-mortem* lignification occurs through the cooperative supply of lignin monomers and ROS from surrounding parenchymatic cells using *Zinnia in vitro* TE differentiating cultures. In **Paper IV** the cooperative supply of lignin monomers by the non-TEs was confirmed using *Arabidopsis* TE differentiating cultures. Characterization of the non-TEs revealed further that some of the non-TEs are in fact differentiated endoreplicated XP. In whole plants of *Arabidopsis* the cooperative lignification was further confirmed by the XP specific complementation of lignin monomer synthesis mutants.

3. **What is the spatial restriction of lignification?**

**Paper III** shows that lignin is restricted to SCWs in *Zinnia* primary xylem vessels of whole plants and *in vitro* TE cultures. These deposition domains are produced by the differential distribution of laccases and peroxidases. Laccases and peroxidases further contribute to the *post-mortem* lignification of TEs in which laccases play a major role. Moreover, laccases were synthesized cell-autonomously by developing TEs, indicating that the deposition domains are defined before cell death. The depositions domains were further produced independently of the monomer availability.
1. Lignification occurs and progresses post-mortem

1.1. Post-mortem lignification in in vitro TE cultures

Lignification of TEs in differentiating *Zinnia* cultures has been investigated in several studies (Fukuda and Komamine, 1982; Hosokawa *et al.*, 2001; Sato *et al.*, 2011). In these studies lignification appeared after 60 hours and progressed up to 120 hours of culture. Groover *et al.*, (1997) showed that TE PCD occurs between 60 and 72 hours after induction, which is just at the beginning of lignification. Therefore these results indicate that lignification occurs mostly after PCD. The exact timing of lignification in relation to PCD has, however, never been defined clearly. Live-cell imaging of TE differentiation in *Arabidopsis* TE differentiating cultures provided the first evidence that lignification directly follows PCD (Pesquet *et al.*, 2010). The post-mortem lignification was further studied in *Zinnia* TE differentiating cultures using the viability stain fluorescein diacetate (FDA) and the UV-CLSM-assisted detection of lignin autofluorescence (Pesquet and Tuominen, 2011; *Paper IV*, Figure 1). To confirm that TEs are able to lignify post-mortem, TEs were initially inhibited to lignify using the lignin monomer synthesis inhibitor PA and supplied with the lignin monomers coniferyl alcohol and/or sinapyl alcohol once all TEs have died. Phloroglucinol-HCl staining and FT-IR microspectroscopy of these TEs revealed that they lignified after TE cell death (*Paper I*, Figure 1A-D and G-K). In the same manner, post-mortem lignification was confirmed by the non-overlapping fluorescent staining of cellulose with Congo Red and lignin autofluorescent analysis using CSLM of dead *Zinnia in vitro* protoxylem and metaxylem TEs inhibited to lignify and supplied with coniferyl alcohol (*Paper III*, Figure 4A-M). Moreover, PA treated *Zinnia* TE differentiating cultures were incubated with NBD-CA before TE PCD (*Paper III*, data not shown). Interestingly, these living TEs were not able to incorporate the fluorescent monomers in their SCWs, which demonstrates that PCD is required for the initiation of lignification. To further investigate the role of PCD during TE differentiation, TEs were inhibited to perform PCD using STS. TE lignification was estimated by UV-CLSM, showing that the TEs were not lignified (*Paper I*, Figure 3H), which confirms that PCD is required for the initiation of TE lignification. To further examine if monomer synthesis and polymerization were inhibited by the STS treatment, the extracellular medium of 120h-old STS treated TE differentiating cultures was supplied to PA treated un lignified TEs. In these cultures TEs became lignified, pointing out that monolignol production was not inhibited by STS (*Paper I*, Figure 3N-3P). Consequently, PCD seems to be required to initiate the polymerization of lignin monomers. ROS have been associated to PCD (Vianello *et al.*, 2007) and are necessary for the oxidation of lignin monomers by phenoloxidases. Therefore, ROS could be released.
during PCD and subsequently induce monomer radical activation and polymerization through phenoloxidases.

The post-mortem lignification of TEs was further demonstrated by Congo Red staining of cellulose and lignin autofluorescence using CLSM of Zinnia in vitro protoxylem and metaxylem vessels before and after PCD (Paper III, Figure 2A). SCW-cellulose fluorescence was correlated with the formation of the secondary thickenings, while lignification appeared 8 hours after initiation of SCW-cellulose deposition and only once PCD has occurred. These results are coherent with those of Groover et al., (1997) who stated that SCW formation requires about 6 hours. To further confirm that the precise deposition of lignin in TE SCWs was not an artefact of the cell wall autofluorescence, lignin was stained with KMnO₄ and visualized by TEM. Lignin was only present in dead 96h-old TEs, whereas living 60h-old TEs did not show any sign of lignification (Paper III, Figure 1C-D). Altogether, these results are in agreement with several other studies in Zinnia TE differentiating cultures (Ingold et al., 1990; Fukuda and Komamine 1982; Hosokawa et al., 2001), which showed that lignin is deposited after the polysaccharidic matrix has been laid down.

![Figure 11. Post-mortem lignification in Arabidopsis TE differentiating cultures. A, Different cell types observed in differentiating TE cultures; nucleus is visualized by Histone (H2B)-RFP fluorescence; cellulose is stained with Evans blue; lignin is detected by UV autofluorescence. B, Viability of TEs and non-TEs along TE differentiation, determined by FDA staining. C, Lignin along TE differentiation quantified by the TGA method. Error bars ± SD, asterisks indicate t-test with Welch correlation (* p < 0.05; ** p < 0.01). Paper IV, Figure 1.](image-url)
To further confirm the *post-mortem* lignification with the *Arabidopsis in vitro* TE differentiation system, lignin was quantified along the TE differentiation time course from 0 to 9 days and at 14 and 21 days, by using the TGA lignin method. In parallel the TE viability was assessed by using the viability stain FDA. TE cell death inversely progressed exponentially from 100% living TEs after 3 days of induction to around 12% after 5 days, whereas TE lignification appeared only after 5 days (Figure 11B-C) (Paper IV, Figure 1C-D). Subsequently, TE lignification progressed linearly, at a rate of 1% per day per TE, up to 16 days after PCD to finally comprise 18% of the TE cell wall, which is comparable to the amount of lignin in stems of whole *Arabidopsis* plants (16-20%) (Eudes *et al.*, 2006; Schilmiller *et al.*, 2009). In order to confirm that the increase in TGA lignin is caused by an increase of TE SCW lignification, the lignin UV fluorescence of TE cell walls was measured by UV-CLSM along the time course, 7, 14 and 21 days after induction. The results confirmed that lignification increased only in the SCW of TEs (Paper IV, Extended Figure 1C-D) and correlated with the amount of lignin determined by the TGA lignin method ($R^2=0.99$, data not shown).

### 1.2. *Post-mortem* lignification in whole plants

To confirm the *post-mortem* lignification in whole plants, early protoxylem and metaxylem vessels from sequential *Zinnia* internode sections were analysed using FT-IR microspectroscopy (Figure 12) (Paper I, Figure 2). These vessels mature and lignify rapidly after their formation and they can be easily distinguished as they are separated from the other lignifying cells, as shown by phloroglucinol-HCl staining (Figure 12C-F). To quantify the lignin in these vessels, the peak area of the $1510 \text{ cm}^{-1}$ peak was measured at four different internodes. The $1510 \text{ cm}^{-1}$ is specific to G-lignin, which is the major type of lignin in vessel elements. The peak area increased from younger to older proto- and metaxylem vessels along the vertical axis of the plant, thereby indicating that these TE lignify *post-mortem* (Figure 12B) (Paper I, Figure 2B). Because protoxylem vessels become crushed during later stages, when the xylem expands, protoxylem vessels could not be measured at the 4th internode. To provide further evidence for the occurrence of lignification after PCD in *Zinnia* plants, polyphenolics were precipitated in cross sections, 1 cm below the shoot apex by using caffeine. Lignification, visualized by TEM, was only restricted to TE SCWs (Paper III, Figure 4N-O), thus showing that lignin deposition domains that facilitate the *post-mortem* lignification are conserved in TE SCWs after PCD. In order to monitor the progression of lignification in newly developing TEs, lignin and cellulose were quantified in serial sections of the shoot apical meristem of *Zinnia* plants by applying Congo Red staining and CLSM of the lignin UV fluorescence. Lignification appeared in the SCW of TEs, once SCW-cellulose has been deposited and increased
while progressing further away from the shoot apical meristem (Paper III, figure 3C,F). After an initial exponential phase between 500-600µm from the meristem, lignification progressed linearly with a rate of 7.5% every 300µm. These results are in agreement with the linear progression observed in Arabidopsis cell cultures and therefore confirm the post-mortem lignification in Zinnia proto- and metaxylem TEs. Altogether, lignification of primary xylem TEs occurs in two phases: an initial exponential phase and a following linear phase. The initial appearance of lignin in the SCW of primary xylem tissues indicates that, unlike in secondary xylem where lignification starts in the middle lamella, lignification begins in the SCW. This further suggests that in primary xylem the middle lamella lignifies later, when multiple TEs assemble to form an intact vascular bundle. The differential quantity and distribution of lignin between different cell types and cell wall compartments supports this hypothesis (Paper II, Figure 1). Hence, these findings indicates that lignification of different cell types and/or cell wall compartments is regulated by different mechanisms (see section 3).

