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Investigations into the Transcriptional Regulation of Wood Formation

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ABSTRACT

Xylogenesis (wood formation) is one of the planet's most crucial biological processes. It is characterised by the complex, multi-stage development of cells commencing with periclinal divisions of cambial initials which subsequently undergo a series of dynamic, differentiation stages including further cell division, xylem cell expansion, secondary cell wall deposition and programmed cell death. Each of these stages is orchestrated by molecular controllers which are strictly regulated by an underlying network of transcription factors, the central regulators of wood formation. These transcription factors determine the morphology, chemical composition and physical properties of xylogenic cells as well as the overall dynamics of the wood formation process including its responsiveness to biotic and abiotic stresses including distinct responses to gravitational stress.

This thesis used wood formed in response to gravitational stress (reaction wood) to dissect the transcriptional regulation of xylogenesis in order to gain detailed insights into the molecular control of wood formation in woody perennials. It does this by examining the current literature on transcriptional regulation of wood and reaction wood formation with specific focus on the roles played by transcription factors. Using this information, nine candidate transcription factors were chosen for subsequent experimentation to add to our current sketchy understanding of their specific functions during wood formation. Selected genes from *Eucalyptus* included *INDOLE-3-ACETIC ACID INDUCIBLE 13* (*EgIAA13*), *KNOTTED-LIKE HOMEODOMAIN 7* (*EgKNAT7*), *MYELOBLASTOSIS 103* (*EgMYB103*), *SECONDARY WALL NAC DOMAIN 2* and *3* (*EgSND2* and *EgSND3*), *BELL1-LIKE HOMEODOMAIN 6* (*EgBLH6*), *EgWRKY2* and *EgWLIM1*. Aiding these functional investigations, also a suite of contemporary microanalytical techniques was reviewed assessing their suitability for phenotyping wood cell morphology and cell wall chemical composition in instances where available wood samples amount to as little as a only a few micrograms, as was the case for the induced somatic sector analysis (ISSA) derived samples analysed in this thesis. In these *in vivo* transformation experiments, up- and down-regulation constructs of candidate transcription factors were used to perturb

wood formation in the ecologically and economically important forest tree species *Eucalyptus* and *Populus*.

Our investigations revealed that EgIAA13 significantly alters xylem fibre and vessel morphology as well as xylem cell division rates, identifying EgIAA13 as a novel regulator of cambium dynamics and xylem formation. Subsequent protein-protein interaction analyses revealed that EgIAA13-EgARF (2, 5, 6 and 19) modules are presumably involved in mediating this dual regulatory role of EgIAA13. Our results also demonstrate that EgKNAT7 and EgMYB103 significantly alter xylem fibre morphology in terms of secondary cell wall thickness and fibre size, confirming their regulatory involvement in mediating secondary cell wall deposition and xylem expansion, respectively.

DECLARATION

This is to certify that:

- I. The thesis comprises only my original work towards the PhD except where indicated in the preface.
- II. Due acknowledgement has been made in the text to all other material used.
- III. The thesis is fewer than 100,000 words in length, exclusive of tables, illustrations, and bibliographies.

Nadeeshani Karannagoda

PREFACE

- Chapter 3 of this thesis was published in IAWA journal.
Karannagoda, N., Spokevicius, A., Hussey, S. and Bossinger, G. (2020)
Microanalytical techniques for phenotyping secondary xylem. *IAWA J.* **41**, 356-389.
My contribution - 80%
NK and AS conceived the ideas and developed the structure. NK led the writing of the manuscript with editorial guidance from GB, AS and SH.
Editorial assistance - IAWA journal
- Chapter 4, 5 and 6 of this thesis contain currently unpublished material which has not been submitted for publication yet.
My contribution for each chapter - 80%
- Some parts of Chapter 4 and 5 have been presented at the following conferences:
Karannagoda, N.N.H., Hussey, S.G., Spokevicius, A.V. and Bossinger, G. (2019) Functional investigations into transcription factors associated with wood formation. International Union of Forest Research Organisations (IUFRO) Tree Biotechnology Conference, North Carolina, USA.

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- I. **Fig. 3.2a** - Donaldson, L.A. (2019) Wood cell wall ultrastructure, The key to understanding wood properties and behaviour. *IAWA J* **40**, 645-672.
- II. **Fig. 3.2b** - provided by Dr Naoki Takata, Forestry and Forest Products Research Institute, Japan and Prof Yuzou Sano, Hokkaido University, Japan.
- III. **Fig. 3.2c** - provided by Gabriele Ehmcke, Holzforschung München, Germany.
- IV. **Fig. 3.2d** - Müller, G. and Polle, A. (2009) Imaging of lignin and cellulose in hardwood using fourier transform infrared microscopy - comparison of two Methods. *N Z J For Sci* **39**, 225-231.
- V. **Fig. 3.2e** - provided by Assoc. Prof Arata Yoshinaga, Kyoto University, Japan.
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1 GENERAL INTRODUCTION

1.1 THESIS INTRODUCTION

Secondary growth in perennial woody plants leads to the formation of secondary xylem, wood, which in mature angiosperm tree stems consist of centrally located dead heartwood surrounded by sapwood composed of living ray and axial parenchyma cells as well as dead fibres, tracheids and vessel elements. Sapwood is the central conductive tissue which transports water and minerals from roots to the crown and provides structural reinforcement to the tree as it continues its growth while facing a plethora of challenges imposed by the environment.

Globally, forests and the wood they produce account for most of the planet's woody biomass amounting to a total of 557 billion m³ (FAO 2020). With an estimated total carbon stock of 662 gigatonnes, this makes forests the world's second-largest carbon reservoir (behind the world's oceans), and defines xylogenesis (wood formation) as one of the planet's most important biological carbon sequestration processes (FAO 2020; Groover *et al.* 2010). Wood provides fuel for energy production, timber for construction and fibre for paper and pulp production (Andersson-Gunneras *et al.* 2006; Dejardin *et al.* 2010; Plomion *et al.* 2001; Rockwood *et al.* 2008; Ye and Zhong 2015). Forests and woody biomass also provide many ecosystem services as they support much of the planet's biodiversity, offering support for about half of the world's species. Finally, they play an important role in alleviating poverty and improving food security (FAO 2011). Thus, wood formation is undoubtably one of the biosphere's most vital biological processes.

Before the background of an exponentially growing world population and the challenges that arise from rapid climatic changes, understanding the molecular basis of wood formation therefore seems crucially important for meeting both commercial and ecological demands. Wood formation is a complex and a continuous differentiation process which commences at the vascular cambium, a single layer of bifacial stem cells that undergo five developmental stages to develop into a mature

wood cell (Bossinger and Spokevicius 2018; Shi *et al.* 2019). Each of these stages is tightly regulated by the combinatorial effect of multiple molecular controllers, including phytohormones, structural genes, peptides, miRNA, and transcription factors (TFs). Since TFs exert a tight regulation on these molecular controllers, they function as central players in the molecular regulation of wood formation (Du and Groover 2010; Hertzberg *et al.* 2001; Hussey *et al.* 2013). Therefore, unravelling its transcriptional regulation will greatly contribute to improving our current knowledge regarding the molecular control of secondary growth and wood formation.

Dissecting the transcriptional regulation of wood formation requires the identification of xylogenesis related TFs followed by investigations into their specific function during xylogenesis. In the absence of significant mutant populations for most forest trees that could support molecular and developmental studies, the most widely used approaches for such functional investigations include various *in vitro* techniques including the generation of callus cultures, apical stem cultures, hairy root cultures, or use of transgenic plants or plant parts. Each of these methods is associated with inherent limitations including low transformation efficiencies, inability to produce certain woody cell types, long regeneration times, long stem maturation timeframes, and high technical and labour demand (Chaffey 1999; Groover *et al.* 2010; Moller *et al.* 2003; Plasencia *et al.* 2016; Spokevicius *et al.* 2016; Spokevicius *et al.* 2007b; Van Beveren *et al.* 2006). Most of these limitations have been successfully overcome by the introduction of Induced Somatic Sector Analysis (ISSA), an *in vivo* transformation method which allows for the generation of transgenic cells and tissues in intact secondary stems of important woody plants including *Eucalyptus*, *Populus*, *Pinus*, *Acacia* and *Corymbia* (Spokevicius *et al.* 2016; Van Beveren *et al.* 2006).

In previous studies, ISSA has been successfully employed to dissect the functions of several xylogenesis related TFs and structural genes (Bossinger and Spokevicius 2018; Creux *et al.* 2013; Hussey *et al.* 2011; MacMillan *et al.* 2015; Melder *et al.* 2015; Spokevicius *et al.* 2007a) and has therefore been selected to again be the method of choice for investigations into the transcriptional regulation behind wood formation in the present thesis. Here, reaction wood (RW) is used as a model to select candidate TFs, by analysing RW related transcriptomic data from *Eucalyptus*, *Populus* and

Betula. Selected candidate TF genes were functionally tested for their roles during wood formation using homologous (*Eucalyptus*) and heterologous (*Populus*) genetic backgrounds. These functional investigations revealed the effects of selected candidate TFs on different stages of wood formation and wood cell morphology. In particular, one candidate TF gene (*EgIAA13*) was discovered as a novel regulator of wood formation and further investigations shed light on its regulatory role, proving further insights into specific aspects relating to the transcriptional regulation of wood formation.

1.2 THESIS AIMS

Wood formation is intricately regulated by an underlying network of hundreds of TFs but our knowledge of the transcriptional regulation of wood formation to date remains sketchy. Although particular roles for some of these TFs have been determined (mostly in model species), specific functions for most of them, and importantly their impact on wood formation, remain obscure. This thesis aims to address some of these knowledge gaps and thereby expand our current understanding of the transcriptional regulation of wood formation. In order to focus the selection of potential candidates, we employed RW as a model to select a set of critical, yet understudied *Eucalyptus* TF genes that are potentially involved in regulating wood formation.

Specifically, this thesis describes how we conducted

- I. analyses of promoter expression patterns and functional characterisation of four potential main drivers of secondary cell wall (SCW) biosynthesis and RW formation (*EgKNAT7*, *EgMYB103*, *EgSND2*, *EgSND3*), during xylogenesis in *Eucalyptus* and *Populus*;
- II. functional characterisation of RW formation related novel candidate TF genes (*EgIAA13*, *EgWLIM1*, *EgBLH6*, *EgWRKY2*), during xylogenesis in *Eucalyptus* and *Populus*; and
- III. further investigations into the novel candidate TF/s (identified in this study) to elucidate how it/they mediate transcriptional regulation of wood and RW formation.

1.3 THESIS OUTLINE

This thesis is structured around a theoretical section and an experimental section.

The theoretical section is composed of two chapters (Chapter 2 and 3), which serve as a review of the pertinent literature covering two distinct fields.

Chapter 2 provides a review on wood and RW formation and explores the feasibility of using RW as a model to dissect the molecular regulation underlying wood formation.

Chapter 3 reviews a collection of microanalytical techniques that can be employed for phenotyping when only minute amounts of xylem tissue (microgram quantities or less) are available or when cellular morphology, cell wall chemical composition and micromechanical properties need to be assessed at a fine scale resolution.

The experimental section is composed of three chapters (Chapter 4, 5 and 6).

Chapter 4 examines a set of TFs which are potentially involved in both SCW formation and RW formation.

Chapter 5 presents investigations into a novel set TFs to determine their effects on wood formation, wood cell morphology and RW formation.

Chapter 6 further examines a novel transcriptional regulator of wood formation, which has been revealed from the above investigations, to elucidate its regulatory role during wood formation.

2 WOOD AND REACTION WOOD FORMATION

2.1 WOOD

2.1.1 A result of secondary growth

Following the establishment of the plant body during primary growth, further development is complemented by differentiation of secondary tissues in both roots and shoots promoted by lateral or secondary meristems, mainly the vascular and the cork cambium. In woody tree species, the vascular cambium produces secondary xylem (wood) centripetally and secondary phloem (bark) centrifugally, while the cork cambium produces the suberised cork. The vascular cambium is a continuous cylinder formed by the activation of cell division in fascicular vascular cambia (procambium in vascular bundles) in coordination with nearby interfascicular regions. A synchronised increase of cambial circumference is ensured by the addition of new radial cell files via anticlinal divisions of two types of cambial initials, isodiametric ray initials and long and narrow fusiform initials, while periclinal divisions provide a cell population which undergoes secondary growth leading to an increase in stem diameter (Barlow *et al.* 2002). The periclinal divisions of short ray initials give rise to ray parenchyma cells, which form a radial system which is horizontal to the long axis of the stem and is responsible for the translocation of nutrients between xylem and phloem tissues (Groover *et al.* 2010; Plomion *et al.* 2001). Divisions of elongated fusiform initials give rise to phloem and xylem mother cells, which then divide into phloem and xylem daughter cells, respectively. These daughter cells ultimately give rise to secondary phloem and secondary xylem, which together comprise the vertical or axial system which is responsible for translocation of water and photosynthates from roots to branches and vice versa, respectively (Bossinger and Spokevicius 2018; Groover *et al.* 2010). To best support these functions, secondary tissues are composed of different types of cells with distinct features.

2.1.1 A dynamic tissue

The basic structure of the secondary xylem is ultimately decided by xylem cell composition, unique features of different xylem cell types and their three-dimensional associations with each other (Plomion *et al.* 2001). Gymnosperm secondary xylem is mostly composed of only one type of tracheary element known as tracheids; however, it may also contain a few parenchyma cells (axial and ray parenchyma) (Dejardin *et al.* 2010; Ye and Zhong 2015). In contrast, angiosperm secondary xylem is a heterogeneous tissue that contains four distinct cell types: tracheids and vessel elements (collectively known as tracheary elements), fibre cells and parenchyma cells. Depending on species and to some degree, environmental factors, xylem cells are present in different proportions. For example, the majority of *Populus* xylem tissue consists of fibres (53-55%) and vessel elements (33%) while parenchyma cells (ray parenchyma ~11-14% and axial parenchyma ~1%) make up only a relatively small proportion (Groover *et al.* 2010; Mellerowicz *et al.* 2001). Xylem cells also differ with respect to their morphology. Xylem fibres are more elongated and have a high capacity for axial expansion leading to ‘intrusive’ growth (Chaffey *et al.* 2002a; Dejardin *et al.* 2010) while the axial expansion of vessel elements is restricted. Vessel elements, however, are characterised by larger diameters due to their increased capacity for radial expansion. Perforations on the axial walls connect vessel elements and thereby form tube-like structures facilitate long-distance water transport. Longitudinal walls contain pits that provide a pathway for cellular communication and short-distance water and nutrient transport between neighbouring vessel elements or parenchyma cells (Dejardin *et al.* 2010).

Each xylem cell type has clearly defined functions. In gymnosperm, tracheids facilitate water and mineral conduction and mechanical support whereas, in angiosperms, tracheids and vessel elements facilitate water and mineral transport while fibres provide mechanical strength. In both gymnosperms and angiosperms, the parenchyma cells facilitate storage, cavitation recovery, synthesis and to a lesser degree, transport (Crang *et al.* 2018; Dejardin *et al.* 2010; Groover *et al.* 2010). Together, these cell types enable the secondary xylem to function as the central structural, transport (water and mineral) and storage tissue of the plant body. Therefore, its ability to dynamically

respond to biotic, abiotic, seasonal and developmental forces is essential to ensure the survival of a tree under ever-changing and potentially extreme conditions.