The results clearly confirm that lignification occurs only post-mortem. In contrast, other previous studies suggested that lignification occurs not only after, but also before PCD (Pickett-Heaps, 1968; Terashima and Fukushima, 1988; Smith et al., 2013). All these studies have in common the use of radiolabeling experiments with 3H labeled monomer precursors (i.e. 3H-cinnamic acid, 3H-phenylalanine or 3H p-coumaric acid). The radiolabeling of monolignols, however, does not allow to decipher between lignin precursors such as monomers or oligomers and the lignin polymer. Therefore, the signal may be due to the accumulation of lignin precursors in the wall prior to polymerization. Our studies are based on the lignin UV fluorescence and on
staining lignin with KMnO₄ in combination with TEM. These methods do not interfere with the detection of lignin precursors, as they cannot be detected by UV fluorescence or TEM. Furthermore, we used biochemical methods such as FT-IR spectroscopy and the TGA lignin method. FT-IR spectroscopy has been shown to specifically detect the lignin polymer (Gorszás et al., 2010) and the TGA method was optimized to eliminate other interfering compounds prior to the extraction and quantification of lignin (see methods section). Another study, also based on radiolabeling experiments, showed label incorporation in the PCW, between the SCW thickenings of developing protoxylem vessels (Woodings, 1968). These results are contradictory to our results (see results section 3) as well as to the results of several other studies (Hepler, 1970; Taylor et al., 1992; Smith et al., 2013) where lignin is only deposited in the secondary thickenings of protoxylem vessels. Altogether, this suggests that feeding radiolabeled monolignols may lead to false interpretations due to the detection of lignin precursors. Furthermore, the results of the Zinnia and Arabidopsis TE differentiation cultures indicated that PCD initiates lignification, possibly by the release of ROS, which are required for lignin monomer oxidizing enzymes and therefore catalyse lignin polymerization. This would imply that lignification can only occur once PCD has been executed.

As previously mentioned TE SCW lignification progresses long after PCD (Paper III, Figure 3; Paper IV, Extended Figure 1). While the SCW does not increase in size after initiation of lignification, this indicates a reduction of the intermolecular spaces between cell wall polysaccharides and lignin. This, in turn, would further results in the reduced accessibility for newly secreted lignin precursors. The results of Nakashima et al., (1997) support this hypothesis, showing that lignin completely fills the intra-microfibrilar spaces during later stages of lignification. In this regard π-π-stacking of aromatic ring structures may contribute to adapt the irregular lignin structure to the aligned microfibrils (Bylin et al., 2014). Based on the hydrophobic nature of the lignin molecule, the increase in lignin causes a decrease in the water content of the SCW, which was formerly bound to polysaccharides as the result of their hydrophilic properties. Thus the overall hydrophobicity of the SCW increases over time, which could have negative effects on the sap transport as it may result in cavitation (Sperry, 2004). Cavitation leads to the formation of small vapour bubbles that generate mechanical friction and therefore reduces the sap flow and possibly damages the conduits. The SCW, however, is estimated to contain up to 25% lignin (Sjöström, 1993), while the rest are hydrophilic polysaccharides that attract water. It therefore appears that SCW lignification is limited by steric hindrance, to maintain optimal properties for sap conduction.
2. TE lignification is a cooperative process

In in vitro cultures, the post-mortem lignification of TEs implies that the lignin monomers are either supplied by the dying TEs or by the surrounding living non-TEs. The progression of lignification long after PCD as shown in the Arabidopsis TE culture system (Paper IV, Figure 1) and in protoxylem vessels of whole Zinnia plants (Paper III, Figure 3) suggests at least a partial supply of monomers by the surrounding non-TEs for post-mortem TE lignification. This is further supported by the prolonged activity of the lignin biosynthesis proteins PAL (Fukuda and Komanine, 1982), C4H (Ye et al., 1996), and CAD (Sato et al., 1997), long after TE cell death in Zinnia TE cultures. Multiplex in-situ RT-PCR expression analysis in Zinnia TE differentiating cultures and in stems of whole plants revealed further that the genes CAD and CCR were expressed in both TEs and non-TEs as well as in XP next to xylem vessels (Paper I, Figure 3, Supplemental Figure 4). Promoter::GUS expression analysis in 5d-old Arabidopsis TE differentiating cultures indicated further that CCR1 is certainly expressed in both TEs and non-TEs (Paper IV, Extended Figure 4). In hypocotyl and stem sections of Arabidopsis, C4H was confirmed to be expressed in XP using the promoter::GUS reporter system (Paper I, Figure 8; Paper II, Figure 3). Promoter::GUS analysis of CCR1 in Arabidopsis stems showed expression in newly forming xylem cells, which does not allow a clear identification of the exact cell type and suggests that they are expressed in all xylem cells (i.e. TEs, XP or XF s). Nevertheless, in the hypocotyl CCR1 was expressed in XP next to TEs (Paper IV, extended Figure 8).

2.1. Cooperative lignification in in vitro TE cultures

More evidence for the production of monomers by the non-TEs came from the accumulation of lignin monomers such as coniferyl and sinapyl alcohol in the extracellular medium of Zinnia TE differentiating cultures beyond TE cell death (Hosokawa et al., 2001; Ito et al., 2004; Tokunaga et al., 2005). The quantification of phenolics in the extracellular medium of Arabidopsis TE differentiating cultures, using the Folin Ciocalteau method, revealed that phenolics were exported even after most of the TEs were dead (after 5 days) (Paper IV, Figure 1). Total phenolics in the extracellular medium increased after 4 days (when TEs start to form), reached the plateau after about 8 days and then decreased up to 21 days, potentially due to the incorporation into lignin. The export of phenolics was specific to the extracellular medium of induced cultures, non-induced control cultures did not show considerable changes along the time course. Interestingly, the amount of intracellular total phenolics was not altered during TE differentiation, supporting that TEs do not alone provide the lignin precursors. Likewise, the intracellular phenolic
content of induced *Zinnia* TE differentiating cultures did not increase substantially, non-induced cultures however did show a slight increase (Fukuda and Komamine, 1982). The export of phenolics by the non-TEs was further tested by using *Zinnia* TE differentiating cultures that were inhibited to lignify by supplying PA. The inhibition was washed away once all TEs were dead and only non-TEs remain alive. The amount of lignified TEs was determined 24h, 48h, 72h and 96h after the wash, by using the lignin stain phloroglucinol-HCl. The results revealed an increase of lignified TEs up to 96h, thereby showing that the non-TEs export lignin monomers for the *post-mortem* lignification of TEs (*Paper I*, Figure 7A). To confirm these results the same experiment was performed with the *Arabidopsis* TE differentiation system. In these cultures the extracellular total phenolics and lignin were quantified 0, 7 and 14d after the wash, using the Folin Ciocalteau and the TGA lignin method, respectively. Both, total extracellular phenolics and lignin increased after 7 and 14d which confirms that the non-TEs in fact export lignin monomers into the newly supplied medium for the *post-mortem* lignification of TEs (Figure 13A,B) (*Paper IV*, Extended Figure 9C-D). Nevertheless, lignification in these cultures only reached about 1.8 mg lignin per g cell wall, which is considerably lower than 7d-old induced cultures (15 mg/g). This small increase in lignin could be explained by the fact that not all non-TEs are XP cells which actively export lignin monomers. The amount of GUS expressing non-TEs argues for this hypothesis, showing that only 2-8% of the non-TEs express the endoreplication specific gene SIAMESE1 (*SIM1*), which is specifically expressed in XP cells (Figure 14). Moreover, not all non-TEs have larger nuclei as shown by the distribution of nucleus area over cell area for non-induced cells, TEs and non-TEs (Figure 16).
(Paper IV, Figure 3). However, the small increase in lignin could further be the result of the removal of the extracellular medium, which may contain signalling molecules that induce the production of monomers by XP. For instance, during TE differentiation or cell death such signalling molecules could be released. This would further suggest the temporal coordination of TE formation and the production of monomers by XP.

To confirm that the non-TEs are required for post-mortem TE lignification, Arabidopsis TE differentiating cultures were inhibited to lignify using PA. When all TEs were dead, the cultures were supplied with saturating amounts of coniferyl alcohol together with PA, in the presence of either living or dead non-TEs. Lignin in these cultures was assessed by the TGA method, revealing that in the presence of dead non-TEs lignification was reduced 10 fold compared to the living control (Figure 13C) (Paper IV, Figure 3). These results indicate that the presence of the non-TEs is crucial for post-mortem lignification of TEs and suggests further that the non-TEs produce other substrates required for lignification, such as ROS. Another possibility is that coniferyl alcohol is not the “real” lignin monomer and is converted by the non-TEs to produce the appropriate monomer or oligomer, which is then incorporated into lignin. The conversion of coniferyl alcohol to other modified precursors appears reasonable when considering that nucleation sites are required for the initiation of lignification. Such nucleation sites can be formed by various modified lignin precursors such as dilignols of coniferyl alcohol and ferulic acid or di-ferulate esters (Ralph et al., 1995, 2004; Terashima et al., 2012). Furthermore, PA only inhibits C4H but not the downstream enzymes of the phenylpropanoid pathway. Consequently, the downstream enzymes could convert coniferyl alcohol to other lignin precursors, e.g. the enzymes F5H and COMT are able to convert coniferyl alcohol to sinapyl alcohol (Figure 4). FT-IR microspectroscopy of dead unlignified TEs, supplied with coniferyl and sinapyl alcohol, showed that FT-IR spectra of such TEs resemble the ones of normally lignified TEs, whereas the spectra of TEs supplied with either coniferyl or sinapyl alcohol were different (Paper II, Figure 2C). Together, this supports the hypothesis that more than one type of monomer is required for lignification and that coniferyl alcohol could be converted to other substrates by the non-TEs. Regarding the lignin quantity in the presence of living non-TEs, lignification could not be fully restored and was about three fourth of the lignin of normally induced control cultures, although saturating amounts of monomers were supplied. Apparently, coniferyl alcohol and eventually modified coniferyl alcohol derivatives are not sufficient to achieve full lignification, probably because the C4H inhibition prevents the production of other downstream substrates and/or lignin precursors that are incorporated during TE lignification under normal conditions.
2.2. Cooperative supply of reactive oxygen species

As previously suggested ROS, i.e. secondary substrates of the phenoloxidases, could be the limiting factor that restricts lignification of TEs. The ROS H$_2$O$_2$, required for peroxidases, has been shown to be produced primarily by non-TEs (Gómez Ros et al., 2006; Ros Barceló et al., 2005) and is potentially produced by the two enzymes NADPH oxidase and SOD (Ogawa et al., 1997; Karlsson et al., 2005). To determine the role of ROS/H$_2$O$_2$ during post-mortem lignification of TEs, enzymes or inhibitors of ROS were applied to Zinnia TE differentiating cultures, such as the general ROS scavenger NAC, the H$_2$O$_2$ hydrolysing enzyme CAT and the NADPH oxidase inhibitor DPI. Zinnia TE differentiating cultures were inhibited to lignify with PA and supplied with coniferyl alcohol when all TEs were dead, in the presence of either of the inhibitors mentioned above (Paper I, Figure 7B). NAC and DPI reduced the amount of lignified TEs drastically, as assessed by phloroglucinol-HCl staining. The supply of DPI together with NBD-CA to PA-treated cultures further confirmed the reduction of lignin in the SCW of TEs (Paper III, Figure 7E). These results confirm that ROS are required for lignification and that NADPH oxidase is indeed involved in post-mortem TE lignification. The treatment with CAT showed only a slight reduction in the amount of lignified TEs. Together, these results are coherent with a similar study using the Zinnia TE differentiation system, which showed that the supply of DPI, the SOD inhibitor DDC as well as the H$_2$O$_2$ scavengers CAT, ascorbate and reduced glutathione, caused a decrease in the presence of H$_2$O$_2$ and TE SCW lignification (Karlsson et al., 2005). Together with our results this approves that non-TEs provide H$_2$O$_2$, which is produced by NADPH oxidase and SOD for the post-mortem lignification of TEs. The slight reduction of lignification by the supply of CAT, which converts H$_2$O$_2$ to oxygen, suggests further that H$_2$O$_2$ is not the only substrate required for post-mortem TE lignification. Because monomer oxidation is not only restricted to peroxidases but also involves laccases, which require molecular oxygen, the presence of oxygen may play an important role as well. In this regard CAT may disturb the balance between the presence of H$_2$O$_2$ and oxygen in the medium, required to allow radical activation by both, peroxidases and laccases.

2.3. Cooperative lignification in whole plants

In order to investigate the cooperative lignification in whole plants, XP/non-TE specific genes were identified for subsequent genetic modulation. The identification of non-TE specific genes was facilitated by analysing the expression of the SSH library genes from Paper I (Pesquet et al., 2013) together with publicly available expression databases of Zinnia and Arabidopsis in vitro TE differentiation time courses (Demura et al., 2002;
Kubo et al., 2005). The criteria for the identification were: i) the gene showed expression after TE PCD (after 72h for Zinnia and after 8d for Arabidopsis) and ii) the expression was at least 75% of the maximal expression before TE PCD. In Zinnia 57.3% fulfilled this criteria while in Arabidopsis 50% of the genes were considered non-TE specific. A set of 20 genes were selected due to their non-TE characteristic expression profiles. To test their contribution to TE lignification, T-DNA mutants of their Arabidopsis homologs were analysed for lignin amount and composition in hypocotyls using pyrolysis-GC/MS. Mutants of the monomer synthesis genes c4h-3 and ccr1-3, which are also considered to be expressed in XP, were included as control. The most interesting results were obtained for the mutant of the transcription factor MYB13 (myb13) and the mutant of RADICAL-INDUCED CELL DEATH1 (RCD1) (rcd1-1) which exhibited significant changes in lignin content and composition. The myb13 mutant showed an overall reduction in lignin caused by a decrease in G-units, whereas the rcd1-1 mutant displayed an increase of lignin due to more G- and H-units (Paper I, Figure 9; Paper II, Figure 3). The XP specific expression of MYB13 and RCD1 was verified by promoter::GUS expression analysis in stems, hypocotyls and 7d-old seedlings of Arabidopsis. Both RCD1 and MYB13 were found to be expressed in the vascular cambium and in XP of 8-weeks old stems and hypocotyls (Paper I, Figure 4; Paper II, Figure 3). In seedlings RCD1 and MYB13 were expressed almost along the entire root. In cotyledons RCD1 expression could not be detected, whereas MYB13 was expressed in the vasculature in which TEs were already dead, as defined by XCP2 expression along a seedling time course (Paper IV, Extended Figure 7), thus confirming the XP specific expression of MYB13.

RCD1 belongs to the poly(ADP-ribose) polymerase superfamily and has been shown to play a role in controlling oxidative stress for example during ozone-induced PCD (Ovemeyer et al., 2000). The rcd1 mutant accumulates ROS and nitric oxide which suggests that the protein prevents the overaccumulation of these oxidizing species. Moreover, RCD1 is involved in regulating cell division for the coordinated differentiation of xylem vessels and fibers (Teotia and Lamb, 2011). As a conclusion, RCD1 functions in a pleiotropic manner to: i) control the overproduction of ROS, to constrain lignin polymerization, and ii) regulate the differentiation of xylem vessels and fibers. This may further explain the expression of RCD1 in two different cell types, i.e. cambial cells and XP. Supposedly, cambial cells are involved in the differentiation of vessels and fibers, whereas XP regulate the production of ROS. MYB13 is a transcription factor that belongs to the R2R3 gene family which shows similarity to MYB58 and MYB63, two major transcription factors that activate the expression of nearly all monomer synthesis genes (Zhou et al., 2009). This suggests that MYB13 is involved in the production and supply of lignin monomers by XP for the post-mortem lignification of TEs. Another
gene that exhibited a non-TE expression profile was COBALAMIN BIOSYNTHETIC PROTEIN (CBP). Mutation of this gene caused an increase of lignin based on the increased abundance of G- and H-units. In prokaryotes cobalamin (vitamin B12) acts as co-factor (Nahvi et al., 2004), consequently mutation of this gene could lead to the inactivation of proteins or transcription factors that negatively regulate lignification. Altogether, these results have shown that these newly identified XP specific genes are implicated in controlling lignification, although their function remains to be elucidated.