This ability of secondary xylem to respond to perturbations is referred to as wood plasticity. It enables adjustment of new growth in response to intrinsic or external forces depending on a plant's developmental stage or environmental factors such as gravity, nutrient availability (especially nitrogen), temperature and drought. Wood plasticity is exemplified by the co-occurrence of different types of wood in a single tree with each type of wood characterised by its unique anatomical, chemical and/or physical features (Paux *et al.* 2005; Plomion *et al.* 2001). For example, seasonal variation leads to the formation of earlywood and latewood, developmental changes cause the production of juvenile wood (during early stages of stem development) and mature wood (produced by older cambia) and some abiotic stresses such as gravitational forces stimulate the production of reaction wood (RW). To ensure the development of woody tissue that is appropriate for each circumstance while at the same time facilitating wood plasticity, the wood formation process has to be dynamic to respond to various developmental and environmental cues swiftly, but also intricate to fine-tune specific wood properties in response to these cues.

2.2 MOLECULAR CONTROL OF WOOD FORMATION

Wood formation progresses through a series of dynamic differentiation events including four successive stages; division of vascular cambial cells and differentiation into xylem cells, expansion and elongation of xylem cells, secondary cell wall (SCW) formation and programmed cell death (PCD) (Bossinger and Spokevicius 2018; Zhang *et al.* 2014). In addition to these stages, maintenance of the vascular cambium and its pluripotent nature, are of central importance for the subsequent stages of xylogenesis, as it is the lateral meristem responsible for secondary growth (Guerriero *et al.* 2014; Miyashima *et al.* 2013). Cambium maintenance and the progression of a cambial initial towards a mature wood cell involves developing new features in its cellular morphology and chemical composition, which are mediated by the interplay between hundreds of molecular controllers. Molecular controllers such as transcription factors (TFs) and phytohormonal regulators, together with peptides and miRNAs, ensure the

precise regulation of each stage while guaranteeing dynamic coordination between consecutive stages as well as their responsiveness towards perturbations (Hussey *et al.* 2013; Mizrachi and Myburg 2016).

2.2.1 Maintenance of vascular cambium

Maintenance of stem cells within the vascular cambium is a crucial aspect of facilitating secondary growth. This maintenance is mediated by several phytohormones and TFs, and crosstalk between them is essential for precise regulation. The central regulatory pathway involved in cambial maintenance is the peptide-receptor-TF signalling pathway, TDIF/CLE-TDR/PXY-WOX. A small peptide called TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF) is formed in the phloem from CLAVATA/EMBRYO SURROUNDING REGION-RELATED (CLE) proteins. The receptor-like kinase, *TDR-PXY* is expressed in procambial cells, and the TF, WUSCHEL RELATED HOMEODOMAIN (WOX) facilitates peptide-receptor signalling to maintain procambial cells and to control the proliferation of vascular cambium. WOX4 is a central cambium regulator that conveys auxin responsiveness to the cambium and upholds the fate of cambial stem cells (Hirakawa *et al.* 2010; Zhang *et al.* 2014). This TDIF/CLE-TDR/PXY-WOX pathway acts downstream of the auxin signalling pathway, and it cross talks with the ethylene signalling pathway (Etchells *et al.* 2012; Seyfferth *et al.* 2018; Zhang *et al.* 2014). In addition, CLASS III HOMEODOMAIN LEUCINE ZIPPER (HD-ZIP III) TFs are also involved in both the establishment and maintenance of vascular cambium (Robischon *et al.* 2011; Smetana *et al.* 2019). Class I KNOX homeodomain TFs ARBORKNOX (KNOX/ARK) is another vital group of TFs involved in vascular cambium maintenance (Groover *et al.* 2006; Liu *et al.* 2015a). Orthologs of the genes of above TFs, which are preferentially expressed in *Arabidopsis* cambium or shoot apical meristems are also preferentially expressed in the vascular cambium of *Populus* and *Eucalyptus*, signifying evolutionary conservation of this pathway between herbaceous and woody genera (Etchells *et al.* 2015; Etchells and Turner 2010; Han *et al.* 2016; Kucukoglu *et al.* 2017; Schrader *et al.* 2004; Zhang *et al.* 2014). In addition to these key TFs, some contributors to phytohormonal signalling pathways, also participate in the regulation of vascular cambium maintenance.

Auxin, cytokinin and ethylene are the key phytohormones contributing to the regulation of cambial maintenance. Certain members of the auxin signalling pathway such as INDOLE-3-ACETIC ACID INDUCIBLE (Aux/IAA) and their downstream targets AUXIN RESPONSE FACTORS (ARFs) act as prevalent cambium regulators. Some of these members interlace transcriptional and phytohormonal regulation. As an example, several studies have shown that the activity of WOX4 depends on ARF5 which can be regulated by auxin via Aux/IAA (Bhalerao and Bennett 2003; Brackmann *et al.* 2018; Hirakawa *et al.* 2010; Nilsson *et al.* 2008; Suer *et al.* 2011; Yu *et al.* 2015; Yu *et al.* 2014). In addition, cytokinin also functions as a cambial regulator which stimulates secondary growth by positively regulating cambial activity and division. CYTOKININ RECEPTOR (CRE), HISTIDINE KINASE (HK), ATP/ADP ISOPENTENYLTRANSFERASES (ATP/ADP IPTs or IPTs) and CYTOKININ OXIDASE are some examples of members of the cytokinin signalling pathway which are involved in cambial maintenance (Immanen *et al.* 2016; Matsumoto-Kitano *et al.* 2008; Nieminen *et al.* 2008). Furthermore, ethylene influences the growth and differentiation of cambial cells. Its positive regulatory role on cambial maintenance has been demonstrated by increased cambial cell proliferation in response to ethylene treatment (Felten *et al.* 2018; Love *et al.* 2009; Seyfferth *et al.* 2018; Zhang *et al.* 2014). Optimal maintenance of vascular cambium by these molecular controllers is crucial for continuous wood formation, which commences by division and differentiation of cambial initials.

2.2.2 Cell division and xylem specification

The division and differentiation of cambial initials into xylem mother cells are regulated by key TFs such as KANADI, HD-ZIP III and ARK (where the latter two TFs function as regulators of both vascular cambium maintenance and cell division and xylem specification), and by phytohormones such as auxin, cytokinin, brassinosteroids and gibberellic acid (GA) (Du *et al.* 2011; Guerriero *et al.* 2014; Miyashima *et al.* 2013; Robischon *et al.* 2011; Smetana *et al.* 2019). A surge in HD-ZIP III on cambial initials serves as the trigger for their differentiation into the xylem. Specifically, HD-ZIPs such as REVOLUTA (REV)/INTERFASCICULAR FIBERLESS (IFL), CORONA/INCURVATA (CAN/ICU), HOMEODOMAIN (HB),

PHAVOLUTA (PHV) and PHABULOSA (PHB) promote differentiation of cambial initials into xylem cells (Carlsbecker *et al.* 2010; Du *et al.* 2011; Guerriero *et al.* 2014; Robischon *et al.* 2011; Zhu *et al.* 2013). In addition to vascular cambium maintenance, KNOX/ARK TFs also act as negative regulators of xylem differentiation (Du *et al.* 2009; Groover *et al.* 2006; Liu *et al.* 2015a; Melder *et al.* 2015; Schrader *et al.* 2004; Zhang *et al.* 2014). Furthermore, some phytohormones are also involved in regulating cell division and xylem specification. Auxin plays a pivotal role in cambial division and differentiation (Groover *et al.* 2003; Tuominen *et al.* 1997). While the crosstalk between ethylene and TDIF/CLE41/CLE44-TDR/PXY regulates the rate of cambial cell division (Etchells *et al.* 2012; Seyfferth *et al.* 2018), GA functions as a positive regulator of xylem specification (Mauriat and Moritz 2009). Finally, brassinosteroids have been identified as activators of HD-ZIP III and therefore, are indirect regulators of xylem differentiation (Ohashi-Ito and Fukuda 2003). Regulated by the described TFs and phytohormones, once a cambial initial differentiates into a specific xylem cell, it enters the cell expansion and elongation stage.

2.2.3 Xylem cell expansion and elongation

The capacity to expand and elongate varies with xylem cell type. For example, xylem fibres are characterised by elongation, while vessel elements feature significant expansion. The combinatorial effects of phytohormones such as auxin, GA, and some TFs determine how and when xylem cell expansion and elongation occurs. A recent study reported ETHYLENE RESPONSE FACTOR (ERF) as a suppressor of radial expansion of vessel elements (Wessels *et al.* 2019). However, our current knowledge of transcriptional regulation behind this developmental stage is rudimentary. In contrast, the phytohormonal regulation behind this stage is better understood. A peak in auxin gradient across the zone of xylogenesis can be observed either in the cambial zone or between the cambial and xylem expansion zones suggesting a potential role of auxin in xylem cell expansion as well. Increasing the width of the radial auxin gradient increases the duration of xylem cell expansion, which results in fibres and vessels with larger cross-sectional areas (Bhalerao and Bennett 2003; Milhinhos and Miguel 2013; Tuominen *et al.* 1997). Finally, GA also influences this stage where a rise in GA levels causes the production of longer xylem fibres (Eriksson *et al.* 2000; Jeon *et al.* 2016;

Mauriat and Moritz 2009; Ragni *et al.* 2011). Once xylem cells reach their maximum size, SCW deposition commences.

2.2.4 Secondary cell wall formation

SCW formation is regulated by an interplay between TFs and phytohormones. The transcriptional regulation behind SCW formation has been extensively studied (for examples see Table 2.1) and reviewed (Dejardin *et al.* 2010; Demura and Fukuda 2007; Du and Groover 2010; Groover and Robischon 2006; Groover *et al.* 2010; Guerriero *et al.* 2014; Hussey *et al.* 2013; Liu *et al.* 2014a; Mellerowicz *et al.* 2001; Yang and Wang 2016; Ye and Zhong 2015; Zhang *et al.* 2014; Zhang *et al.* 2018; Zhong and Ye 2014; Zhong and Ye 2015). A three-tiered TF network regulates this stage by orchestrating the expression of downstream genes involved in the biosynthesis of SCW polymers including lignin, cellulose, and hemicellulose. This transcriptional regulation of SCW deposition has been extensively reviewed . Some main drivers of this three-tiered TF network are VASCULAR RELATED NAC DOMAIN (VND), NAC SECONDARY WALL THICKENINGS (NST), SECONDARY WALL NAC DOMAIN (SND), XYLEM NAC DOMAIN (XND), KNOTTED-LIKE HOMEODOMAIN (KNAT), R2R3-type MYELOBLASTOSIS (MYB) and C3H-TYPE ZINC FINGER (C3H) and the functions of some key SND, MYB and KNAT TFs are listed in Table 2.1 (Bhargava *et al.* 2013; Cassan-Wang *et al.* 2013; Chai *et al.* 2014; Hussey *et al.* 2011; Legay *et al.* 2010; McCarthy *et al.* 2009; Ohman *et al.* 2013; Zhang *et al.* 2018; Zhong *et al.* 2008). As reviewed by Didi *et al.* (2015), the phytohormonal regulators central to SCW formation include auxin, GA, abscisic acid (ABA), cytokinin, salicylic acid (SA) and brassinosteroids. ABA positively regulates NST and some other VND TFs (Jensen *et al.* 2010). Similarly, GA is also known as a positive regulator of SCW formation (Nunez-Flores *et al.* 2010). In addition, brassinosteroid acts as an activator of SCW biosynthesis genes of all three cell wall polymers (Kubo *et al.* 2005; Xie *et al.* 2011). In contrast, auxin negatively regulates certain MYB and VND TFs (Didi *et al.* 2015; Johnsson *et al.* 2019). Cytokinin plays a dual role as it imparts both negative and positive effects on SCW deposition, negatively regulating some VND TFs and biosynthetic genes of cellulose and hemicellulose while positively regulating lignin biosynthetic genes (Jung *et al.*

2008; Kubo *et al.* 2005). The combinatorial effects of these phytohormones and TFs ensure the precise deposition of a multilayered, elaborate SCW and the completion of its deposition leads to the final stage of xylogenesis, which is PCD.

Table 2.1 Roles of some key *Arabidopsis* SND, MYB and KNAT TFs in SCW deposition

TF	Ortholog in other species	Role in SCW biosynthesis (activator/repressor)	Reference
KNAT7 ^{a,p}	-	Repressor Activator Activator (xylan biosynthesis)	(Bhargava <i>et al.</i> 2013) (Li <i>et al.</i> 2012a) (Liu <i>et al.</i> 2014b) (Ruzicka <i>et al.</i> 2015) (Yang and Wang 2016) (Zhong <i>et al.</i> 2008) (He <i>et al.</i> 2018) (Wang <i>et al.</i> 2019b)
MYB4 ^{a,p}	PdMYB221 EguMYB1 PtoMYB156 AmMYB308 AmMYB330	Repressor (cellulose, xylan, lignin)	(Tang <i>et al.</i> 2015) (Yang <i>et al.</i> 2017) (Legay <i>et al.</i> 2007) (Legay <i>et al.</i> 2010) (Tamagnone <i>et al.</i> 1998)
MYB5 (MYB82) ^e	EguMYB88	Activator (phenylpropanoid-derived secondary metabolites, lignin)	(Soler <i>et al.</i> 2016)
MYB43 ^p	PtrMYB152	Activator	(Wang <i>et al.</i> 2015b)
MYB46 ^{a,p,e} (MYB83)	EgMYB2 OsMYB46 ZmMYB46 PtrMYB021	Activator	(Goicoechea <i>et al.</i> 2005) (Zhong <i>et al.</i> 2011) (Ko <i>et al.</i> 2009) (McCarthy <i>et al.</i> 2009) (Wang <i>et al.</i> 2016)
MYB54 ^a	-	Activator	(Zhong <i>et al.</i> 2008)
MYB52 ^a	PttMYB21a	Activator Repressor (lignin)	(Karpinska <i>et al.</i> 2004) (Zhong <i>et al.</i> 2008)
MYB58 ^a	-	Activator (lignin)	(Zhou <i>et al.</i> 2009)
MYB63 ^a	-	Activator (lignin)	(Zhou <i>et al.</i> 2009)
MYB75 ^a	-	Repressor	(Bhargava <i>et al.</i> 2013)

MYB85 ^a	-	Activator	(Zhong <i>et al.</i> 2008)
MYB103 ^a		Activator Activator (Syringyl lignin)	(Zhong <i>et al.</i> 2008) (Ohman <i>et al.</i> 2013)
SND1 ^{a,e} (NST3/ANAC012)	EgrNAC61	Activator (cellulose, xylan, lignin)	(Mitsuda <i>et al.</i> 2007) (Zhong <i>et al.</i> 2006) (Zhong <i>et al.</i> 2007) (Laubscher <i>et al.</i> 2018)
SND2 ^{a,e,o}	-	Activator (cellulose, xylan, mannan, lignin polymerisation)	(Zhong <i>et al.</i> 2008) (Hussey <i>et al.</i> 2011)
	OsSND2		(Ye <i>et al.</i> 2018)
SND3 ^a	-	Activator	(Zhong <i>et al.</i> 2008)

^{a,p,e,o} Species from which the genes are characterised where a denotes *Arabidopsis*, p denotes *Populus*, e denotes *Eucalyptus* and o denotes *Oryza*.

2.2.5 Programmed cell death

Our present understanding of the transcriptional and phytohormonal regulation of PCD is in its infancy. Nevertheless, certain genes involved in the biosynthesis of digestive enzymes like proteases and nucleases have been identified as some of the main drivers of PCD (Zhang *et al.* 2014). In addition, some studies identified VND6 and XND1 as potential regulators of PCD in *Arabidopsis* xylem (Ohashi-Ito *et al.* 2010; Zhao *et al.* 2008). However, much remains to be explored in order to unravel the molecular regulation of PCD in woody trees.

Although, the roles of certain molecular regulators during wood formation have been determined (as seen in the above sections), the challenge of understanding the entire regulatory network underlying wood formation is far from complete. Many knowledge gaps remain in terms of elucidating the functions of other participating TFs, phytohormonal regulators and their potential interactions with each other and with genes involved in the biosynthesis of SCW polymers. Some common approaches that can be used to address these knowledge gaps include transcriptomic studies (based on RNA sequencing), gene discovery using gene tagging, traditional breeding and quantitative genetics, use of transgenic systems to elucidate gene function and

assessment of DNA-protein and protein-protein interactions (Groover *et al.* 2010) and a number of model systems have been employed for this purpose as discussed below.