To further confirm that XP contribute to xylem vessel lignification by supplying lignin monomers, T-DNA insertion mutants of the monomer synthesis gene CCR1 were complemented with the full-genomic clone of CCR1 driven by a XP specific promoter. Because the previously identified XP specific genes RCD1 and MYB13 were also expressed in other tissues or cell types, these genes did not seem to be the right candidates for this approach. The SIM1 gene on the other hand appeared to be a much better candidate. qRT-PCR expression along the Arabidopsis TE differentiation time course showed an XP characteristic expression profile with expression beyond PCD (Figure 14A) (Paper IV, Extended Figure 3B). Promoter::GUS expression in Arabidopsis TE cultures confirmed that the SIM1 promoter (2000 bp upstream of the gene) was only expressed in non-TEs (Figure 14B) (Paper IV, Figure 3B). Moreover, promoter::GUS expression in 6-weeks old stems and hypocotyls of Arabidopsis plants verified that SIM1 is specific to XP, except for some expression in the phloem (Figure 14C) (Paper IV, Extended Figure 9). Therefore, the SIM1 promoter has been selected for the XP specific complementation of two mutant alleles of CCR1 (ccr1-3 and ccr1-6). To
confirm the function of the native CCR1 promoter, the CCR1 mutants were also complemented with the CCR1 gene, driven by its native promoter (500 bp upstream of the gene). Phenotypic characterisation of the SIM1 driven CCR1 complemented mutants as well as mutants complemented with CCR1 driven by its native promoter, revealed that the severe phenotypic defects of the CCR1 mutant (reduced stem height, silique length, and lignification as well as collapsed xylem vessels) were nearly fully restored to the wild type (Figure 15A-B) (Paper IV, Figure 4A-B and Extended Figure 11A-C). Determination of the lignin quantity and composition in 8-weeks old hypocotyls by pyrolysis-GC/MS revealed that the total amount of lignin was restored to 70-80% of the wild type (Paper IV, Figure 4C). The restored lignification was due to an increase in both G- and S-units with an S/G-ratio that is completely restored (Figure 15C). These results indicate that XP provide both G- and S-lignin precursors. Lignification was further analysed in stems using the TGA method, which showed that the lignin content in stems was completely restored (90-100%) (Paper IV, Extended Figure 11H). Extraction and quantification of total phenolics from these stems using the Folin Ciocalteau method revealed further an increase of total phenolics in the mutant plants (Paper IV, Extended Figure 11G). This increase is suggested to be related to a redirection of the lignin monomer synthesis pathway to the production of normally less abundant phenolic compounds such as feruloyl-malate (Mir Derikvand et al., 2008). The XP specific complementation restored the phenolic content to the wild type levels. Altogether these results confirm that the promoters used are fully functional and that XP provide lignin precursors to TEs.
To further identify the distribution of lignin in the complemented mutants, stem sections were analysed by UV-CLSM (Paper IV, Extended Figure 11I-J). Mutants exhibited a decrease in lignification of the SCW and middle lamella of IFs, which has already been reported previously (Jones et al., 2001; Smith et al., 2013). In contrast, xylem vessel SCWs appeared to have proportionally more lignin than the wild type. However, it needs to be emphasised that this is only a proportional comparison with other cell types, the absolute lignin quantity is lower in the mutant. The XP specific complementation recovered the proportional change in xylem vessel lignification of mutants by nearly 100%. IF lignification was also restored although the recovery was only about 60%. The lignin of CCR1 mutants has specific characteristics and exhibits a fluorescent shift of the lignin UV emission spectra probably caused by the incorporation of ferulic acid into the lignin polymer (Vanholme et al., 2010). To further confirm that xylem vessel lignification is restored by the XP specific complementation, the UV fluorescent spectra were analysed by UV-CLSM. Figure 14D (Paper IV, Figure 4E-F) shows that the lignin properties in xylem vessels were completely recovered by having normal wild type UV emission spectra. Overall these results provide evidence for the XP mediated supply of lignin precursors to xylem vessels for lignification of the SCW. These results further confirm the
results obtained by the \textit{in vitro} TE differentiation system. In addition, these results are in agreement with another study, in which \textit{CCR1} specific artificial microRNA was expressed specifically in SCW forming cells using the \textit{IRX3} promoter, leaving the \textit{CCR1} expression in XP unaffected (Smith \textit{et al.}, 2013). In these plants, phloroglucinol-HCl staining revealed that xylem vessels appeared to have normal wild type lignification and the \textit{irx} phenotype of the mutant was recovered. Furthermore, these plants showed a normal wild type phenotype, except for a higher flexibility of the stem, which has been associated to reduced IF lignification (Smith \textit{et al.}, 2013). Altogether these results support that XP contribute to xylem vessel lignification.

In the \textit{SIM1} complemented mutants, lignification was also partially restored in the middle lamella and SCW of IFs, showing that XP are able to supply fibers with lignin precursors as well, although to a slightly lower extent. Smith \textit{et al.}, (2013), however, states that IF lignification is cell-autonomous, based on the results obtained from the \textit{IRX3} driven \textit{CCR1} artificial microRNA, which is expressed in IFs. Similarly to the \textit{CCR1} mutants, the downregulation of \textit{CCR1} caused a decrease in lignification mostly in IFs as shown by phloroglucinol-HCl staining. Nevertheless, phloroglucinol-HCl staining does not allow the quantification of lignin and is dependent on the lignin structure, \textit{i.e.} phloroglucinol-HCl reacts only with hydroxycinnamaldehyde end groups (Weng \textit{et al.}, 2010). Moreover, the IFs in these plants still appear lignified which suggests that XP supply lignin precursors to IFs as well. The XP expression is not affected by the \textit{IRX3} driven \textit{CCR1} downregulation, promoter::GUS expression of \textit{IRX3} in stems and hypocotyls did not reveal any expression in XP cells (\textbf{Paper IV}, Extended Figure 8F). Additionally, the artificial microRNA expressing plants exhibit a reduction in G-units and an increase of S-units (Supplemental Table 1, Smith \textit{et al.}, 2013). This indicates further that the lignin content of fibers, which are mostly composed of S-units, is not as strongly affected as believed. Altogether, the study of Smith \textit{et al.}, (2013) does not exclude the partial cooperative lignification of IFs through the supply of monomers by XP. The lignin of XFs that are next to vessels has an intermediate lignin composition enriched in G-units, thereby indicating that these fibers lignify through the partially cooperative supply of lignin precursors from neighbouring living vessel elements (Gorszás \textit{et al.}, 2011). It therefore appears that fibers in general have the capacity to lignify through the partial cooperative supply of lignin monomers by other cell types, \textit{i.e.} XP and living xylem vessels. The complementation of \textit{C4H} knock-out mutants with the \textit{VND6}-promoter driving \textit{C4H}, restores the lignification in IFs and XFs (Yang \textit{et al.}, 2013). Because \textit{VND6} is expressed in the vascular cambium and in metaxylem vessels, this further supports that fiber lignification is facilitated by the partially cooperative supply of lignin precursors by living metaxylem vessels and potentially cambial cells as well.
2.4. Characteristics of the non-TEs

As previously shown, XP cells specifically express the SIM1 gene. SIM1 is localized to the nucleus and suppresses mitosis, hence leading to endoreplication of the nucleus in trichomes (Walker et al., 2000; Churchman et al., 2006). SIM1 has a cyclin binding motif and associates to D-type cyclin dependent kinases (CDKs) and CDKA;1. Consequently, SIM1 is considered to be a CDK-inhibitor that mediates the transition from mitosis to endoreplication (Churchman et al., 2006). In Chruchman et al., (2006) qRT-PCR expression analysis revealed that SIM1 is mostly expressed in stems and roots of Arabidopsis, which is consistent with our observations of the promoter::GUS activity (Paper IV, Extended Figure 8). The specific role of SIM1 together with the XP specific expression indicates further that XP are endoreplication. To analyse the replication of genomic DNA during TE differentiation, DNA was extracted and quantified along the Arabidopsis TE differentiation time course (Paper IV, Figure 9A). The DNA content followed TE appearance and remained elevated even after all TEs were dead, indicating that the non-TEs have a higher DNA content (4-fold at 14d) compared to uninduced cells. Obara et al., (2001) reported that the nucleus of TEs is degraded within 10 minutes after initiation of PCD, which excludes the possibility that this DNA derives from remnants of dead TEs. To further confirm the endoreplication of XP, HISTONE2B (H2B)-RFP lines were generated to measure the nucleus area of non-TEs, TEs and uninduced (basal) cells after 5 days of induction. Figure 16A-B (Paper IV, Figure 3E-F) shows that the nucleus area is larger in non-TEs as well as in TEs compared to uninduced cells, which confirms that non-TEs and TEs are endoreplicated. Measurements of the cell density along the TE differentiation time course revealed that the induced cells exhibit a very low growth rate: the doubling
time for induced cells is 10.6d compared to non-induced cells with 2.1d (Paper IV, Extended Figure 1A). The slow cell division rate together with the endoreplication of the nucleus approves that the non-TEs are differentiated and avoid mitosis by the suppression of CDKs. Altogether, these findings provide substantial evidence that the non-TEs are in fact differentiated endoreplicated XP cells. Thus, the specific cell culture conditions not only allow the differentiation of TEs but also XP.