2.3 MODELS FOR STUDYING THE MOLECULAR CONTROL OF WOOD FORMATION

Studying the molecular control of wood formation involves the identification of participating transcriptional and phytohormonal regulators and elucidation of their functions. Such studies often employ different model species/systems or different woody tissues. Examples for the use of model species include the use of non-woody herbaceous model plants such as *Arabidopsis*, *Nicotiana*, *Oryza* and *Zinnia* (Creux *et al.* 2013; Spokevicius *et al.* 2007b; Van Beveren *et al.* 2006). Studies on xylem development in these species provide valuable tools to mine for molecular regulators of xylogenesis using conserved regulatory pathways between non-woody herbaceous models and woody plants. Such studies have been greatly benefitted by the assembly of complete genome sequences of several woody tree species such as *Populus trichocarpa*, *Populus alba*, *Eucalyptus grandis*, *Picea abies*, *Picea glauca* and *Betula pendula* (Birol *et al.* 2013; Myburg *et al.* 2014; Nystedt *et al.* 2013; Salojärvi *et al.* 2017; Tuskan *et al.* 2006). The gene family expansions observed in particular gene families when comparing *Arabidopsis* to woody genera and gymnosperms to angiosperms, point towards increasing complexity of woody tissue (Hussey *et al.* 2015; Oakley *et al.* 2007; Ye and Zhong 2015; Zhang *et al.* 2014). Herbaceous model plants also serve as model systems for *in planta* functional characterisation of candidate TFs/phytohormonal regulators and for the determination of the regulatory networks that are associated with them. The second most commonly used type of model system is the comparison between different types of woody tissue. Since the co-occurrence of different types of woody tissue within a single tree is a result of variations in the expression patterns (differential expression) of genes and proteins caused by alterations in the regulatory mechanisms in response to environmental or developmental stimuli, these different types of woody tissue provide opportunities for the dissection into their respective molecular regulation (Plomion *et al.* 2001). Examples include comparisons between early and latewood; juvenile and mature wood; and between normal and RW.

2.4 REACTION WOOD AS A MODEL

2.4.1 Reaction wood

Different plants use different mechanisms to respond to gravitational stresses. While annual herbaceous plants differentially elongate in response to gravitational stress, lignified woody stems and branches which are incapable of further elongation, maintain tree posture by reorienting displaced stems and branches through the formation of RW (Gerttula *et al.* 2015; Ruelle 2014). RW in angiosperms is called tension wood (TW), and that in gymnosperms is called compression wood (CW). TW forms on the upper side of displaced stems and branches whereas CW forms on the lower side. CW generates compressive forces to push stems and branches to the optimal orientation while TW reorients displaced stems and branches by pulling them using tensile forces (Donaldson and Singh 2016; Groover 2016; Mellerowicz and Gorshkova 2012; Ruelle 2014; Timell 1986). These compressive and tensile forces are generated by characteristic alterations to cellular morphology and chemical composition of CW and TW cells compared to cells found in normal wood (NW; wood found in an uninclined portion of a stem or branch) and opposite wood (OW; wood on the opposite side of RW) (Donaldson *et al.* 1999; Mizrachi *et al.* 2015; Timell 1982). The characteristic features in cellular morphology and chemical composition can be attributed to various wood formation related processes such as gravitational stress response, cell division, vessel and fibre development, microfibril angle (MFA) determination and biosynthesis of cell wall biopolymers, as all of these processes undergo alterations during RW formation.

2.4.2 Reaction wood as a model system

When an immature xylem cell develops into a wood/mature xylem cell, a large number of changes take place with respect to its morphology, cell wall chemical composition and physical properties. The molecular regulators responsible for these changes include hundreds of TFs that are differentially expressed between immature and mature xylem. However, pinpointing the molecular regulators responsible for each change requires the use of a method/model that allows for easy connection of differential expression of TFs to specific morphological and/or chemical features. Since RW has some characteristic features which can be easily attributed to the

differentially expressed TFs during RW formation, RW can serve as such a model. RW can also be used to study other wood formation related processes which undergo alterations during RW formation (mentioned above), making RW an excellent model to study molecular mechanisms behind some of these processes and their transcriptional regulation (Jin *et al.* 2011; Kwon 2008).

2.4.2.1 *Gravitropic stress sensors*

RW has been used as a model to study the mechanisms behind graviperception. Using *Populus* RW Gerttula *et al.* (2015) demonstrated that initial graviperception in woody trees occurs through sedimentation of starch-filled amyloplasts in a group of specialised cells (statocytes). These statocytes are found in the endodermis of young *Populus* stems and in the secondary phloem of the mature woody trees (Gerttula *et al.* 2015; Groover 2016; Leach and Wareing 1967; Toyota *et al.* 2013). Thus, studies of RW formation revealed that woody stems could be able to sense gravitational stress directly.

2.4.2.2 *Phytohormonal regulators*

Several RW based studies provide further insights into the regulatory roles of phytohormones. Changes in the levels of phytohormones such as auxin, GA and ethylene have been observed in TW, OW and NW (Chen *et al.* 2015; Gerttula *et al.* 2015; Mizrachi *et al.* 2015). As an example, in response to gravitational stimuli, on the upper (tension) side, a flow of auxin is generated towards the cambium, and on the lower (opposite) side, the auxin flow is directed away from the cambium towards the cortex engendering a radial auxin gradient. Gerttula *et al.* (2015) confirmed the directionality of this auxin flow by demonstrating the reorientation of auxin polar transport protein PIN-FORMED3, towards the ground in both upper and lower parts of the bent stem. Groover (2016) suggested that this radial auxin gradient could be the ‘switch’ that turns on the transcriptional machinery for the initiation of RW formation. Also, GA is involved in RW formation by enhancing the radial flow of auxin (Gerttula *et al.* 2015) and Andersson-Gunneras *et al.* (2003) described a variation in ethylene concentration across TW, OW and NW, which they attributed to TW formation. Other RW studies showed that ethylene is involved in regulating the formation of TW fibres,

the establishment of the gelatinous layer (G layer), MFA and chemical composition of TW fibres (Felten *et al.* 2018; Seyfferth *et al.* 2019), however, the exact ethylene driven mechanisms behind these processes are yet to be determined.

Some members of auxin, GA, ethylene, cytokinin, brassinosteroids, ABA, SA and jasmonic acid (JA) signalling pathways are differentially expressed in TW, OW and/or NW (Chen *et al.* 2015; Mizrachi *et al.* 2015). Some of the TFs that show such differential expression include *Aux/IAA*, *ARF*, *ERF* and *Ethylene Response TFs* in *Populus*, *Eucalyptus*, *Betula* and *Liriodendron* (Andersson-Gunneras *et al.* 2003; Andersson-Gunneras *et al.* 2006; Chen *et al.* 2015; Mizrachi *et al.* 2015; Wang *et al.* 2014; Yu *et al.* 2015). For example, following artificial bending, the transcript levels of *EgIAA9A* increase during the first six to 24 hours and then decrease to the level of control between 24 hours to one week. Thus, *EgIAA9A* is a potential regulator of the initial stage of TW formation (Paux *et al.* 2005; Yu *et al.* 2015). Similar expression patterns were found for its *Populus* ortholog *PttAux/IAA2* (Moyle *et al.* 2002). Therefore, the expression levels of some TFs in phytohormonal signalling pathways seem to fluctuate throughout the course of RW formation. Since these members of phytohormonal signalling pathways include genes and TFs which are sensitive to alterations in phytohormone levels, they are potentially involved in integrating the transcriptional and phytohormonal regulation during RW formation.

Transcriptomic studies conducted during RW formation serve as a valuable resource for studying this process in order to identify potential genetic regulators (structural genes and TFs). RW transcriptomic studies have been conducted in *Eucalyptus*, *Populus*, *Betula*, *Acacia* and *Liriodendron* (Andersson-Gunneras *et al.* 2006; Azri *et al.* 2014; Cai *et al.* 2018; Chen *et al.* 2015; Gerttula *et al.* 2015; Jin *et al.* 2011; Mizrachi *et al.* 2015; Nugroho *et al.* 2018; Paux *et al.* 2005; Qiu *et al.* 2008; Wang *et al.* 2014; Yu *et al.* 2015; Zinkgraf *et al.* 2018). Based on the observation of a large number of differentially expressed genetic regulators in OW and NW in *Populus* RW, Groover (2016) hypothesised that although OW is expected to be similar to NW, OW might be having a significant role in gravibending (reaction to gravitational stress). In addition, some of these potential genetic regulators show differential expression during RW formation in more than one species and hence, they are considered as ‘strong

candidates' for contributing to transcriptional regulation of RW formation. Furthermore, several structural genes and TFs, which are known to participate in the regulation of wood formation, are also differentially expressed during RW formation (Pilate *et al.* 2004; Tocquard *et al.* 2014). Thus, it is likely that the network of genetic regulators behind RW formation is not a completely new one, but a network which is composed of several members of the existing regulatory network underlying wood formation.

2.4.2.3 Structural genes as regulators

Several structural genes have been highlighted by above transcriptomic studies as potential regulators of RW formation. For example, some members of single *FAS* group (group A) *FASCICLIN-LIKE ARABINOGALACTANS (FLA)* gene family, show differential expression between TW and OW (Lafarguette *et al.* 2004; MacMillan *et al.* 2015; Qiu *et al.* 2008). Lafarguette *et al.* (2004) found that ten *FLA* genes (*PopFLA1-10*) show differential expression between *Populus* TW and OW. Gerttula *et al.* (2015) reported that during TW formation, *FLA* expression is positively regulated by GA. Also, in *Eucalyptus* branches, *EgrFLA1* and *EgrFLA2* are upregulated in TW, whereas *EgrFLA3* is upregulated in OW (MacMillan *et al.* 2010; Qiu *et al.* 2008). Therefore, FLAs are potential regulators of differentiation and formation of *Populus* and *Eucalyptus* TW, most likely via influencing stem mechanics through altering MFA and cellulose deposition (Hobson *et al.* 2010; Lafarguette *et al.* 2004; MacMillan *et al.* 2010; MacMillan *et al.* 2015; Wang *et al.* 2015a; Wang *et al.* 2017). In addition, certain α -*TUBULIN (TUA)* and β -*TUBULIN (TUB)* members of the *TUBULIN* gene family have been identified as potential regulators of MFA during TW formation. In *Populus*, the *TUA1*, *TUA5*, *TUB9*, *TUB15* and *TUB16* genes are preferentially upregulated in TW (Oakley *et al.* 2007; Qiu *et al.* 2008; Spokevicius *et al.* 2007a). In *Eucalyptus*, the downregulation of *EgrTUB1* gene significantly increases the MFA in transgenic xylem fibres. Therefore tubulins are potentially involved in regulating mechanical properties of the cells during TW formation (Spokevicius *et al.* 2007a). Furthermore, certain *XYLOGLUCAN ENDO-TRANSGLYCOSYLASE/HYDROLASE (XTH)* genes show a higher expression in TW compared to OW and/or NW. As an example, in *Populus*, the *PtxtXTH14*, *PtxtXTH21* and *PtxtXTH36* genes are upregulated in TW compared to NW (Nishikubo *et al.* 2007). In addition, several

studies have suggested the potential involvement of XTH and XYLOGLUCAN ENDO-TRANSGLYCOSYLASE (XET) in G layer maintenance (Mellerowicz and Gorshkova 2012; Mellerowicz *et al.* 2008). XET is proposed to facilitate G fibre shrinkage via maintaining the connection between G and S2 layers by repairing xyloglucan crosslinks that break during shrinkage (Nishikubo *et al.* 2007). Therefore, using RW as a model, further insights have been gained into the regulatory roles of several structural genes during wood and RW formation.

2.4.2.4 Transcriptional regulators

Transcriptomic studies also point towards several TFs as potential regulators of RW formation. Examples comprise TFs including *WOX*, *Zinc fingers*, *WRKY*, *NST*, *SND*, *MYB*, *KNAT*, *VND*, *VND-INTERACTING (VNI)*, *WLIM*, *XND*, *TEOSINTE BRANCHED1/CININNATA/PROLIFERATING CELL FACTOR (TCP)*, *BASIC HELIX-LOOP-HELIX (bHLH)*, *BELL1-LIKE HOMEODOMAIN (BLH)*, *BELL1-LIKE FAMILY (BEL)*, *SHOOT MERISTEMLESS (STM)*, *ARBORKNOX (ARK)*, *COPI INTERACTING PROTEIN (CIP)*, and *HD-ZIPs* such as *HB* (Andersson-Gunneras *et al.* 2006; Azri *et al.* 2014; Cai *et al.* 2018; Chen *et al.* 2015; Gerttula *et al.* 2015; Grant *et al.* 2010; Groover 2016; Hussey *et al.* 2015; Mizrachi *et al.* 2015; Morita *et al.* 2006; Moyle *et al.* 2002; Paux *et al.* 2005; Pilate *et al.* 2004; Wang *et al.* 2014; Yu *et al.* 2015; Zinkgraf *et al.* 2018). Some of these TFs are known to regulate various stages of wood formation, in particular, SCW deposition and some have been found to be differentially expressed in TW or OW relative to NW in several woody trees. *ARK2*, for example, has been found to influence the expression of TW related genes (Du *et al.* 2009). Gerttula *et al.* (2015) studied the role of *ARK2* during RW formation and discovered that it enhances gravibending and accelerates G layer maturation while negatively regulating TW fibre production. Expression of *ARK2* is also sensitive to GA since increased levels of *ARK2* correlate with reduced levels of GA (Gerttula *et al.* 2015).

Although there is a long way to go in terms of identifying the RW specific functions of all the other TFs and in understanding their role within a generalised transcriptional network regulating RW formation, some predictions can be made based on the observed common patterns in their expression during RW formation and SCW

deposition. Based on these predictions, a hypothetical generalised transcriptional network regulating RW formation is presented below (Fig. 2.1).

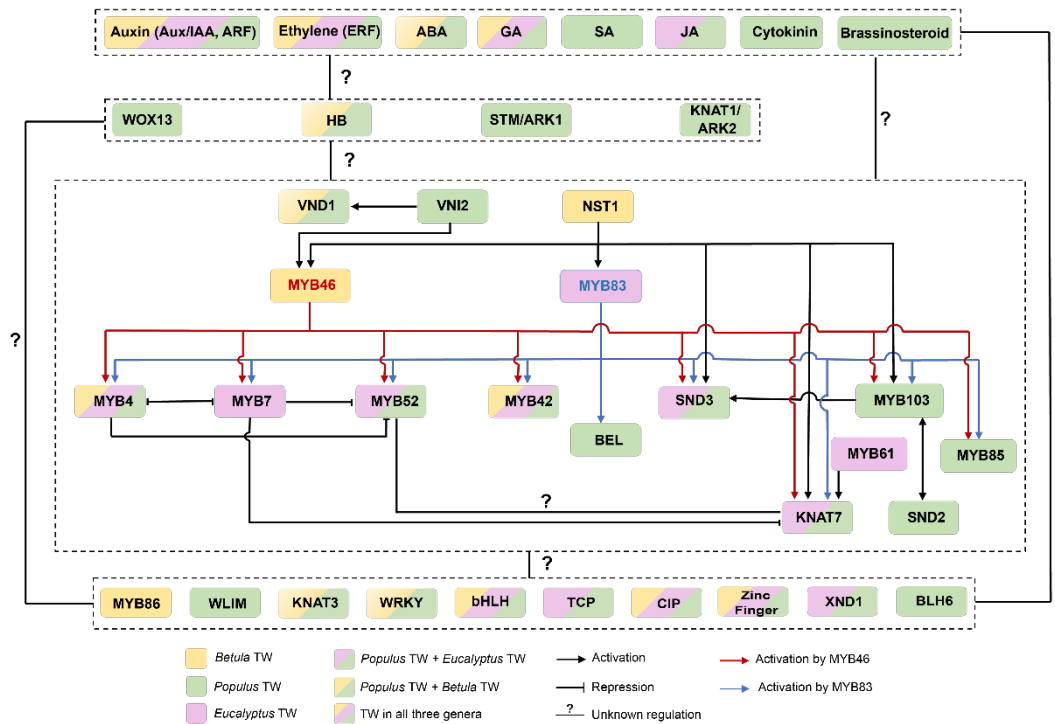


Figure 2.1 A hypothetical generalised network regulating RW formation. The candidate TFs (nodes) are selected based on the differential expression of their genes in TW, OW and NW. Dashed lines are used to group TFs semi-hierarchically based on their currently known functions during wood/TW formation. TFs with an unknown semi-hierarchical position have been grouped at the bottom of the figure. Only the names of phytohormones are mentioned when no specific information exists in the literature regarding the potential involvement of their TFs in RW formation. The name of the TF family or subfamily is used in instances where one or several members of a TF family or a subfamily are potential involved; however, their interactions with other TFs are unknown.