2.5. Coordination of TE and XP differentiation

The differentiation of the non-TEs to endoreplicated XP followed the differentiation of TEs, demonstrated by the expression profile of SIM1 and the increase in DNA quantity along the TE differentiation time course, following the appearance of TEs (Figure 14A; Paper IV, Extended Figure 9A). This is further supported by the coexpression analysis during TE differentiation (section 4), in which SIM1 was confined to the same cluster (B/B’) as TE SCW synthesis and autolysis genes (Paper IV, Figure 2). In addition expression along the TE differentiation time course and promoter::GUS expression of SIM1 in cotyledons of 7d old seedlings overlapped with the expression of genes involved in TE SCW synthesis and autolysis (IRX3, LAC17 and XCP2) with the difference that SIM1 was expressed beyond the TE lifespan (Paper IV, Extended Figure 3, 6, 7). Consequently, the differentiation of TEs and XP is a temporally coordinated process in which both cells differentiate simultaneously.

The coordination of TE and XP differentiation can be further explained by the controlled expression of the master regulator VND7, which is specifically expressed in protoxylem vessel. VND7 activates the transcription of genes involved in TE differentiation, i.e. SCW synthesis and autolysis (Figure 9) (Kubo et al., 2005; Yamaguchi et al., 2010) and therefore induces protoxylem vessel differentiation. The NAC transcription factor VNI2 represses genes activated by VND7 and is specifically expressed in XP. This suggests that the differentiation of either TEs or XP is defined by the coordinated expression of VND7 and VNI2, thus leading to the activation or suppression of genes involved in TE differentiation. Because VND7 does not induce the expression of lignin monomer synthesis genes, the XP specific suppression of VND7 by VNI2 does not affect the monomer production by XP (Figure 9). This is consistent with the cooperative supply of lignin monomers by the XP cells. Together, it appears that the transcriptional regulation of TE and XP differentiation is temporally coordinated to enable the formation of a dead hollow vascular conduit, supplied with lignin monomers from adjacent XP for post-mortem lignification.
3. Lignin is spatially restricted to deposition domains

Lignin is differentially distributed between primary and secondary xylem tissue. In primary xylem tissue, *i.e.* protoxylem and metaxylem, lignin appears to be only restricted to TE SCWs (Hepler *et al.*, 1970; Taylor *et al.*, 1992; Schuetz *et al.*, 2014). Contradictory results have however been observed where lignin appears to be deposited in PCWs of protoxylem vessels (Woodings, 1968). In secondary xylem lignin is deposited in both, the PCW (*i.e.* the middle lamella and the cell corner) and the SCW (Donaldson *et al.*, 2001; Fromm *et al.*, 2003; Gierlinger *et al.*, 2014). This differential distribution of lignin suggests the presence of specific lignin deposition domains that control the spatial restriction of lignification. To confirm the differential distribution of lignin in xylem cells of *Arabidopsis* hypocotyls and TEs of *Zinnia* cell cultures, the lignin distribution was visualized by UV-CLSM (Figure 17A-B) (*Paper II*, Figure 1A,B). In fibers of the hypocotyl, lignin was highest in cell corners and the middle lamella, whereas the SCW had lower amounts of lignin (*Paper II*, Figure 1C). These results correspond to the lignin distribution observed in other species such as spruce, beech wood and pine (Donaldson, 2001; Fromm *et al.*, 2003). Furthermore, TE SCWs exhibited higher amounts of lignin compared to xylem fiber SCWs (Figure 17C), which has also been observed in poplar secondary xylem (Donaldson *et al.*, 2001). The reason therefore are presumably the high tensional forces that vessels need to withstand during sap conduction, which can be compensated by an increase in SCW lignification. In *Zinnia in vitro* TEs, which resemble protoxylem vessels, the
lignin UV autofluorescence was only detected in the SCW (Figure 17B) (Paper II, Figure 1B). To further confirm the specific deposition of lignification in both protoxylem and metaxylem vessels, these cell types were produced using the Zinnia in vitro TE differentiation system. UV-CLSM in combination with non-overlapping cellulose staining using Congo Red revealed that lignin was only confined to TE SCWs and was deposited after SCW-cellulose deposition (Paper III, Figure 2A). To further verify that the observed lignin distribution is not an artefact of the UV fluorescence of the cell wall, lignin and polysaccharides were stained with KMnO$_4$ and PATAg, respectively, for the subsequent detection by TEM (Figure 18A) (Paper III, Figure 2B-E). The results confirmed that in protoxylem and metaxylem in vitro TEs, lignin is restricted to the SCW and deposited after SCW-cellulose formation. Moreover, epicotyl sections of 4-weeks old Zinnia plants were observed using TEM, while staining lignin with KMnO$_4$. The primary xylem vessels in these sections were only lignified in the SCW (Figure 18C) (Paper III, Figure 2F-H). The detection of lignin only once SCW-cellulose is deposition suggests the formation of specific lignin deposition domains. This is further supported by the strict dependency of lignin deposition on SCW-cellulose formation. Genetic or pharmacological inhibition of SCW-cellulose synthesis during TE differentiation resulted in the displacement of lignin (Taylor et al., 1992; Suzuki et al., 1992; Kiedaisch et al., 2002; Endo et al., 2008). The cellulotic SCW therefore appears to exhibit lignin nucleation sites that spatially direct lignification to the SCW.

As previously mentioned, lignification occurs only once TEs are dead. In order to test if the spatial deposition sites are conserved after PCD, dead un lignified protoxylem and metaxylem TEs were produced using the monomer synthesis inhibitor PA and supplied with coniferyl alcohol when all TEs were dead (after 120h). In these TEs, lignin was only deposited in the SCW and absent from the PCW, thus confirming that the subcellular deposition domains are conserved after TE cell death (Paper I, Figure 1; Paper III, Figure 4A-M). In parallel, caffeine precipitation of polyphenolics together with the visualization by TEM was performed in Zinnia epicotyl stem section. Polyphenolics were only present in the SCW of TEs and could not be detected in the cytoplasm, PCW or the surrounding XP cells (Paper III, Figure 4N-O). Altogether, this confirms that subcellular deposition domains are conserved after TE PCD, formed independently of monomer availability and incorporate extracellular lignin monomers.
3.1. Structural characteristics of lignin

The subcellular localization of lignin by TEM further revealed distinct structural characteristics, depending on the position within the SCW. In primary xylem TEs of the *Zinnia in vitro* TE differentiation system, lignin appeared as linear structures aligned with the polysaccharide matrix in the majority of the SCW bulging into the lumen, whereas lignin formed spherical structures farther away from the lumen, where the SCW merges with the PCW (Figure 18B) (Paper III, Figure 2E). This structural appearance was confirmed in primary xylem TEs of epicotyl cross sections of *Zinnia* plants (Figure 18D) (Paper III, Figure 2H). These results are partially coherent with TEM observations of secondary xylem cells in pine, which showed that lignin forms tubular structures in the different SCW layers and globular structures in the middle lamella and cell corners (Donaldson, 2001). In our case we observed globular structures also in the SCW but near the PCW. This difference is potentially due to the lack of the middle lamella in primary xylem TEs. The middle lamella is possibly formed by the adherence of multiple TEs, during later stages of development. The laminar structure in the SCW is the result of the densely aligned cellulose microfibrils coated by hemicelluloses, which restrict the lignin deposition to the narrow space between the microfibrils (Terashima et al., 2012). The globular lignin structure in the middle lamella is thought to be the result of the random orientation of the cellulose microfibrils in the PCW, allowing the presence of larger intermolecular spaces. The lignin structure has further been proposed to be affected by its supramolecular arrangement due to π-π-stacking of the aromatic ring structures (Bylin et al., 2014). Hence, the observed differences in the lignin structure may be the result of the
supramolecular assembly. Overall these results highlight a differential structural appearance of lignin between primary and secondary xylem TEs. In secondary xylem, lignification starts in the cell corner and the middle lamella (Terashima and Fukushima, 1988). According to our results this is not the case for primary xylem: in both protoxylem and metaxylem vessels, lignification was only confined to TE SCWs. Furthermore, Donaldson (2001) mentions that the spherical lignin structures may represent the initiation sites for lignification, which would mean that in primary xylem lignification starts near the PCW. The nature of these nucleation sites is still a matter of debate but the presence of peroxidases in complex with calcium pectate, which is abundant in PCWs and in the middle lamella, appears likely (Goldberg et al., 1996; Boerjan et al., 2003). Altogether, these observations point out that in primary xylem TEs lignification is initiated in the SCW, near the PCW, while in secondary xylem lignification is initiated in the cell corner and the middle lamella.