This hypothetical generalised transcriptional network (Fig. 2.1) includes several TFs whose genes are differentially expressed in TW compared to OW/NW in *Eucalyptus*, *Populus* and *Betula*. In this diagram, the closest *Arabidopsis* homolog of each *Eucalyptus* (pink), *Populus* (green) and *Betula* (yellow) TF is used as a node, demonstrating that homologs of their genes, *AtMYB42*, *AtMYB4* and members of *AtbHLH*, *AtCIP* and some *zinc fingers* show differential expression across TW, OW and NW in *Eucalyptus*, *Populus* and *Betula* (Cai *et al.* 2018; Chen *et al.* 2015; Mizrahi *et al.* 2015; Paux *et al.* 2005; Wang *et al.* 2014; Zinkgraf *et al.* 2018). Homologs of *HB*, *VND1*, *XND1*, *SND3*, *MYB52*, *KNAT7*, *KNAT3* and some *WRKY* and *TCP* TFs

show differential expression across TW, OW and NW tissues at least in two woody species (Andersson-Gunneras *et al.* 2006; Cai *et al.* 2018; Chen *et al.* 2015; Grant *et al.* 2010; Hussey *et al.* 2015; Mizrachi *et al.* 2015; Wang *et al.* 2014; Zinkgraf *et al.* 2018). Interactions between them have been hypothesised based on evidence of their interactions during SCW deposition (Hussey *et al.* 2013).

Based on previously observed functions of the described TFs in *Arabidopsis*, certain predictions can be made on how these TFs might be involved in RW formation. For example, VND1 acts as a positive regulator of xylem vessel formation and SCW deposition of *Arabidopsis* xylem vessels (Endo *et al.* 2015; Tan *et al.* 2018; Zhou *et al.* 2014) whereas XND1 acts as a repressor of xylem vessel formation, SCW deposition and PCD (Zhao *et al.* 2008). Therefore, VND1 and XND1 homologs are presumably involved in regulating xylem vessel-specific RW features such as reduced vessel density and more elongated and less porous vessels (Jourez *et al.* 2001; Ruelle *et al.* 2006). Also, members of MYB, KNAT and SND subfamilies are known regulators of SCW deposition in *Arabidopsis*. Therefore, their homologs in woody species are presumably responsible for the RW specific features related to alterations in SCW deposition and chemical composition of RW cells (Cai *et al.* 2018; Mizrachi *et al.* 2015; Wang *et al.* ; Zhong *et al.* 2008; Zinkgraf *et al.* 2018). As an example, activators of SCW deposition such as MYB46, MYB52, MYB61, MYB83, MYB85, MYB103, KNAT3, SND2 and SND3 are most likely to be the regulators of characteristic TW features such as deposition of G layer, increased lignin monomer (syringyl:guacyl, (S:G)) ratio and increased cellulose and galactose (a type of hemicellulose) contents. In contrast, repressors of SCW deposition such as MYB4 and MYB7 could be the potential regulators of TW features such as reduced lignin content, elevated S:G ratio, and reduced xylose and mannose (types of hemicelluloses) contents (Mizrachi *et al.* 2015). Furthermore, KNAT7 has been known as a repressor of lignin and cellulose biosynthesis, and it has been recently identified as an activator of hemicellulose biosynthesis (He *et al.* 2018; Li *et al.* 2012a; Liu *et al.* 2014b). Therefore, the observed upregulation of KNAT7 during TW formation could be regulating some of the above-mentioned TW specific SCW features. In addition to regulating SCW biosynthesis, MYB46 also functions as a potential regulator of *FLA* genes in *Arabidopsis* (Ko *et al.* 2009). Therefore, during TW formation, MYB46 could

be a potential regulator of cellulose deposition and MFA-mediated alterations in stem mechanics.

Although not a member of the *Arabidopsis* SCW network, certain WRKY TFs in some species (e.g. *Vitis vinifera*) are known to regulate xylem formation, vessel dimensions and SCW lignin composition (Andersson-Gunneras *et al.* 2006; Guillaumie *et al.* 2010; Mizrachi *et al.* 2017). Therefore, differentially expressed WRKY TFs in *Betula* and *Populus* TW could be potential regulators of TW features including altered vessel morphology, reduced lignin deposition and increased S:G ratio. Similarly, the expression of WLIMs is reduced in TW compared to NW and WLIMs are known to be co-expressed with lignin biosynthetic genes (Andersson-Gunneras *et al.* 2006; Azri *et al.* 2014; Chen *et al.* 2015). Therefore, WLIM is potentially involved in regulating lignin-related TW features such as reduced lignin deposition and increased S:G ratio. However, to corroborate or negate the above-hypothesised roles of these TFs during RW formation, further functional investigations need to be undertaken.

2.4.3 Challenges in using reaction wood as a model

Use of models is convenient; however, like any other model, using the RW model too is not without its complications. When using RW as a model, a range of issues need to be considered due to a number of inconsistencies that have been observed between different experiments, presenting significant drawbacks associated with using RW as a model system. Especially, a fact that has often been overlooked is that even though RW from different species share some similarities, RW is not identical across all species (Jourez *et al.* 2001; Mizrachi *et al.* 2015; Ruelle *et al.* 2006). In addition, comparing and using the results from various RW-based studies, need to be done with caution, mostly due to the variations in experimental designs (Qiu *et al.* 2008). For example, while some researchers studied naturally formed RW in stems or branches (Chen *et al.* 2015) others studied artificially inclined stems or branches (Moyle *et al.* 2002; Wang *et al.* 2017; Zinkgraf *et al.* 2018), where the latter imposes an external force in addition to gravitational stress. Therefore, results generated from such studies may not accurately depict natural graviresponses (Li *et al.* 2013). Tissue samples for various gene expression studies have been collected at various time points during the

RW formation process, ranging from hours to months or even years since the artificial or natural inclination of stems or branches. However, some previous RW studies have shown that during RW formation, the expression of certain genes and TFs varies in a temporal manner. Therefore, the time of sample collection may influence the gene expression data arising from different studies. Furthermore, RW studies reveal expression levels of candidate genes and TFs comparing different tissues such as TW vs OW, TW vs NW or TW vs OW vs NW. These variations need to be taken into consideration, particularly when drawing conclusions about the up or down-regulation of a selected gene or TF during RW formation. Although not yet proven, these discrepancies in experimental design might have influenced the observed changes in the level of expression of certain TFs. Thus, these variations need to be taken into account when elucidating the role of a particular molecular controller during RW formation.

3 MICROANALYTICAL TECHNIQUES FOR PHENOTYPING SECONDARY XYLEM TISSUE

3.1 INTRODUCTION

The secondary tissues wood and bark play significant roles in plant development. Secondary phloem is responsible for translocation of photosynthates and it shields and protects the plant from extreme temperatures, pests and pathogens. Secondary xylem is responsible for the transport of water and minerals from roots to crown, providing both rigidity and flexibility to allow trees to grow while adjusting to different environmental stimuli (such as gravity, wind and light) and acting as one of the major carbon sinks on earth (Dejardin *et al.* 2010). As a renewable material, secondary xylem has numerous commercial applications including energy production (firewood and second generation biofuels), construction (timber and plywood for buildings and furniture) and pulp and paper production (Andersson-Gunneras *et al.* 2006; Dejardin *et al.* 2010; Ye and Zhong 2015). Because of its biological and economic significance, secondary growth has been extensively studied both in annuals such as *Arabidopsis* and in woody tree species (Chaffey *et al.* 2002b; Groover and Robischon 2006; Zhang *et al.* 2018; Zhong *et al.* 2010; Zhong and Ye 2013; Zhong and Ye 2014; Zhong and Ye 2015).

Much research has been directed at understanding how cell/tissue structure, morphology, and chemical composition changes in response to extrinsic (biotic and abiotic stresses) and intrinsic (genetic and epigenetic) factors. Also, in recent years, a substantial amount of research has focussed on lignocellulosic biomass due to the increasing attention on the use of lignocellulose in biofuel production. Examples include large scale population level experiments as well as fine scale experiments such as tissue or cellular level mapping and analysis of changes in cell morphology, cell wall chemical composition and/or micromechanical properties. Such efforts reveal how secondary xylem differentiation responds to seasonal, environmental and genetic variations, with respect to its physiology, molecular responses or plant growth

regulator concentrations (Felten *et al.* 2018; Gerber *et al.* 2016; Gierlinger *et al.* 2010; Kong *et al.* 2018; Spokevicius *et al.* 2016; Taguchi *et al.* 2010; Tuominen *et al.* 1997; Wood *et al.* 2017). One common challenge faced by such studies is the selection of suitable methods to determine phenotypic changes specific to secondary xylem.

Selection of a suitable phenotyping method depends on the objective of the study, the nature of the sample (fresh or dried/preserved tissue), cost, number of samples and on the amount of tissue that is available for phenotyping. Of course, at the organismal level, individual trees can produce large amounts of tissue (gram or kilogram quantities), whereas, in the case of fine scale (micrometre) mapping across secondary xylem, the use of small tissue sectors derived from methods such as Induced Somatic Sector Analysis (ISSA) or when young plants are sampled, only microgram amounts of tissue are available for phenotyping (Gerber *et al.* 2016; Spokevicius *et al.* 2016). Compared to phenotyping unlimited amounts of tissue, only a few methods have proven suitable when analysing tissue samples in limited or small amounts or in cases where studying sample heterogeneity is important. In these instances, microanalytical characterisation becomes the only option.

Microanalytical techniques provide insights into morphological and chemical properties of secondary xylem where samples are limited to microgram quantities or less and can be broadly categorised into (i) methods for phenotyping morphological features (morphotyping) and (ii) methods for phenotyping chemical properties (chemotyping). Morphotyping can reveal anatomical features of wood cells such as cellular diameter, perimeter, cell size, cell length, cell shape, cell wall thicknesses, cell wall area and microfibril angle (MFA), and ultrastructural details such as different secondary cell wall (SCW) layers and their distinct features. In contrast, chemotyping can provide details on chemical composition and the abundance and distribution of cell wall biopolymers (cellulose, lignin and hemicelluloses) and wood extractives (phenolics, tannins, terpenes, flavonoids and lignans). However, some specific techniques can be used both for morphotyping and chemotyping. Accordingly, this review describes some of the most widely used microanalytical techniques based on their ability to use small (μg or less) amounts of tissue for morphotyping, chemotyping or both (Fig. 3.1). We begin this review by highlighting some key aspects of sample

preparation related to microanalytical techniques discussed below. We provide examples of how these microanalytical techniques have been successfully used to phenotype woody tissue and present a comparative assessment of key microanalytical techniques together with a guide for the selection of techniques for specific applications.

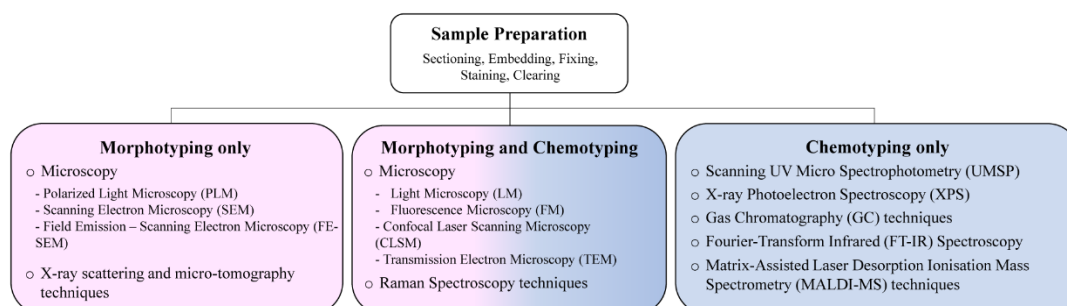


Figure 3.1 A summary of the key microanalytical techniques discussed in this review. Pink demonstrates microanalytical techniques for morphotyping, blue demonstrates microanalytical techniques for chemotyping, and pink/blue denotes dual micro morpho and chemotyping techniques. The pink and blue shading is consistent with that in Fig. 3.4.

3.2 SAMPLE PREPARATION

3.2.1 Sectioning, embedding, and fixing

In this section, we outline some of the most used sample preparation methods and provide a summary relating them to the microanalytical techniques described in later sections (Table 3.4 and 3.5). Appropriate sample preparation is crucial to ensure that the quality and quantity of the sample suit the intended phenotyping method (Verhertbruggen *et al.* 2017). Depending on the intended phenotyping method, sample preparation could involve either a single step such as sectioning, grinding and milling or a sequence of steps such as fixing, embedding and sectioning. In preparation for certain applications like electron microscopy, the sectioned sample is subjected to additional steps such as dehydration, drying and sputter coating (with metals such as chromium, gold and/or platinum). Tissue maceration is another sample preparation method in which the cells/fibres are separated from one another by dissolving the middle lamella using chemicals such as glacial acetic acid, hydrogen peroxide, or nitric acid to name a few (Mahesh *et al.* 2015). Furthermore, for chemotyping purposes, samples can be ground to a powder (using a ball mill or similar) and/or chemically degraded/hydrolysed to separate soluble and insoluble fractions. For example, the

chromatographic techniques such as gas chromatography (GC), require a sequence of labour-intensive sample preparation steps, generally starting from extraction followed by processing via hydrolysis, reduction and derivatisation (chemical reactions which can enhance the volatility of the sample components) (Angeles *et al.* 2006; MacMillan *et al.* 2015).

Tissue sections can be preserved by ‘fixation’ which terminates all cellular processes and immobilises cellular components to ensure that tissue sections remain close to their native state with all cells intact. Depending on the type of plant tissue and intended type of phenotyping, the requirement of a fixation step and appropriate type of fixation can vary. Fixation is only required in cases where intact cells/tissues are needed to analyse fine cellular features at the subcellular/organellar level. Fixation can be achieved either by chemical or physical means (i.e. dehydration), with the former using chemicals such as acetone, acetic acid, ethanol, methanol, glutaraldehyde, formaldehyde, glacial acetic acid or osmium tetroxide and the latter employing cryopreservation or microwaves (Bao *et al.* 2012; Begum *et al.* 2012; Yeung *et al.* 2015). With either method, consideration must be given to the potential introduction of fixation artefacts (cell shrinkage, cell swelling) which might influence the final outcome (Begum *et al.* 2012). One method of avoiding fixation artefacts is adjusting the osmolarity of fixation reagents to match that of the tissue (Loqman *et al.* 2010).