3.2. Enzymes defining the lignin deposition domains

The lignin deposition domains are suggested to be defined by phenoloxidase enzymes, because the lignin monomer availability did not appear to affect the restriction of lignin deposition. Moreover, lignin monomer oxidizing enzymes, such as the *Arabidopsis* peroxidase PRX72 and the laccases LAC4 and LAC17, were transcriptionally coregulated with SCW biosynthesis genes and localized to TE SCWs (Herrero et al., 2013; Schuetz et al., 2014). In Zinnia, the peroxidases ZPO-C, homolog of *Arabidopsis* PRX66, was also coregulated with SCW biosynthesis and localized to TE SCWs (Sato et al., 2006). Mutation of *PRX72*, *LAC4* or *LAC17* leads to collapsed xylem vessels, thus indicating the specific role of these enzymes for TE lignification. However, laccases and peroxidases do not seem to act redundantly and are suggested to operate in a sequential order with laccases being involved in earlier stages of lignification and peroxidases being active during later stages (Sterjiades et al., 1993). This hypothesis is mostly build on different substrate specificities between laccases and peroxidases during DHP formation: laccases are only able to oxidize lignin monomers, while peroxidases can oxidize both lignin monomers and oligomers. This is further supported by the observation that peroxidases can form cross-linkages between lignin, hemicelluloses and extensins in SCWs during later stages (Lamport, 1986; Lagrimini et al., 1987; Passardi et al., 2004). To investigate whether laccases and peroxidases have different functions during TE lignification, the enzymatic activity was analysed in isolated cells and the extracellular medium along the *Zinnia* TE differentiation time course. The activity was measured by using coniferaldehyde or 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) in the presence or absence of CAT, to decipher between laccases or peroxidases, respectively.
Both, laccase and peroxidase activities were detected throughout the entire time course, even after TE PCD. Laccase activity was restricted to the isolated cells while peroxidase activity was present in both isolated cells and the extracellular medium (**Paper III**; Figure 5A,C). To confirm these results in whole plants, laccase and peroxidase activities were analysed in *Zinnia* epicotyl and hypocotyl sections, which revealed that laccases were only present in TE SCWs while peroxidases were found in both TE SCWs and the apoplastic space (**Paper III**; Figure 5E-M). These results are coherent with Sato *et al.*, (2011) who detected the enzyme activity of *Zinnia* peroxidase ZPO-C in the cell wall and in the extracellular medium of TE differentiating cultures. Overall, these results clearly confirm that laccases and peroxidases define the lignin deposition domains. Their differential localization further supports that laccases and peroxidases do not have redundant function but act in distinct subcellular domains of the cell wall, *i.e.* SCWs and the apoplast respectively. The previous results showed that lignification starts first in the SCW, whereas the middle lamella is lignified during later stages. Together with the differential localization this could explain the sequential intervention of laccases and peroxidases, active during earlier and later stages of lignification, respectively.

The contribution of laccases and peroxidases to the *post-mortem* lignification of TEs was further addressed by supplying coniferyl alcohol to dead unlignified (PA-treated) *Zinnia* TEs together with one of the following inhibitors: the general phenoloxidase inhibitor NaN₃ or HL, the laccase-specific inhibitor CTAB or TGA, and the peroxidase specific inhibitor SHAM. The quantification of the cellulose and lignin fluorescence by non-overlapping cellulose staining with Congo Red and UV-CLSM showed that TE SCW lignification was reduced by all inhibitors (**Paper III**; Figure 6A). NaN₃ and CTAB, however, caused the formation of stress lignin in the extracellular medium and reduced the viability of non-TEs, which is why no conclusions could be drawn from these inhibitor treatments. To further confirm these results, lignin was characterized by FT-IR spectroscopy of the corresponding cell wall extracts. Data analysis using OPLS-DA, which allows to highlight proportional differences of the lignin specific peaks at 1510 cm⁻¹ and 1595 cm⁻¹ between the inhibitor treatments, revealed that *post-mortem* TE SCW lignification was more dependent on laccases than on peroxidases (Figure 19) (**Paper III**, Supplementary Figure 2). These results are based on the proportional differences between the inhibitors TGA and SHAM, which are specific to laccases and peroxidases, respectively. By comparing the correlation scale loadings of these two conditions, differences between the 1510 cm⁻¹ peak were observed, while the band at 1595 cm⁻¹ did not show substantial changes (**Paper III**, Supplementary Figure 2D,E,F). This suggests that the reduction in lignin is mainly due to a decrease in G-units and that laccases are primarily involved in G-lignin formation. This is in agreement
with the fact that TE SCWs are almost exclusively composed of G-units (Boerjan et al., 2003). The contribution of laccases to the formation of G-units is further supported by the double knock-out mutant of LAC4 and LAC17 which exhibits a considerable decrease in G-lignin, while the S-lignin composition is not affected, leading to an increased S/G-ratio (Berthet et al., 2011). Overall, the inhibitor experiment confirms that both laccases and peroxidases are involved in the post-mortem lignification of TEs, where laccases play a key role.

Figure 19. FT-IR spectroscopy of Zinnia TE cultures, inhibited to lignify (PA), treated with inhibitors for laccases (TGA), peroxidases (SHAM) or both (Hylan), and supplied with conifer alcohol (CA) to induce lignification once all TEs are dead. (A-C) OPLS-DA score plots showing the separation between different inhibitors. (D-F) Correlation-scaled loadings plots for predictive component 1, showing factors separating the different inhibitors. The marked bands at 1510 cm\(^{-1}\) and 1595 cm\(^{-1}\) indicate more G-lignin in SHAM compared to TGA and in SHAM compared to Hylan, while S-lignin is not affected. Paper III, Supplementary Figure 2.
The previous results together with the literature suggests that laccases are predominantly responsible for TE SCW lignification. To further investigate how laccases participate in forming the deposition domains, the gene expression of laccases was studied in Zinnia TE differentiating cultures and whole plants. Laccase mRNAs were identified by degenerative primer design on conserved laccase sequences. Together with the four known laccases from genomic EST databases, three additional laccases were identified, leading to a total number of seven Zinnia laccases, called LACA to LACF. The comparison of these laccases to the Arabidopsis laccases (in total 18) revealed that LAC4, LAC11 and LAC17 were the closest homologs to Arabidopsis LAC4, LAC11 and LAC17. As previously mentioned these laccases have been found to be implicated in TE lignification. RT-PCR expression of LACC and LACD during the Zinnia TE differentiation time course showed specific expression during TE differentiation and coregulation with the SCW-cellulose synthesis gene CS811 and the TE PCD related gene ZC4 (Paper III; Figure 8). In situ RT-PCR expression analysis in Zinnia epicotyl cross sections confirmed that LACC and LACD were specifically expressed in the cambium and in developing TEs, while no expression was detected in the surrounding XP cells (Paper III, Figure 9). The expression of LAC17 was investigation by RT-qPCR along the Arabidopsis TE differentiation system and by promoter::GUS expression in Arabidopsis seedlings. LAC17 was specifically expressed during TE formation (day 4 and 5), the time when most of the TEs are alive, and showed specific expression during proto- and metaxylem vessel formation in roots and cotyledons of 7d old seedlings (Figure 20) (Paper IV, Extended Figure 3D and 7). The coexpression network analysis of Paper IV (Figure 2) revealed further that the expression of LAC4, LAC11 and LAC17 was coregulated with TE SCW biosynthesis and autolysis genes. Taken together, these results indicate that Zinnia LACC, LACD and Arabidopsis LAC4, LAC11, LAC17 are cell-autonomously produced by developing TEs and deposited in the SCW before PCD. These results are coherent with the observed irx phenotype in lac4 single and lac4 lac17 double mutants (Berthet et al., 2011), indicating their importance for xylem vessel SCW lignification. Previous studies also showed promoter::GUS expression of LAC4 and LAC17 in the cambium but, in contrary to our results, these genes were expressed in XP and IFS of Arabidopsis stems, and not in xylem vessels (Berthet et al., 2013; Turlapati et al., 2011). In the protoxylem of Arabidopsis, on the other hand, fluorescent labeling of LAC4 and LAC17 revealed the specific localization to TE SCW (Schuetz et al., 2014). This suggests that LAC4 and LAC17 are differently expressed during early and later stages of plant development, and/or a different mechanism for metaxylem vessels, in which these laccases are supplied by XP to TE SCWs or deposited already during early stages of
differentiation near the cambial zone. Altogether, these results indicate the *pre-mortem* cell-autonomous production of laccases by TEs, to contribute to the formation of the SCW specific lignin deposition domains.

In summary, we have identified specific lignin deposition domains in SCWs of proto- and metaxylem TEs, which are defined by laccases and peroxidases. *Post-mortem* TE lignification is predominantly controlled by the cell-autonomous production of laccases by developing TEs before cell death. The localization and the cell-autonomous deposition of laccases further supports the sequential intervention theory in which laccases are deposited during earlier stages of development for lignification of the TE SCW. Some peroxidases may have partial redundant functions with laccases, based on the detection of peroxidase activity in the SCW of TEs. At the same time, peroxidases were found in the apoplast, which suggests that these peroxidases are involved in lignification of the middle lamella, during later stages when multiple TEs assemble to form the vascular bundle.

4. Transcriptional regulation of lignification

The results of Paper III indicate that the transcriptional regulation of TE lignification is largely defined by the TE cell-autonomous production of phenoloxidases. To further investigate the transcriptional regulation of genes during TE differentiation, *in silico* analysis of microarray experiments from two different *Arabidopsis in vitro* TE differentiation systems (Derbyshire et al., 2015; Kubo et al., 2005), were performed. In total 21,639 genes were analyzed, with only 3.9% showing differential expression during induced conditions. These genes formed two distinct clusters, either associated to stimuli/hormone response or SCW formation and TE autolysis (cluster A and B, Paper IV, Figure 2A and Extended Figure 2A-D). Genes associated to stimuli/hormone response typically exhibited elevated expression in uninduced cells, indicating the stem cell nature of these cells, whereas SCW and TE autolysis genes peaked during TE formation. The microarray expression analysis was further validated by constructing a coexpression network which was compared to the coexpression of 1436 publically available datasets. The coexpression network revealed two discrete clusters (cluster A’ and B’, Paper IV, Figure 2D and Extended Figure 5A), with high levels of coexpression, that overlap in terms of gene identity with clusters A and B from the microarray experiments. This confirms the presence of two distinct cell states, one defining stem-cells and the other one defining TE differentiation. Concerning genes implicated in TE lignification, the laccases *LAC4*, *LAC11*, and *LAC17*, the peroxidase *PRX66* and the lignin monomer biosynthesis genes *CCoAMT1* and *PAL4* were coexpressed with SCW and TE autolysis genes, confined to cluster B/B”. These results are in agreement with Paper III
(Figure 8) showing that the Zinnia homologs of LAC4 and LAC17 (LACC and LACD) are coregulated with TE SCW polysaccharide deposition and autolytic processes. **Paper IV** (Extended Figure 3 and 7) confirmed further that LAC17 is specifically expressed during TE differentiation (Figure 20). The presence of PRX66 in cluster B/B’ is coherent with previous publications, which showed that PRX66 is expressed in xylem vessels (Tokunaga et al., 2009) and the homologous Zinnia protein (ZPO-C) is localized to TE SCWs (Sato et al., 2006). This suggests that PRX66 contributes to the lignification of TE SCWs. The triple mutant lac4 lac11 lac17 exhibited severe growth defects and strongly reduced xylem lignification compared to the double mutants of LAC4 and LAC17 indicating that LAC11 is involved in xylem lignification as well (Berthet et al., 2011; Zhao et al., 2013). The overexpression of the protoxylem vessel specific master regulator VND7 causes an increase in expression of LAC4, LAC11 and PRX66 (Yamaguchi et al., 2010), which provides further evidence for regulation of these phenoloxidases by the transcriptional control of protoxylem vessel lignification.

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**Figure 20.** Promoter::GUS expression in 7d old cotyledons and RT-qPCR expression along the Arabidopsis TE differentiation time course of CCR1 (A) and LAC17 (B). CCR1 is ubiquitously expressed while LAC17 is restricted to differentiating TEs; black bracket indicates LAC17 expression in the midrib. Bars 1 mm; error bars ± SD, t-test with Welch correlation (* p < 0.05, ** p< 0.01). **Paper IV**, Extended Figure 3, 6 and 7.
Concerning *PAL4*, VND7 has been reported to repress the expression of the transcription factor REVOLUTA (REV), which is involved in SCW synthesis and represses *PAL4* expression by binding to its promoter (Taylor-Teeples *et al.*, 2015). As a result, an increased VND7 expression would repress REV expression and consequently result in increased *PAL4* expression, which could explain the correlation of *PAL4* with TE SCW synthesis and its presence in cluster B/B’. The knock-out mutant of *PAL4*, however, did not show any phenotypic change, which suggest that *PAL4* is not directly involved in xylem lignification (Huang *et al.*, 2010). Lignin monomer synthesis gene expression was not induced by VND7 overexpression and, except for *CCoAMT1* and *PAL4*, monomer synthesis genes were not correlated with TE SCW synthesis and autolysis (Paper IV, Extended Figure 2A-D). This is coherent with the promoter::GUS expression of the monomer synthesis genes *C4H*, *CCR1* and *CAD5*, which did not show TE differentiation specific expression patterns: *C4H* was expressed in the cortex of hypocotyls and along the entire root of 7d-old seedlings, while *CCR1* was expressed in the cortex of young stems, ubiquitously in leaves of seedlings and in non-induced cell cultures (Figure 20) (Paper I, Figure 8; Paper IV, Extended Figure 4, 6, 7, 8). *CAD5* was also found to be expressed unspecifically in roots of 7d-old seedlings (Paper IV, Extended Figure 7). Therefore, lignin monomer synthesis is not restricted to one specific cell type and is not regulated by the transcriptional control of TE SCW lignification. However, this does not exclude the possibility of a cell specific post-transcriptional regulation of lignin monomer synthesis, for example by the formation of multi-enzyme complexes that lead to metabolic channelling (suggested by Eckardt, 2002) or by regulatory modifications of enzymes such as phosphorylation (shown for COMT, Wang *et al.*, 2015). Altogether, the transcriptomic analysis supports that phenol oxidases are regulated by the transcriptional control of TE SCW lignification, whereas lignin biosynthesis genes are not.

To further investigate the coordination of transcriptional regulation between TE and non-TE/XP, a microarray experiment was conducted with 14d-old induced and uninduced control cultures from the *Arabidopsis in vitro* TE differentiation time course (Paper IV, Figure 1). By superimposing the gene expression of the 14d-old cells onto the coexpression network, the gene expression was assign to the two previously defined clusters A/A’ and B/B’ (Paper IV, Figure 3D). Genes expressed in the 14d-old uninduced cultures were mostly coregulated with genes associated to hormone response (cluster A/A’), while genes expressed in 14d-old induced conditions were predominantly coregulated with genes expressed during TE SCW formation and autolysis (cluster B/B’). These results indicate that the transcriptional regulation of XP differentiation is coordinated with the differentiation and maturation of TEs. This further confirm that the post-mortem TE SCW formation is a non-cell autonomous process guided by XP cells.
5. Evolutionary and physiological considerations

In a physiological perspective post-mortem lignification becomes obvious when considering the tension associated to the hydro-mineral sap rising. During plant growth the stem elongates and with increased stem height the negative xylem pressure decreases (Koch et al., 2004), which in turn generates an increase in hydraulic tension. This means that older (dead) xylem vessels are exposed to higher tensional forces, which need to be compensated by an increase in rigidity. In this regard, Gibson (2012) reported that SCW lignification results in an additional tensile strength of 25-75 MPa and a Young’s modulus of 2.5-3.7 GPa. Hence, the post-mortem lignification increases the mechanical strength and therefore enables the maintenance of a functional xylem conduit. This is further supported by the phenotype of several irx mutants affected in either lignin biosynthesis or SCW synthesis, which have collapsed xylem vessels (Turner and Somerville, 1997; Jones et al., 2001). The collapse is associated to a reduction in mechanical resistance against the hydraulic tension and is caused by the modification of the SCW chemistry. Furthermore, in vitro TEs inhibited to lignify using PA did not exhibit an irx phenotype, indicating that the hydro-mineral sap transport induces the collapse of the vessels due to the hydraulic tension.