Tissue samples are often embedded to prevent distortion during subsequent sectioning steps. Use of a suitable embedding medium ensures that samples are sufficiently stabilised to withstand physical forces applied when sectioning using microtome-based methods or with laser microdissections. Commonly used embedding media include agarose, paraffin, LR-white resin, polystyrene, polyethylene glycol, Spurr’s resin and epoxy resin (Angeles *et al.* 2006; Bao *et al.* 2012; Begum *et al.* 2012; Sato *et al.* 2001; Wu *et al.* 2009; Yang *et al.* 2017). However, it must be noted that some embedding agents may not be suitable in all instances since they may interfere with a specific microanalytical technique where the embedding agent might become a contaminant (Foston *et al.* 2011). In such cases where embedding is not desirable, either a removable embedding medium (post sectioning) can be used or a sectioning method

that does not require embedding can be adopted, e.g. cryotome sectioning, which involves freezing tissue to increase its rigidity for sectioning (Foston *et al.* 2011).

Sectioning can be preceded by fixation, embedding, or a combination of both depending on the intended phenotyping method and required phenotypic data. Obtaining high quality sections with appropriate thicknesses is crucial for phenotyping since the quality of the section will have a considerable impact on the result. Sectioning can be performed by hand, by microtome (sliding microtome, rotary microtome, ultramicrotome, vibratome or cryotome) or by using laser microdissection, where methods like preparative laser capture microdissection have the potential to isolate microgram amounts of tissue potentially consisting of only a few cells (Angeles *et al.* 2006; Foston *et al.* 2011; Gerber *et al.* 2014; Lima *et al.* 2014; Wu *et al.* 2009; Yang *et al.* 2017). However, an alternative approach has been suggested by Dickson *et al.* 2017, which involves embedding the sample in resin and polishing it with a few different grinding papers. This approach precludes the need for sectioning and has proven to generate smooth surfaces which enable observation of subtle structures such as the cambium. The sectioned samples can then either be directly phenotyped or first subjected to a staining step.

3.2.2 Staining

Staining allows rapid visual assessment of the integrity of tissues, cellular features and specific cell wall depositions. It has both morphotyping and chemotyping applications as stains can be used for visualisation of different chemical components in cell walls (e.g. polysaccharides, differences in lignin composition, differential staining of polysaccharides and lignin) and thereby allow for assessment of certain morphological features including cell wall thickness and cell size (Anderson *et al.* 2010; Dogu and Grabner 2010; Eckert *et al.* 2019; Kidner *et al.* 2016; Lafarguette *et al.* 2004; Mitra and Loque 2014; Patten *et al.* 2005; Qiu *et al.* 2008; Smetana *et al.* 2019; Verbelen and Kerstens 2000; Wessels *et al.* 2019; Wood 1980; Yuan *et al.* 2019; Zhong *et al.* 2019). Commonly used stains include histological stains, fluorescent stains, immunological stains and metal-based stains where the choice of stain/staining

depends on the features of interest and phenotyping method. Since some of these stains have toxic effects; special care must be taken when handling them.

Histological stains are the most used type of stains, and some of them are employed in light microscopy (LM) (Table 3.1). These histological stains can be used either for single or double staining (use of two stains to differentially stain two different types of cell wall polymers in contrasting colours) depending on cellular features of interest.

Table 3.1 Commonly used histological stains for visualising cell wall polymers

Histological stain	Chemical target	Colour after staining	References
Safranin [†]	Lignin	Red	Begum <i>et al.</i> 2012, Carrillo <i>et al.</i> 2008, Gerber <i>et al.</i> 2014, Qiu <i>et al.</i> 2008, Sato <i>et al.</i> 2001, Smetana <i>et al.</i> 2019, Zhong <i>et al.</i> 2019
Phloroglucinol-HCl (Wiesner reagent)	Lignin	Characteristic pink	Araujo <i>et al.</i> 2014, Bao <i>et al.</i> 2012, Eckert <i>et al.</i> 2019, MacMillan <i>et al.</i> 2010, Wang <i>et al.</i> 2014, Yang <i>et al.</i> 2017
Mäule stain	Syringyl (S) lignin	Red	MacMillan <i>et al.</i> 2010, Patten <i>et al.</i> 2005
Toluidine blue O (TBO) (Stevenel's blue)	Pectin Lignin Tannins	Pinkish purple Greenish or bright blue	Bao <i>et al.</i> 2012, Chai <i>et al.</i> 2015, Kidner <i>et al.</i> 2016, MacMillan <i>et al.</i> 2010, Manabe <i>et al.</i> 2013, Mitra and Loque 2014, Patten <i>et al.</i> 2005, Yang <i>et al.</i> 2017, Wu <i>et al.</i> 2009
Congo red ^{†*}	β -(1→4)-glucans Cellulose Xyloglucan	Red	Mitra and Loque 2014, Verbelen and Kerstens 2000, Anderson <i>et al.</i> 2010, Wood 1980

Methylene blue	Cellulose	Blue	Al-Haddad <i>et al.</i> 2013, Chaffey <i>et al.</i> 2002, Kidner <i>et al.</i> 2016, Lafarguette <i>et al.</i> 2004, Qiu <i>et al.</i> 2008
Fast green	Cellulose	Green	Yuan <i>et al.</i> 2019, Zhong <i>et al.</i> 2019
Alcian blue	Insoluble carbohydrates Non-lignified tissue	Blue	Smetana <i>et al.</i> 2019, Wessels <i>et al.</i> 2019, Al-Haddad <i>et al.</i> 2013, Chaffey <i>et al.</i> 2002, Kidner <i>et al.</i> 2016, Lafarguette <i>et al.</i> 2004, Qiu <i>et al.</i> 2008
Astra blue	Non-lignified polysaccharides	Blue	Carrillo <i>et al.</i> 2008, Groover 2016, Dogu and Grabner 2010

† Safranin and congo red also has some fluorescent properties. * Congo red is considered as a weak stain for cellulose.

Fluorescent stains are another type of stain which are employed in fluorescence and confocal microscopy (Anderson *et al.* 2010; Bond *et al.* 2008; Brundrett *et al.* 1988; Brundrett *et al.* 1991; Kitin *et al.* 2000; Kraus *et al.* 1998; Moller *et al.* 2006; Morris *et al.* 2018; Pendle and Benitez-Alfonso 2015; Schenk *et al.* 2018; Siebers 1960; Sotiriou *et al.* 2016; Stone *et al.* 1984; Thomas *et al.* 2017; Thomas *et al.* 2013; Ursache *et al.* 2018; Wang *et al.* 2019a; Wood 1980; Yeung *et al.* 2015). The fluorescence emitted from these stains can be easily distinguished from the scattering of the incident light, particularly if the incident light is monochromatic. Hence, if the sample itself possesses only a little or no fluorescence at a specific wavelength, then the entire intensity detected at that wavelength can be attributed to the fluorescent stain (Hubbe *et al.* 2019).

Table 3.2 Commonly used fluorescent stains for visualising cell wall polymers

Chemical target	Fluorescent stain	References
Callose	Aniline blue	Brundrett <i>et al.</i> 1988, Stone <i>et al.</i> 1984, Sotiriou <i>et al.</i> 2016, Pendle and Benitez-Alfonso 2015
Cellulose	Calcofluor white (fluorescent brightener)	Thomas <i>et al.</i> 2012, Anderson <i>et al.</i> 2010, Wood 1980
Cellulose	Pontamine fast scarlet 4B	Thomas <i>et al.</i> 2017, Thomas <i>et al.</i> 2012
Cellulose, Xyloglucan	Congo red [†]	Thomas <i>et al.</i> 2012, Anderson <i>et al.</i> 2010, Wood 1980
Lignin	Safranin [†]	Bond <i>et al.</i> 2008, Kitin <i>et al.</i> 2000
Lignin, Callus	Basic fuchsin	Kraus <i>et al.</i> 1998, Moller <i>et al.</i> 2006, Ursache <i>et al.</i> 2018
Lignin, Suberin	Berberine	Brundrett <i>et al.</i> 1988, Yeung <i>et al.</i> 2015
Pectin	Coriphosphine	Schenk <i>et al.</i> 2018, Morris <i>et al.</i> 2018, Siebers 1960
Suberin	Fluorol yellow	Brundrett <i>et al.</i> 1991, Wang <i>et al.</i> 2019

[†] Safranin and congo red also function as histological stains.

The challenging task of developing methods to selectively target specific cell wall polymers *in-muro*, has been significantly addressed by carbohydrate binding modules and immunostaining. Immunostaining techniques use antibodies for detection of specific cell wall polymers and even specific decorations on polysaccharide backbones (Altaner *et al.* 2010; Arend 2008; Clausen *et al.* 2003; Gritsch *et al.* 2015; Kim and Daniel 2017; Knox 1997; Liebsch *et al.* 2014; Liu *et al.* 2013; Meikle *et al.* 1991; Obro *et al.* 2007; Pattathil *et al.* 2010; Pendle and Benitez-Alfonso 2015; Potocka *et al.* 2018; Qi *et al.* 2013; Snegireva *et al.* 2010; Willats *et al.* 1998; Zhang *et al.* 2003). Carbohydrate binding modules can detect polysaccharides and oligosaccharides such

as cellulose and mannan (Blake *et al.* 2006; Filonova *et al.* 2007; Hilden *et al.* 2003; McCartney *et al.* 2004). Some of the most commonly used antibodies and carbohydrate binding modules are listed in Table 3.3. The unique binding specificities of antibodies or carbohydrate binding modules towards cell wall polymers provide an excellent platform to determine the abundance and distribution of a polymer in cell walls. However, the signal reliability and interpretation can be influenced when certain antibodies fail to bind the targeted cell wall polymers either due to a structural alteration in the targeted cell wall polymer, due to masking of targeted cell wall polymer by other cell wall polymers or due to inaccessibility (Knox 2008; Sutherland *et al.* 2009).

Table 3.3 Commonly used antibodies and Carbohydrate binding modules in cell wall polymer detection

Specificity to epitopes in cell wall polymers	Antibody or Carbohydrate binding module	References
Callose ((1-3)- β -glucan)	(1-3)- β -oligoglucos-ides antibody, Anti-1,3- β -glucan	Meikle <i>et al.</i> 1991, Pendle and Benitez-Alfonso 2015, Altaner <i>et al.</i> 2010
Cellulose (anti-amorphous)	CBM28	Blake <i>et al.</i> 2006, Liu <i>et al.</i> 2013, Ruel <i>et al.</i> 2012
Cellulose (crystalline)	CBM3a	Blake <i>et al.</i> 2006, Liu <i>et al.</i> 2013, Qi <i>et al.</i> 2013, Ruel <i>et al.</i> 2012, Yang <i>et al.</i> 2013
Cytoplasmic portion	JIM13	Yang <i>et al.</i> 2013
β -1,3-1,4-glucan	MLG	Liu <i>et al.</i> 2013
Galactan ((1-4)- β -galactan)	LM5	Arend 2008, Kim and Daniel 2017, Verhertbruggen <i>et al.</i> 2017, Willats <i>et al.</i> 2000

Mannan	LM21	Kim and Daniel 2017,
Heteromannan	BGM C6, LM22	Verhertbruggen <i>et al.</i> 2017
Xylan (4-O-methylglucuronoxylan), Heteroxylan (low substituted (1-4)- β -D-xylan)	LM10, LM11	Kim and Daniel 2017, Liu <i>et al.</i> 2013, Verhertbruggen <i>et al.</i> 2017, Yang <i>et al.</i> 2013
Xyloglucan	LM15	Kim and Daniel 2017
Arabinan in pectin (α (1-5) arabinan)	LM6, LM13, LM16	Snegireva <i>et al.</i> 2010, Arend 2008, Kim and Daniel 2017, Willats <i>et al.</i> 1998, Verhertbruggen <i>et al.</i> 2017
De-esterified homogalacturonan	PAM1, CCRC-M38	Gritsch <i>et al.</i> 2015, Pattathil <i>et al.</i> 2010, Willats <i>et al.</i> 2000
Homogalacturonan in pectin - high degree methylation - low degree methylation - partial methylation	JIM7, LM20 JIM5, LM19 LM18	Arend 2008, Clausen <i>et al.</i> 2003, Liu <i>et al.</i> 2013, Obro <i>et al.</i> 2007, Snegireva <i>et al.</i> 2010, Verhertbruggen <i>et al.</i> 2017, Yang <i>et al.</i> 2013
Arabinogalactan protein	JIM8, JIM13	Knox 1997, Liebsch <i>et al.</i> 2014, Potocka <i>et al.</i> 2018, Zhang <i>et al.</i> 2003
Arabinogalactan protein (tend to label cell walls or extracellular spaces)	Anti-AGPB	Zhang <i>et al.</i> 2003

Transmission Electron Microscopy (TEM) often employs metal stains such as lead citrate, uranyl acetate, osmium tetroxide and potassium permanganate. Lead citrate alters the contrast and thereby enhances structures in the final image to visualise cell wall polymer architecture. Uranyl acetate functions as a negative stain when looking at cellulose microfibrils whereas potassium permanganate functions as a positive stain

for lignin (Bao *et al.* 2012; Lehringer *et al.* 2009; Sato *et al.* 2001). Osmium tetroxide, in particular, provides dark contrast areas which correspond to lignin or to its precursors on cell walls (Fig. 3.2h) (Borgin *et al.* 1975; Hepler and Newcomb 1963; Reza *et al.* 2015; Reza *et al.* 2014). Sometimes these stains are used in combination, e.g. the routine use of uranyl acetate and lead citrate in combination to achieve the highest contrast (Bao *et al.* 2012).

When observing secondary xylem under a microscope, 'clearing' of tissue can enhance transparency of the specimen and reduce background signal, thereby circumventing light scattering which is the main hindrance to obtaining clear microscopic observations (Ariel 2017; Gray *et al.* 1999; Kitin *et al.* 2000; Richardson and Lichtman 2015; Running *et al.* 1995). Clearing agents are of two types; clearing agents like ethanol are used to remove the cellular cytoplasmic content whereas other clearing agents like lactic acid are used due to their ability to reduce refraction and light scattering during microscopy (Lux *et al.* 2005). However, it is crucial to choose a compatible solvent (glycerol, acetone, ethanol) for clearing since the use of unsuitable solvents can result in removal of the stain (Kitin *et al.* 2002; Kitin *et al.* 2003). Similarly, the potential impact of certain solvents used in sample pre-treatment, such as excess stain removal, on wood properties, particularly the removal of soluble fractions via organic solvents, needs to be considered.

3.3 MORPHOTYPING MICROANALYTICAL TECHNIQUES

Micro morphotyping techniques can reveal anatomical details such as cell size, cell shape, SCW thickness, MFA, and ultrastructural details such as different SCW layers and their distinct features (Donaldson 2019). In this section, we describe some of the most widely used micro morphotyping techniques like polarised LM, electron microscopy and X-ray based techniques. For ease of reference, we summarise the features, inherent limitations, requirements and advantages of each technique in Table 3.4 and Fig. 3.4 to assist with the selection of a suitable micro morphotyping technique.

Polarised Light Microscopy (PLM) is a simple method that uses thinly sectioned or macerated (see section 3.2.1) samples and polarised light to observe cell wall layering

and to inspect SCWs which are birefringent owing to the orientated deposition of cellulose in contrast to the primary cell walls which are non-birefringent as a result of random cellulose deposition. Hence, polarised light circumvents the requirement for stains to distinguish cellular features (Oldenbourg 2013; Preston 1974; Zhang *et al.* 2003). Some applications of PLM include observation of different xylem cell types at varying developmental stages, MFA, variations in SCW thickening and cellulose crystallinity (Fig. 3.2a) (Andersson *et al.* 2000; Donaldson 2019; Oldenbourg 2013; Olins *et al.* 2018; Zhang *et al.* 2003). PLM has been successfully used to measure MFA, for example, in ISSA studies involving as little as 30 - 80 µg of wood sector material, demonstrating the high sensitivity of this method (Lima *et al.* 2014; Spokevicius *et al.* 2007a). Similarly, polarised fluorescence microscopy also has been successfully used to measure MFA (Thomas *et al.* 2017; Verbelen and Kerstens 2000). In addition, polarised fluorescence microscopy is also capable of estimating cell wall anisotropy (Altartouri *et al.* 2019).