In terms of evolution PCD occurs in hydroids (primordial water-conducting elements) of mosses that do not have SCW, which suggests that PCD appeared before SCW formation and lignin biosynthesis. Therefore, PCD is considered to be the initial step for the generation of a sap conducting unit (Friedman and Cook, 2000). Xu et al., (2014) showed that mutation or overexpression of homologs of the NAC transcription factors (SNDs, NSTs and VNDs) in the moss P. Patens, resulted in abnormal or ectopic hydroid development, respectively. This indicates that, already in mosses, the transcriptional regulation of hydroid differentiation was facilitated by the NAC transcription factors. Moreover, the nine core lignin monomer synthesis genes were found in P. Patens, though hydroids do not possess lignified SCWs (Persson, 2012). On the other hand P. Patens produces smaller phenolic compounds related to pathogenic defense and UV protection such as lignans and flavonoids (Weng and Chapple, 2010). This clearly illustrates that lignin biosynthesis genes were originally not intended for lignin biosynthesis but potentially to produce phenolic compounds that protect against UV radiation and pathogen infection (Kawasaki et al., 2006). It is therefore not surprising that lignin biosynthesis genes are expressed in cells others than TEs, such as XP. The “modern” type of lignin appeared later in tracheids of lycophytes with S- or G-type SCW-like structures (Friedman and Cook, 2000), which supports that the lignin deposition followed the evolution of SCWs. Plants have possibly redirected the phenylpropanoid pathway towards lignin biosynthesis along with the specific deposition of laccases and peroxidases in SCWs, together
with an increase in the amount of laccase and peroxidase isoforms during evolution.

The height of plants has substantially increased along evolution, together with the transpiration rate associated with an increase in leaf size (Sperry, 2004). This may have lead to an overall increase in hydraulic tension within the water conducting elements. To withstand this tension the initial conduits (tracheids) developed the SCW. In these early S-, G- or P-type tracheids, lignin or lignin-like substances are deposited in the cell wall layer bordering the lumen, while the rest of the SCW contains a degradation prone substance (Friedman and Cook, 2000), possibly composed of polysaccharides. The deposition of lignin near the lumen is suggested to be more susceptible to cavitation as it generates a hydrophobic surface that is in contact with the sap (Sperry, 2004). The lycophyte Huperzia, which is slightly different from the ancient S-, G- or P-type tracheids develops a SCW that is completely lignified except for the part where the SCW joins the PCW (Friedman and Cook, 2000). This SCW type appears to be the transition towards contemporary SCWs, in which the lignin concentration decreases towards the outer S3 layer, closest to the lumen. In physiological means this “modern” lignin distribution is more efficient to prevent cavitation. In addition, the region where the SCW joins the PCW contains lignin in form of globular structures (Paper III, Figure 1P-Q). This points out that lignification in this part of the cell wall has evolved later potentially to reinforce the junction between SCW and PCW. Overall, the differential localization of lignin during SCW evolution suggests that the lignin initiation sites sequentially evolved from being closest to the lumen to being farthest away from the lumen (middle lamella). The cooperative supply of lignin precursors could have participated in this process and may have evolved in parallel to the differential deposition of lignin in TE SCW.

**Conclusion**

Higher plants have developed a vascular system to transport hydro-mineral sap over long vertical distances for photosynthetic purposes. TEs are key features of this transport system that allow the sap conduction based on their specific characteristics: an ornamented lignified SCW that withstands the negative pressure, provides hydrophobicity and resists pathogen invasion. The development and maintenance of an intact TE structure requires a specific spatio-temporal coordination of lignification together with the assistance of neighbouring XP cells. This thesis provides substantial evidence for the regulation of this process.
**Paper I, III and IV** revealed that TE lignification occurs *post-mortem* using *in vitro* cultures of *Zinnia* and *Arabidopsis* as well as whole *Zinnia* plants. In both cell suspension cultures and plants, lignification progressed long after PCD and this progression was only associated to an increase of lignin in the TEs’ SCW. Moreover, TE lignification was shown to require PCD and it therefore appears that PCD initiates lignification, potentially by the release of ROS which are required for monomer oxidation by phenoloxidases. The exact mechanism lying behind the initiation of lignification remains, however, to be elucidated.

**Paper I, II, and IV** have shown that the *post-mortem* lignification of TEs is mediated by the cooperative supply of lignin precursors and secondary substrates from non-TEs/XP, in both *Zinnia* and *Arabidopsis*. Secondary substrates were H₂O₂ required for peroxidases, molecular oxygen for radical activation by laccases and possibly other ROS as well. Besides TEs, fibers were found to have the ability to lignify through the cooperative supply of monomers as well, at least partially. Non-TEs were shown to represent differentiated XP cells that exhibit an enlarged endoreplicated nucleus. Their differentiation and transcriptional regulation was further temporally coordinated with TE formation. Moreover, XP expressed specific genes (*e.g.* MYB13, RCD1 and SIM1) as well lignin biosynthesis genes (*e.g.* C₄H, CCR and CAD) that affect TE lignification when mutated. The nature of the cooperatively supplied lignin precursors and their subcellular deposition requires further investigation.

**Paper II and III** addressed the control of the spatial restriction of TE lignification. In protoxylem and metaxylem TEs, lignin was deposited only in the SCW. Lignin exhibited further distinct subcellular structures, appearing spherical in the SCW near the PCW and tubular in the other SCW layers. The spatially restricted deposition of lignin was regulated by specific phenoloxidase enzymes, *i.e.* laccases and peroxidases. Peroxidases were detected in both the apoplastic space and SCWs, whereas laccases appeared only in SCWs, indicating that laccases and peroxidases define different lignin deposition domains. Laccases appeared to be primarily responsible for TE SCW lignification and were cell-autonomously produced by TEs before PCD. These results suggest that these phenoloxidases act during different stages of development, starting with laccases during TE SCW lignification and continuing with peroxidases at later stages when the middle lamella is formed. This hypothesis however needs further support. The results further indicate that the transcriptional control of TE SCW lignification regulates phenoloxidases and not lignin monomer synthesis proteins. However, lignin monomer synthesis could be controlled on the post-transcriptional level, which needs further scientific evidence.
References


*Phytochemistry* 57: 859–873.


Acknowledgements

First of all, I want to thank my supervisor Edouard, for giving me the opportunity for this PhD and for guiding me throughout the entire project. Thank you for taking your time and patience to provide me with plenty of ideas and scientific expertise.

Further I would like to thank my co-supervisors Gunnar, Hannele and Markus. Thank you for the helpful discussions during our reference group meetings and for critical comments on my project.

Special thanks to all the current group members: Delphine, Emiko, Raphaël and Haris. Thank you all for reading and making comments on manuscripts, figures etc. I deeply appreciate your efforts and your time spent. Thank you Delphine, for taking care of the cell cultures when I was on vacation and for providing help and materials. Thank you Emiko and Raphaël for all the help and the interesting discussion.

Further I want to thank all the previous lab members. Thank you Irene, Jaime, Walter and Anke for your help and support. Thanks to all the project and Master students: Anaxi, Arnaud, Anna, Oliver, Christopher, Linus, Vimal, Hanna, Jenny, Guillaume, Steven, and Lennart for all your efforts during your projects. You contributed a lot to my project as well.

Thanks also to Sacha, Bo, Jakob, Daniel, Sergiu, Ogonna, Sabine, Bas, Márcia and Christian for your help and support whenever there were questions or problems. Thank you Jakob for providing sequencing labels.

In addition I would like to thank David, Melis, Jimmy, Zsofia, Barbara and Bernard for their contributions to organise lab practicals. Thank you for your help with preparing the labs, teaching students and for your valuable input.

András, thank you for your help with the FT-IR and for your critical comments on figures and manuscripts as well as for the support with data interpretation.

Thanks to Junko and all the members of the cell wall facility, for keeping the pyrolysis-GC/MS running and for the support for the data analysis.
Gunilla, Eva and Ken, thank you for showing me the HPLC and for helping me whenever I had problems with the machine.

Thanks to all the members of the BioImprove programme, including all the other graduate students. Thanks for constructive questions and discussions during our meetings, congratulation for the ones that have already finished and good luck for the ones that have not finished yet.

I would also like to thank the Kempe foundation and the Carl Tryggers foundation for financially supporting this work.

Moreover, I want to thank all the members of the UPSC, everyone was very kind and helpful. Thanks to Inger, Ylva, Karin, and Jenny B., for all the administrative support. Special thanks also to Siv and Rosi who did a great job on taking care of all the glassware, it was a lot especially from our group. Further I want to thank Janne and Simon for solving technical and computer related issues as well as Thomas for safety advices. Britt-Marie and Jenny L., many thanks for taking care of the greenhouse facilities and for all the pesticide treatments.