Scanning Electron Microscopy (SEM) is the most commonly used microanalytical technique for observing surface features and anatomy of wood tissue sections and cells, typically at high spatial resolution and high magnification. Generally, in conventional SEM, achievable spatial resolution and magnification fall between 10 - 100 nm and 20× - 30,000×, respectively (Begum *et al.* 2012; Foston *et al.* 2011; Spokevicius *et al.* 2016). SEM fires a focused beam of electrons onto the surface of the sample and detects signals generated by electrons interacting with the sample to obtain information about the topography of the sample surface. Sample preparation can involve drying, fixing, sectioning and sputter coating or it can be as simple as just sectioning either by hand or by a microtome (Begum *et al.* 2012; Melder *et al.* 2015; Spokevicius *et al.* 2016). Sputter coating with gold, platinum or chromium makes samples more conductive and reduces charging, however, un-coated samples can be used if imaging can be done at low voltage mode, e.g. studies on xylem cell morphology that measures xylem cell features such as cross-sectional area, cell wall thickness and lumen area (Begum *et al.* 2012; Kanbayashi and Miyafuji 2016; Spokevicius *et al.* 2016; Yeung *et al.* 2015). SEM provides a pragmatic and relatively high throughput morphotyping platform to observe the ultrastructure of the SCW, the anatomy of wood cells and to accurately measure cellular dimensions.

Field Emission-Scanning Electron Microscopy (FE-SEM) is a versatile, high-resolution microanalytical technique for morphotyping both surface and subsurface layers of secondary xylem. In particular, it can be used to morphotype tissue samples which are damaged due to mechanical handling (Singh and Dawson 2014). Compared to conventional SEM, FE-SEM employs a low acceleration voltage, a brighter electron source and a smaller beam size which typically improve the magnification and resolution up to $50,000\times$ - $80,000\times$ and 1 - 10 nm, respectively. However, the sample resolution also depends on sample preparation. As an example, sputter coating, which takes place after microtome sectioning, dehydration and drying of the samples, potentially adds ~10 - 20 nm to small structures. Heu *et al.* (2019) provide an excellent analysis of coating metals as a guide to select an optimum metal for sputter coating. As an example, the most suitable coating metals for high magnification and high-resolution FE-SEM imaging are chromium and tungsten. Although sputter coating may be disregarded, in the absence of sputter coating FE-SEM analysis may require a higher voltage and compared to a metal-coated surface the electron emission from a non-coated carbon surface is generally low. Hence an appropriate coating metal should be selected depending on the nature of the required analysis, which could be either microanalysis or imaging at high or low magnification (Heu *et al.* 2019).

In electron microscopy, charging artefacts often occur due to the tendency of electrons to accumulate within the sample resulting in an excess local signal. However, since FE-SEM can perform at low acceleration voltages, these charging artefacts can be reduced (Donaldson *et al.* 2007). When combined with a Focused Ion Beam (FIB), FE-SEM has the potential to provide micrographs with a resolution of as little as few angstroms, similar to that of a conventional transmission electron microscope. FE-SEM often serves as the method of choice for fibre cell analysis as it can be used to assess topography and anatomy of wood fibres (Fig. 3.2b), the arrangement of cellulose microfibrils and MFA (Derba-Maceluch *et al.* 2015; Foston *et al.* 2011; Lehringer *et al.* 2009; Takata *et al.* 2019). Although a higher magnification ($> 80,000\times$) cannot be obtained without causing radiation damage and the delicate matter can be quickly degraded by the electron beam, FE-SEM has the potential to serve as a reliable and successful nanoscale platform for the analysis of tissue samples (Lehringer *et al.* 2009).

X-ray based techniques; Wide Angle X-ray Diffraction (WAXD), Small Angle X-ray Scattering (SAXS) and X-ray micro-computed tomography (μ CT) are some of the most commonly used X-ray based techniques. The WAXD and SAXS techniques are applied for elucidating structure and orientation of cellulose microfibrils, providing insights into the mechanical properties of tissue samples (Reiterer *et al.* 1999; Ruggeberg *et al.* 2013). X-ray scattering permits the use of samples in their native state rather than prepared samples, provides robust measurements and is high throughput, requiring about two minutes for measuring each angular step of 1.6° (Gerber *et al.* 2014; Ruggeberg *et al.* 2013). By adjusting the size of the beam, SAXS can be used at a spatial resolution on a micrometre scale or a bigger sample area comprising of several cells. However, the latter requires significant expertise in data interpretation as SAXS cannot differentiate between two cell types with different MFAs or the same cell type with two MFAs (Andersson *et al.* 2000; Reza *et al.* 2015). Radiation-induced damage is another drawback of X-ray based methods (Saxe *et al.* 2014). Nevertheless, SAXS and WAXS methods can provide insights into the cellulose arrangement of xylem cells. Some studies have used SAXS and/or WAXS methods to elucidate average MFA in different SCW layers (e.g. S1 and S2), the average diameter of cellulose crystallites and the degree of crystallinity of cell wall layers (Andersson *et al.* 2000; Gerber *et al.* 2014; Maloney and Mansfield 2010; Svedstrom *et al.* 2012). However, such applications are not restricted to samples with secondary growth as SAXS can be successfully employed to elucidate MFA in primary cell walls as well (Saxe *et al.* 2014). Furthermore, these techniques are often employed in X-ray based wood microdensitometry devices that analyse wood density and related anatomical features (Jacquin *et al.* 2017). For example, SilviScan is a device that combines an image analyser, an X-ray densitometer and an X-ray diffractometer which allows investigation of fibre morphology, wood density and MFA, respectively (Evans 1994; Evans and Ilic 2001; Evans *et al.* 2000). In addition, μ CT is a high-resolution and non-destructive technique for studying the 3D structure of wood which helps to visualise the spatial distribution and associations between different xylem cell types (Brodersen 2013; Jacquin *et al.* 2017; Mayo *et al.* 2010). Brodersen (2013) provides an excellent review of μ CT in relation to wood anatomy.

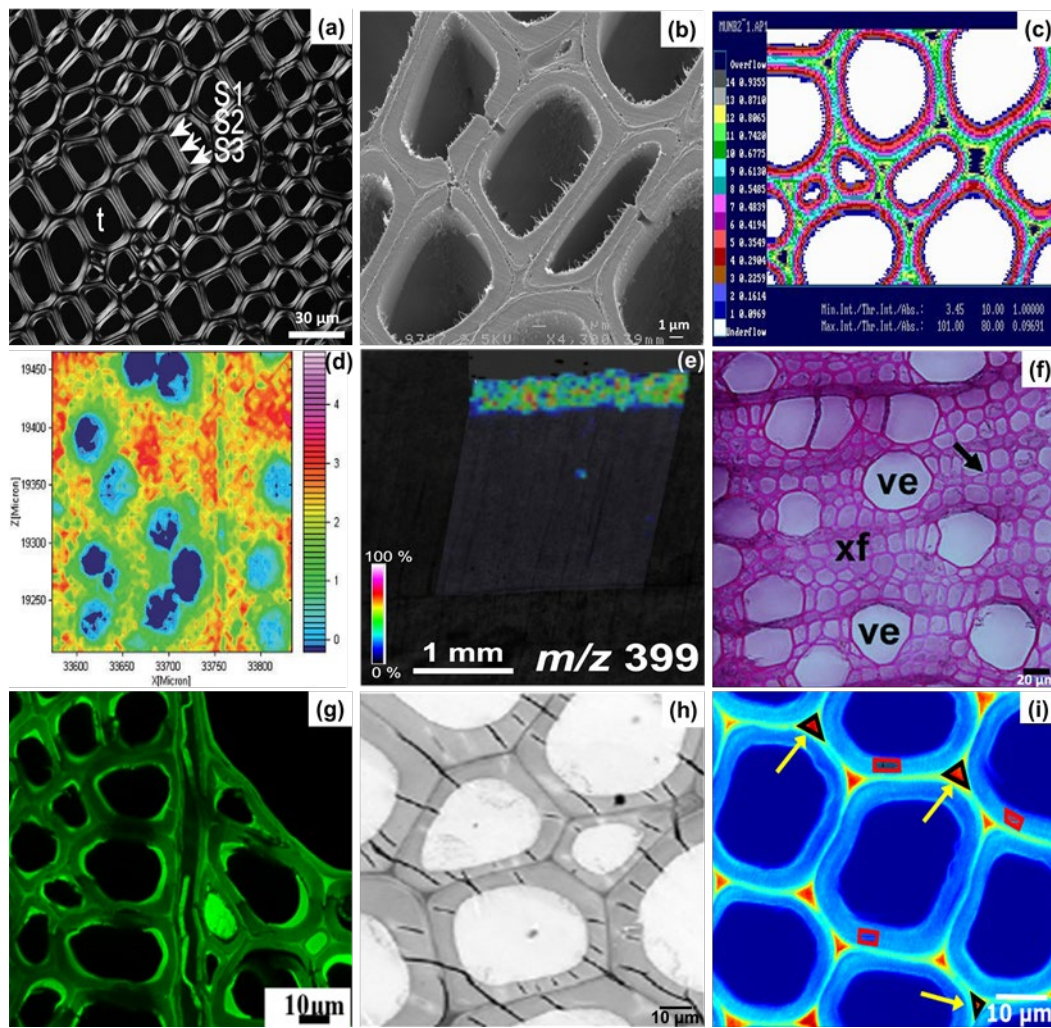


Figure 3.2 Examples of wood samples subjected to different micro phenotyping methods. (a) PLM image of a transverse section of *Pinus radiata* juvenile wood demonstrating SCW layers (S1, S2 and S3), t denotes tracheid (adapted and reproduced with permission from Donaldson 2019), (b) FE-SEM micrograph of *Populus tremula* × *Populus tremuloides* stem cross section showing SCWs of xylem fibres (kindly provided by Naoki Takata, Forestry and Forest Products Research Institute, Japan and Yuzou Sano, Hokkaido University, Japan), (c) UMSP scanning profile of *Acer* stem cross section showing lignin on xylem fibre and parenchyma cells, where the coloured pixels denote the absorbance intensity at 280 nm (kindly provided by Gabriele Ehmcke, Holzforschung München, Germany), (d) FT-IR spectral image showing lignin distribution in wood cells of *Fagus sylvatica* L., the colour scale demonstrates the lignin content from low (blue) to high (pink) (adapted and reproduced with permission from Müller & Polle 2009). (e) MALDI-MS ion image of a transverse section of differentiating *Chamaecyparis obtusa* xylem showing the distribution of coniferin (possible lignin precursor), where the colour scale indicates coniferin percentage (kindly provided by Arata Yoshinaga, Kyoto University, Japan). (f) LM image of *Populus tomentosa* stem cross section stained with phloroglucinol HCl, where the arrow indicates lignification and ve and xf denote vessels and xylary fibres, respectively (adapted and reproduced with permission from Yang *et al.* 2017). (g) CLSM image of poly(furfuryl alcohol)-treated *Populus euramevicana* stem cross section where fluorescence indicates the distribution of poly(furfuryl alcohol) on xylem cells (reprinted with permission from Kong *et al.* 2018, copyright (2018) American Chemical Society). (h) TEM micrograph of *Populus tremula* × *Populus tremuloides* stem cross section showing SCW thickness of xylem cells (adapted and reproduced with permission from Felten

et al. 2018). (i) Raman spectroscopy image of *Pinus sylvestris* stem cross section; red squares in the cell walls denote the areas from where the cell wall spectra were extracted, while the triangles in the cell corners (pointed to by yellow arrows) denote the areas from where the cell corner spectra were extracted (adapted and reproduced with permission from Belt *et al.* 2017).

Table 3.4 Comparative assessment of microanalytical techniques that can be used to morphotype the secondary xylem

Properties	LM/ PLM/ FM	CLSM	SEM	FE- SEM	TEM	X-ray scatter ing/ μ CT	Raman spectro scopy
1. Sample thickness	μm	μm	μm	μm	nm	μm	μm
2. Sample preparation Hand sectioning (H), Microtomy (M)	H or M	H or M	H or M	M	M	M	M
3. Intact cellular structures after sample preparation	✓	✓	✓	✓	✓	✓	✓
4. Staining Chemical Stains (CS), Immuno Stains (IS), Fluorescent stains (FS)	LM - all, FM - FS, PLM - x	CS, IS	-	-	CS, IS	-	-
5. Technical and labour demand	Low	Low	Low - Med	Med - High	Med - High	Med - High	Med - High
6. Potential throughput (relative to each technique)	High	High	Med - High	Med - High	Med - High	Med - High	Med - High
7. Chemotyping ability	✓	✓	x	x	✓	x	✓
8. Possible sample damage during imaging/analysis	x	✓	✓	✓	✓	✓ ¹	x
9. Output Qualitative (QL), Quantitative (QN)	QL	QL	QL	QL	Both	QL	Both
10. Structural data Surface Structures (SS), Beneath Surface Structures (BSS)	SS	Both	SS	Both	Both	SS, BSS ¹	SS
11. Additional benefits i = Optical sectioning, ii = Single cell resolution, iii = 3D images, iv = Micromechanical properties	-	i, ii, iii	ii	i, ii, iii	ii, iii	iii ¹ , iv	ii, iv
12. Key references [#]	[1], [2], [3]	[4], [5], [6]	[7], [8], [9]	[10], [11], [12]	[13], [14], [15]	[16], [17], [18]	[19], [20], [21]

The above-discussed properties of LM are similar to those of PLM and FM except in the use of stains where PLM does not employ any stains and FM can employ fluorescent stains as well as fluorescently labelled immunostains. ¹Between X-ray scattering and μ CT, only μ CT is non-destructive, can analyse both surface and beneath the surface structures and generate 3D images. However, unlike X-ray scattering techniques, μ CT cannot provide information on micromechanical properties. [#][1] Donaldson *et al.* 2010, [2] Oldenbourg 2013, [3] Verherbruggen *et al.* 2017, [4] Bao *et al.* 2012, [5] Liu *et al.* 2017, [6] Kong *et al.* 2018, [7] Melder *et al.* 2015, [8] Yeung *et al.* 2015, [9] Kanbayashi and Miyanfuji 2016, [10] Lehringer *et al.* 2009, [11] Foston *et al.* 2011, [12] Derba-Maceluch *et al.* 2015, [13] Donaldson and Xu 2005, [14] Reza *et al.* 2015, [15] Xu *et al.* 2018, [16] Brodersen 2013, [17]

Each micro morphotyping method has inherent constraints, requirements and potentials. Briefly, although PLM is convenient and straightforward, it fails to provide high resolution and magnification, which is crucial for morphotyping fine cellular structures. Though conventional SEM successfully addresses this drawback, it fails to reveal structural information beneath solid surfaces; however, this has been overcome by FE-SEM, as it enables imaging subsurface features. In addition to morphotyping, X-ray scattering has the potential to serve as a platform for investigating micromechanical properties of tissue specimens. Consequently, the selection of a suitable micro morphotyping method mainly depends on properties such as the required type of information, throughput/number of samples, physical effort, cost and skills required for data analysis and interpretation. Fig. 3.4 provides a decision guide that helps to select a suitable micro morphotyping method based on some of these properties.

3.4 CHEMOTYPING MICROANALYTICAL TECHNIQUES

In addition to the need for qualitative or quantitative information, the selection of a micro chemotyping method depends on the scope of the study, the throughput of sample preparation, the complexity of data analysis and the ease of data interpretation. Further consideration must be given to the toxic nature of some chemicals, and how both chemicals and instruments can be handled safely. Micro chemotyping methods discussed under this section are employed to investigate the chemical characteristics of secondary xylem samples, such as particular polysaccharides and phenolics present in SCWs (Willats *et al.* 2000), without yielding morphotypic data.

Scanning Ultraviolet Microspectrophotometry (UMSP) generates spectra for selected areas (usually resolutions of $0.25 \times 0.25 \mu\text{m}$) on tissue sections and the data derived from them can be employed in *in-situ* characterisation and/or semi-quantification of lignin and phenolics (Carrillo *et al.* 2008; Ehmcke *et al.* 2017; Foston *et al.* 2011; Fukazawa 1992; Irbe *et al.* 2006; Koch and Grunwald 2004; Koehler and Telewski 2006; Sandquist *et al.* 2015). Generally, sample preparation involves fixing,

embedding and then sectioning ($\sim 1 \mu\text{m}$ thickness) (Carrillo *et al.* 2008; Irbe *et al.* 2006). For lignin detection, UMSP utilises the UV absorbance spectrum of lignin. As an example, absorbance at 273 nm, 278 nm and 280 nm wavelengths are used to semi-quantify S lignin monomers, hardwood lignin and G lignin monomers, respectively (Irbe *et al.* 2006; Koehler and Telewski 2006; Sandquist *et al.* 2015; Sarkanen and Hergert 1971). Different colours represent the absorbance intensities at different wavelengths and this information can be used to visualise the distribution of different lignin monomers, as can be seen, for example, across the SCW for various fibre cells in a xylem cross-section in Fig. 3.2c (Carrillo *et al.* 2008; Irbe *et al.* 2006). Similarly, UMSP can also reveal the subcellular distribution of phenolic extractives (Carrillo *et al.* 2008; Koch and Kleist 2001). Although the instrument that can perform UMSP is not readily available, UMSP is a useful microanalytical technique both for qualitative and semi-quantitative chemotyping of secondary xylem.

X-ray Photoelectron Spectroscopy (XPS) can be used for both qualitative and semi-quantitative assessment of secondary xylem. Microtome sectioning is the only sample preparation step required, and samples with thicknesses in the millimetre or micrometre range are used. XPS is used to analyse cell wall structure and chemical composition and to semi-quantify cell wall polymers such as cellulose, hemicellulose and lignin, by analysing X-ray photoelectron spectra (Fig. 3.3a) (Banuls-Ciscar *et al.* 2016; Foston *et al.* 2011; Sinn *et al.* 2001; Zuo *et al.* 2012). Contemporary XPS analyses have the potential to provide both transverse and longitudinal (depth) distribution information for chemotyping (Zuo *et al.* 2012). In addition to information about cell wall polymer distribution, XPS also detects extractives and inorganic elements such as calcium, potassium, phosphorus, nitrogen, carbon and oxygen (Banuls-Ciscar *et al.* 2016; Inari *et al.* 2006; Shchukarev *et al.* 2002; Zuo *et al.* 2012). Analysis of resulting spectral data provide qualitative information such as chemical structures and chemical bonds, and quantitative information such as changes in molecular concentrations (Foston *et al.* 2011; Zuo *et al.* 2012). According to Inari *et al.* (2006), results produced by XPS are in good agreement with those acquired by other spectroscopic methods. Therefore, XPS is an applicable, complementary, reliable method for chemotyping secondary xylem, and it delivers reproducible results.

Gas Chromatography (GC) techniques are well-known and widely used micro chemotyping technique for identification and quantification of individual compounds in a mixture, usually in the form of an extraction. After preparation (see section 3.2), the sample is introduced to the GC set-up, where a carrier gas conveys the sample molecules to the injector port which volatilises the sample and introduces/injects it to the column for subsequent separation of sample components. Since the temperature of the injection port generally varies between 200 - 300°C, GC techniques are only informative for the compounds that can be vaporised below 300°C without decomposing. This limitation of GC techniques can be overcome by increasing the number of detectable compounds, through derivatising the sample to increase the volatility of polar fragments. However, this increases the labour-intensiveness of the sample preparation process. For quantification of monomers of cell wall polysaccharides (e.g. carbohydrates such as glucose, xylose) usually a dry weight of about 70 - 100 µg of cell wall material is used for the derivatisation-based sample preparation process (Angeles *et al.* 2006; MacMillan *et al.* 2015). After sample processing, as little as 1 µl of the processed sample can be injected into a capillary column (Biermann and McGinnis 1989).

GC can be coupled with one or more other instruments such as an autosampler, Mass Spectrometer (MS), Flame Ionisation Detector (FID) or pyrolyser, to reduce sample size and to enhance throughput, resolving power, compound detection sensitivity and data quality (Al-Haddad *et al.* 2013; Gerber *et al.* 2012; Lourenço *et al.* 2013). The use of an autosampler improves the throughput as it substitutes for slower manual injection of samples, and some autosamplers can even perform automated derivatisation of the sample. Coupling GC to MS (GC-MS) enables compound separation, identification and quantification of for example, different cell wall-derived monosaccharides such as arabinose, xylose, mannose, galactose and glucose (Angeles *et al.* 2006). When the objective is to analyse organic material, FID is the detector of choice. One common application of the Gas Chromatography/Flame Ionisation Detector (GC/FID) method is the analysis of monosaccharide compositions and lignin monomer ratios. GC analysis requires the construction of calibration curves for each monosaccharide (standard curves from pure monosaccharides), while for monosaccharides for which calibration samples are unavailable, the detector response

of a suitable calibration sample is used to construct the calibration curve (Al-Haddad *et al.* 2013; Gerber *et al.* 2014).

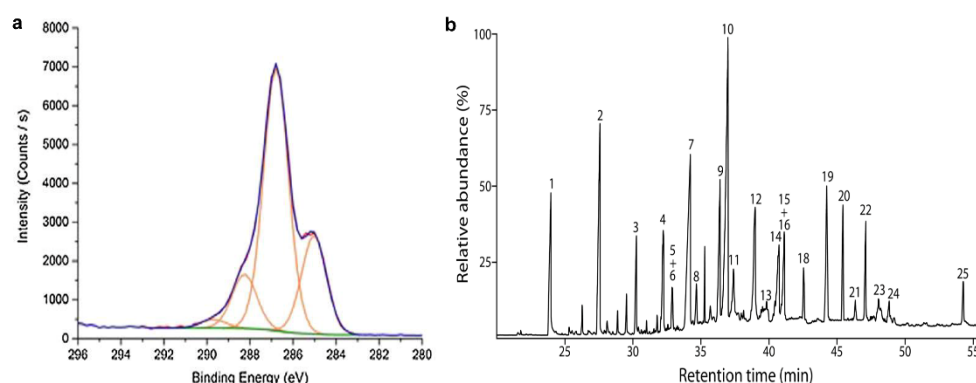


Figure 3.3 Examples of non-imaging techniques used for micro phenotyping. (a) an XPS high resolution spectra of *Pinus sylvestris* extracted hardwood (adapted and reproduced with permission from Bañuls-Ciscar *et al.* 2016), (b) Py-GC/MS chromatogram of *Quercus suber* L. xylem samples, the peaks labelled with numbers represent relative abundances of various lignin-derived compounds (adapted and reproduced with permission from Lourenço *et al.* 2016).

Pyrolizers can thermally decompose material at high temperatures. Thus, when a pyrolizer is coupled to GC, labour-intensive sample preparation steps like extraction and hydrolysis become unnecessary, making Pyrolysis-Gas Chromatography (Py-GC), a convenient and high throughput method. Pyrolizers can also be coupled to techniques such as GC-MS and GC-FID, resulting in Pyrolysis-Gas Chromatography/Mass Spectrometry (Py-GC/MS) and Pyrolysis-Gas Chromatography/Flame Ionisation Detection (Py-GC/FID). Pyrolysis requires only a small amount of sample (1 - 40 µg) either in the form of a ball-milled powder or as a thin tissue section (Gerber *et al.* 2016; Gerber *et al.* 2014; Lourenço *et al.* 2013). Pyrolysis brings two significant advantages; pyrolytic reactions are applied to cell wall polymers in their *in-situ* context without any chemical separation or chemical modification, and it allows quantification of polysaccharides and lignin. Compared to Klason and acid-soluble lignin quantification methods (standard wet chemistry analytical methods for lignin), Py-GC/FID is more advantageous for evaluation of total lignin in particular since it accurately takes acid-soluble lignin into account (Lourenço *et al.* 2013). The chemotypic data generated from Py-GC/MS can be used either to semi-quantify cell wall polymers such as cellulose, hemicellulose and lignin or to fingerprint the sample. These data can also be used to obtain detailed information such

as S, G and H lignin ratios (Gerber *et al.* 2016; Lourenço *et al.* 2013; Lourenço *et al.* 2016; Lupoi *et al.* 2015). Although Py-GC/MS is convenient in terms of sample preparation, the resulting chromatograms are complex due to the wide range and variety of decomposition products that can be formed (Fig. 3.3b). Several MS libraries are available via National Institute of Standards and Technology (NIST), to assist in the identification of these decomposed compounds. Overall, Py-GC/MS and Py-GC/FID provide high throughput and sensitive microanalytical techniques for chemotyping cell wall polymers in secondary xylem.

Fourier-Transform Infrared (FT-IR) spectroscopy is a simple, convenient and non-destructive method to obtain structural and chemical fingerprints of cell walls. Chemotyping can be extended to detect conformational changes and putative cross-links between cell wall polymers (Alonso-Simón *et al.* 2011; McCann *et al.* 1992; Ohman *et al.* 2013). It uses an infrared (IR) beam to obtain a spectrum of absorption or emission of the samples. Wood samples can either be cryo-sectioned ($\sim 20\ \mu\text{m}$ thickness) and then dried or ground depending on the FT-IR instrument being used. Ground samples can be placed directly into the IR beam, whereas the chemistry of secondary xylem sections can be mapped with a microscope (microspectroscopy) which has the potential to analyse areas as small as $50 \times 50\ \mu\text{m}$ effectively. Contemporary instruments in transmission mode can provide chemical fingerprints of cell walls with spatial resolutions in the $5 - 20\ \mu\text{m}$ range (influenced by the wavelength of the radiation) (Gorzsas *et al.* 2011; Lasch and Naumann 2006). Smaller areas require an IR source that possesses a high brilliance (synchrotron), however, this might prove time-consuming and challenging (Gou *et al.* 2008). Several studies have provided detailed information on different FT-IR wavelengths assigned to different cell wall polymers including cellulose, lignin and hemicelluloses (Fig. 3.2d) (Alonso-Simón *et al.* 2011; Derba-Maceluch *et al.* 2015; Gorzsas *et al.* 2011; Müller and Polle 2009). In addition, near-infrared (FT-NIR) spectroscopy facilitates rapid determination of wood extractives and phenolic content in heartwood (Gierlinger *et al.* 2002). The use of multivariate data analysis enables FT-IR to elucidate patterns of lignin composition in wood fibres which could not be detected by traditional data analysis (chemical imaging by heat mapping) (Gorzsas *et al.* 2011). However, FT-IR spectroscopy is only capable of semi-quantification since absolute quantification

would require internal standards which at this stage are not available for this type of tissue analysis.

Matrix-Assisted Laser Desorption Ionisation Mass Spectrometry (MALDI-MS)

is an ionisation method where the sample is mixed or coated with an energy-absorbing matrix such as silica to support the ionisation process. When a laser beam hits a target on the sample within the matrix, desorption and ionisation generate ions from the sample which can then be detected by a mass analyser such as MS (Araujo *et al.* 2014; Liu *et al.* 2007; Lunsford *et al.* 2011; Singhal *et al.* 2015). Sample preparation for MALDI-MS typically involves sectioning, drying and spraying the matrix onto the sections (Araujo *et al.* 2014; Yoshinaga *et al.* 2016). Through combination with sophisticated equipment (e.g. LTQ-Orbitrap MS, Time-Of-Flight (TOF) analyser) and by altering laser optics, MALDI can be extended to single cell level chemotyping of cell wall polymers at a high resolution (ranging from 5 μm - few mm) (Araujo *et al.* 2014; Feenstra *et al.* 2017; Li *et al.* 2016; Obel *et al.* 2009; Qin *et al.* 2018). The resulting techniques, such as MALDI-TOF-MS, are high throughput microanalytical techniques for obtaining chemical fingerprints of cell walls (Fig. 3.2e) using wood samples as small as 100 ng (Bauer 2012; Boughton *et al.* 2016; Persson *et al.* 2010; Sturtevant *et al.* 2016). MALDI-MS can also determine S to G lignin ratios, and it also has the potential to provide semi quantitative information about some extractives such as terpenes (Sturtevant *et al.* 2016). When MALDI-MS is coupled with Oligosaccharide Mass Profiling (OLIMP), semi-quantitative data on cell wall oligosaccharides can be obtained from samples of around 1 μg (Obel *et al.* 2009; Persson *et al.* 2010). MALDI permits quantification only if a library of MS/MS data is available or if a calibration curve has been created. However, if such information is unavailable, the literature on ion fragmentation data (MS/MS) can be used (Araujo *et al.* 2014). MALDI methods serve as valuable microanalytical techniques for micro chemotyping secondary xylem.

Table 3.5 Comparative assessment of microanalytical techniques that can be used to chemotype the secondary xylem

Properties	UMSP	XPS	GC-MS	Py-GC (MS or FID)	FT-IR	MALDI-MS
1. Sample thickness or weight	µm	µm-nm	µg	µg	µm-µg	µm-ng
2. Sample preparation Hand sectioning (H), Microtomy (M), Processing Steps (PS) [^] , Laser Dissection (LD)	M	M	PS	H or LD	M	H or M
3. Intact cellular structures after sample preparation	✓	✓	×	✓	✓×*	✓
4. Technical and labour demand	Med - High	Med - High	High	Med - High	Med - High	Med - High
5. Potential throughput (relative to each technique)	Med - High	Med - High	Low	High	Med - High	High
6. Possible sample damage during imaging/analysis	✓	✓	✓	✓	×	✓
7. Output Qualitative (QL), Quantitative (QN)	Both	Both	QN	QN	Both	Both
8. Structural data Surface Structures (SS), Beneath Surface Structures (BSS)	SS	SS, BSS (<5 nm)	-	-	SS	SS
9. Additional benefits i = Optical sectioning, ii = Single cell resolution	-	i	-	-	ii	ii
10. Key references [#]	[1], [2], [3]	[4], [5], [6]	[7], [8], [9]	[10], [11], [12]	[13], [14], [15]	[16], [17], [18]

[^] denotes processing steps such as extraction, hydrolysis, reduction and derivatisation. *✓× denotes instances in which sample can be used for analysis in several different forms such as sections or ground tissue. [#][1] Koehler and Telewski 2006, [2] Carrillo *et al.* 2008, [3] Sandquist *et al.* 2015, [4] Inari *et al.* 2006, [5] Zuo *et al.* 2012, [6] Banuls-Ciscar *et al.* 2016, [7] Angeles *et al.* 2006, [8] Al-Haddad *et al.* 2013, [9] MacMillan *et al.* 2015, [10] Brodersen 2013, [11] Lourenco *et al.* 2013, [12] Lupoi *et al.* 2015, [13] Alonso-Simón *et al.* 2011, [14] Gorzsas *et al.* 2011, [15] Derba-Maceluch *et al.* 2015, [16] Obel *et al.* 2009, [17] Araujo *et al.* 2014, [18] Sturtevant *et al.* 2016

Although micro chemotyping methods have many similarities among them in terms of requirements, restrictions and potentials, there are slight differences which make them unique. GC-based micro chemotyping methods can only identify cell wall polymers and provide quantitative information; however, these methods fail to demonstrate the distribution of cell wall polymers. In contrast, other techniques like XPS, FT-IR, MALDI-MS and UMSP can provide information which is both quantitative and qualitative (able to identify the cell wall polymers and to demonstrate their distribution on cell walls). Although XPS can perform optical sectioning and reveal information about subsurface structures (< 5 nm), it cannot achieve single cell resolution, which

can be achieved by techniques such as FT-IR and MALDI-MS. A further comparison between these micro chemotyping techniques and an extensive summary are given in Table 3.5. Fig. 3.4 provides a decision guide to help the selection of a suitable micro chemotyping method based on some of the properties discussed in Table 3.5.

3.5 DUAL MICRO MORPHOTYPING AND CHEMOTYPING TECHNIQUES

Microanalytical techniques discussed in this section have the capacity to perform aspects of both morphotyping and phenotyping, allowing data on both tissue/cell morphology and chemical composition to be obtained from a single technique.

Light microscopy (LM) is inarguably the mainstay of phenotyping techniques, as it provides a straightforward method for both morphotyping and chemotyping. It uses thin sample sections ($\sim 1 - 30 \mu\text{m}$ thickness), which are prepared by either hand or microtome sectioning before being mounted on glass slides. Morphotyping can be done either with or without staining, at magnifications between $40\times - 1000\times$, to view cellular features such as size, diameter, lumen size, MFA, cell wall thickness and cell wall area. However, stained secondary xylem sections are often required for chemotyping as it helps to observe the presence or distribution patterns of cell wall polymers (Fig. 3.2f) (MacMillan *et al.* 2010; Manabe *et al.* 2013; Sato *et al.* 2001; Wu *et al.* 2009; Yang *et al.* 2017). Some frequently used stains in LM are listed in Table 3.1. Micrographs obtained by LM can be further analysed using a range of computer software packages such as ImageJ, Fiji, BioImageXD, and Icy among others (Eliceiri *et al.* 2012). LM has for example successfully revealed changes to wood cell morphology and chemical composition in response to changing environmental conditions or tree genetics (Bao *et al.* 2012; Begum *et al.* 2012; Carrillo *et al.* 2008; Chai *et al.* 2015; Groover 2016; Kanbayashi and Miyafuji 2016; Yang *et al.* 2017). Compared to other advanced microscopy techniques, LM is inexpensive, sample preparation is relatively simple (if fixation/embedding are not required), it does not require much expertise, provides a method for visualising both live and dead cells and, if unstained, provides a means for observing a sample's natural colour. However, two

main drawbacks of LM are low resolution (upper limit ~ 250 nm) and comparatively low magnification (upper limit ~ 1000×).

Fluorescence Microscopy (FM) is a type of LM that utilizes the fluorescence properties of the native sample or such properties introduced to a sample by fluorescent labels/stains, for visualisation of cell walls and cell wall polymers. Some of the most frequently used fluorescent stains are listed in Table 3.2. Lignin autofluorescence facilitates identification and visualisation of lignified cell walls and can be used to semi-quantify lignin between different cells, between distinct cell wall layers and different regions of the cell wall (Ma *et al.* 2013a). Cellulose in cell walls can be visualised by staining with calcofluor white which is one of the most commonly used fluorescent stains (Chai *et al.* 2015; Mitra and Loque 2014; Sato *et al.* 2001). In addition, FM is often employed to screen compression and normal wood (Donaldson *et al.* 2010; Donaldson and Knox 2012; Donaldson *et al.* 2015; Donaldson and Singh 2016). As an example, FM can be used to quantify the severity of compression wood using the ratio between the fluorescence produced at blue and violet wavelengths (Donaldson *et al.* 2010). Furthermore, FM is frequently used in immunohistochemistry using fluorescently labelled antibodies or fluorescently labelled carbohydrate binding modules (McCartney *et al.* 2004; Yang *et al.* 2013). Sample preparation is often as simple as sectioning (~ 20 µm thickness) except in cases where a fluorescent stain is required in which case there are additional staining steps.

Confocal Laser Scanning Microscopy (CLSM) performs non-destructive optical sectioning (~ 0.5 µm thickness) which enables observation of a relatively thick specimen (50 - 200 µm) at various depths, precluding the need for fine manual sectioning (Dickson *et al.* 2017; Minsky 1988; Taguchi *et al.* 2010; Thorn 2016). It enables both surface observations as well as the construction of 3D structures by using computer software to stack optical sections to generate a projection image (Kitin *et al.* 2003). CLSM allows the re-use of samples and precludes the introduction of artefacts that could arise from uneven thickness of sections and manual stacking of a series of sections. CLSM is often employed to observe changes taking place in secondary tissue/cell morphology and chemical composition following chemical treatment and

as a result of altering tree genetics and/or environmental conditions (Fig. 3.2g) (Kong *et al.* 2018; Liu *et al.* 2017).

CLSM is also used to analyse stem sections stained with antibodies or carbohydrate binding modules (Bao *et al.* 2012). For example, CLSM has been used to observe Sitka spruce compression wood sections labelled with LM5 antibody which provided insights into the synthesis of (1→4)- β -galactan as an initial response to compression wood formation (Altaner *et al.* 2007). Similarly, using CLSM and various antibodies (Table 3.3) the spatial distribution of non-cellulosic polysaccharides including distinct xyloglucan, heteroxylan, galactoglucomannan, and pectin epitopes have been observed in the secondary xylem of *Pinus radiata* (Donaldson and Knox 2012; Putoczki *et al.* 2008). Therefore, CLSM can be used for both morphotyping and chemotyping (Bao *et al.* 2012; Donaldson *et al.* 1999; Knebel and Schnepf 1991; Liu *et al.* 2017; Miller and Johnson 2017).

Transmission Electron Microscopy (TEM) has the highest resolving power of any of the methods described in this review (Duchesne and Daniel 1999; Reza *et al.* 2015). It facilitates tomography (a method for computing 3D structural data from a series of 2D images obtained at different angles), imaging (2D images) and analysis of cell wall polymers (Ercius *et al.* 2015). TEM can be coupled with analytical methods like Electron Energy-Loss Spectrometry (EELS) and Energy Dispersive X-ray Spectroscopy (EDX or EDS), to characterise chemicals or to analyse elements of samples (Reza *et al.* 2015). However, the requirement of ultra-thin samples (nanometre thicknesses), tedious sample preparation and the possibility for radiation damage are significant drawbacks of TEM (Reza *et al.* 2015; Wang *et al.* 2015b). Sample preparation often involves embedding, ultramicrotomy and staining using some of the stains mentioned under staining in section 3.2.2. TEM and advanced versions of TEM, such as TEM-EDX, facilitate chemotyping by demonstrating the distribution of different polymers such as hemicellulose, lignin and even different monomers of lignin in cell walls (Reza *et al.* 2015). Compared to SEM, TEM micrographs are a lot ‘cleaner’ due to the absence of cell wall artefacts that could be introduced by sectioning. Some typical applications of TEM include morphotyping different cell types such as xylem fibre, vessels, axial and ray parenchyma (Fig. 3.2h), providing

insights into fine structural details such as pit membrane, presence/thickness of different cell wall layers (S1, S2 and S3 layer), molecular structure of microfibrils, MFA in different cell wall layers and different structures formed by microfibrils (e.g. bundles, aggregates) (Donaldson and Xu 2005; Duchesne and Daniel 1999; Ma *et al.* 2013a; Reza *et al.* 2015; Wang *et al.* 2015b; Wilson *et al.* 2005; Xu *et al.* 2004; Xu *et al.* 2006; Xu *et al.* 2011; Xu *et al.* 2009). In addition, TEM can be used to visualise the distribution of immunogold-labelled lignin, cellulose, (1→4)-β-galactan, arabinogalactan proteins and heteroxylans and (1→3)-β-glucan in wood cell walls (Altaner *et al.* 2010; Kim and Daniel 2014; Kim and Daniel 2017; Ruel *et al.* 2012). Furthermore, lead citrate staining enables TEM to reveal the distribution of phenolic extractives such as tannins in the lumina and cell walls of xylem vessels, fibres and parenchyma cells (Streit and Fengel 1994) and potassium permanganate staining reveals the distribution of lignin where noticeable changes can be correlated to the degree of lignification in each cell wall layer (Lehringer *et al.* 2009). Reza *et al.* (2015) provide an excellent review of TEM, where more examples can be found on how TEM can be employed for morphotyping and chemotyping.

Raman spectroscopy allows label-free, non-destructive and high-resolution imaging of plant tissues at sub micrometre levels (< 0.5 μm). It exploits vibrational characteristics of molecules by utilising a monochromatic light source (laser) to measure the shift in energy of scattered photons which resembles the vibrational energy of the functional group that it is interacted with (Agarwal 2006; Foston *et al.* 2011; Gierlinger *et al.* 2012; Ma *et al.* 2013b; Schmidt *et al.* 2010; Zhang *et al.* 2017). Sample preparation often involves hand sectioning or microtomy and may or may not involve an embedding step depending on the features of interest. Secondary xylem sections (~ 3 μm - 0.5 mm thickness) with intact cell walls and flat surfaces can be used for Raman imaging. Nevertheless, the sample impurities or lignin autofluorescence or both could lead to false fluorescence which could hamper the measurements or conceal the actual Raman spectra; however, lignin autofluorescence can be mitigated by selecting a suitable excitation wavelength (Gierlinger *et al.* 2012; Gierlinger and Schwanninger 2007; Zhang *et al.* 2017).

Several modifications have been made to Raman spectroscopy to address issues associated with fluorescence, long image acquisition times and to enhance sensitivity and resolution further. Confocal Raman micro-spectroscopy is one such approach that combines confocal microscopy with Raman spectroscopy to improve resolution and reduce fluorescence (Agarwal 2006; Gierlinger *et al.* 2012; Ma *et al.* 2013a). Near Infrared-Fourier Transform (NIR-FT) Raman spectroscopy utilises lasers in the IR range instead of visible light and coupling it with Fourier transformation successfully addresses the issues caused by fluorescence (Gierlinger and Schwanninger 2007). Coherent Anti-Stokes Raman Spectroscopy (CARS) employs two laser beams instead of one, resulting in reduced image acquisition times (Pohling *et al.* 2014). UV Resonance Raman Spectroscopy (UVR) utilises lasers in the UV range thereby improving sensitivity. By introducing controlled micro-displacement (a microscopic movement) of the sample, this UVR method can be extended to study single wood cells at the microscopic scale (Czaja *et al.* 2006). Specific band regions observed in the Raman spectrum can be used to assess and simultaneously map the distribution of cell wall polymers such as cellulose, lignin (S and G monomers), pectin and hemicellulose (Agarwal 2006; Chu *et al.* 2010; Gierlinger *et al.* 2012; Gierlinger and Schwanninger 2006; Ma *et al.* 2013b; Ozparpucu *et al.* 2017; Richter *et al.* 2011; Schmidt *et al.* 2010; Zhang *et al.* 2017). In addition, Raman spectroscopy also has the potential to reveal the cellular level distribution of wood extractives (Fig. 3.2i) (Belt *et al.* 2017). Band intensities in Raman spectroscopy are directly proportional to the analyte concentration, and therefore, semi-quantification can be achieved (Agarwal 2006). Raman spectroscopy is often used to assess cellular properties such as cell wall thickness, micromechanical properties of fibres (by analysing band shift patterns), MFA, variability of cellulose microfibrils (at micron level) in different SCW layers and spatial distribution of carbohydrates (mainly cellulose) and lignin in cell walls (Gierlinger *et al.* 2010; Ma *et al.* 2013b; Perera *et al.* 2012; Schmidt *et al.* 2010). Hence, Raman spectroscopy serves as an efficient microanalytical technique for morphotyping and chemotyping secondary xylem in its native state without the need for complex sample preparation.

Micro chemotyping techniques such as LM, CLSM, TEM and Raman spectroscopy can be used for both morphotyping and chemotyping. Although TEM and Raman

spectroscopy provides both qualitative and quantitative information, LM and CLSM can only provide qualitative information. Compared to LM, CLSM has more properties such as optical sectioning, achieving single cell resolution and generation of 3D images. Raman spectroscopy also can achieve single cell resolution, and it can even reveal the micromechanical properties of the sample. Although TEM cannot reveal micromechanical properties, it can provide a resolution higher than that of any other dual micro morphotyping and chemotyping techniques.

Phenotyping of limited or small amounts of secondary xylem is challenging since only a few microanalytical techniques can successfully accommodate small samples. In this review, we aimed to provide a concise summary of some of the most commonly used microanalytical techniques highlighting their features, advantages and constraints in phenotyping secondary xylem. We have further simplified the task of selecting a suitable microanalytical technique by providing a comprehensive comparison and selection guide (Table 3.4, Table 3.5 and Fig. 3.4) between these microanalytical techniques based on eleven critical properties that need to be taken into consideration such as amount of sample/sample thickness, sample preparation, technical and labour demand, tissue/cellular integrity after sample preparation, possibility of sample damage during imaging/analysis, ability to reveal details on surface structures and beneath surface structures, and the qualitative/quantitative nature of the output. However, the ultimate selection of a suitable technique is at the user's discretion and depends on the scope of the study, cost and resource availability.

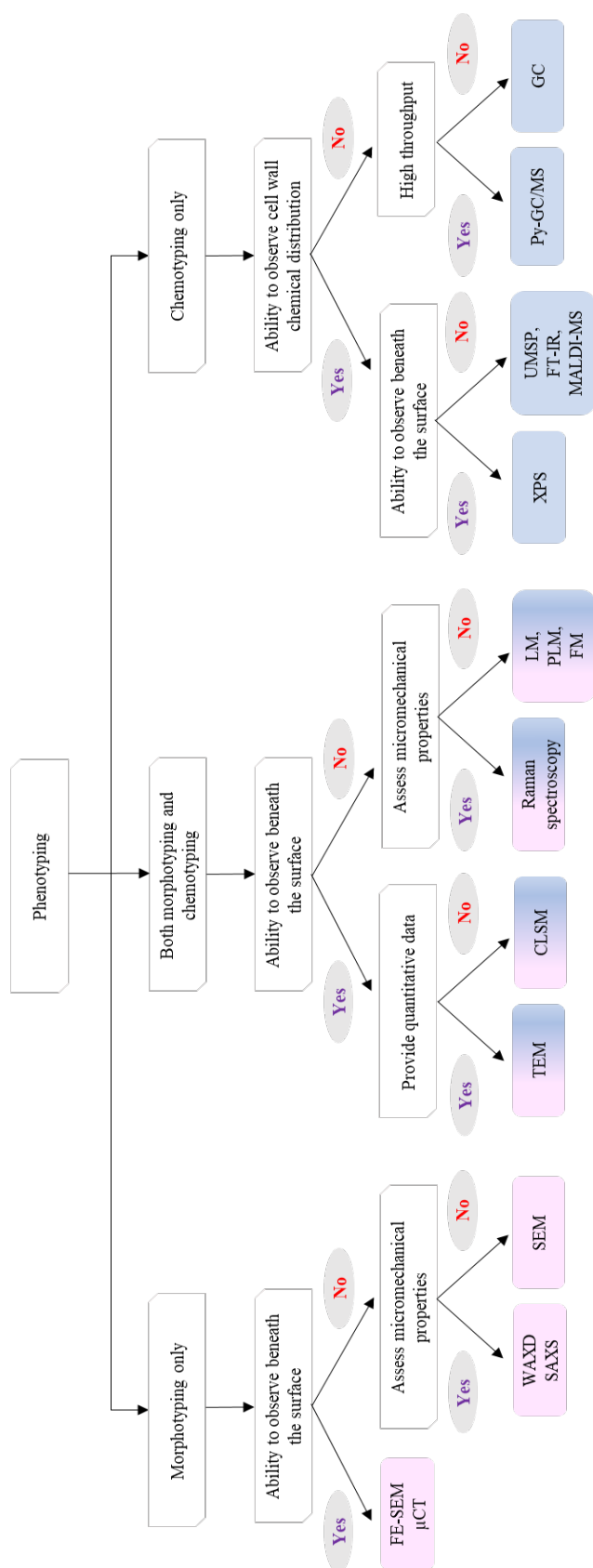


Figure 3.4 Selection guide for microanalytical techniques. Pink and blue shading is consistent with that in Fig. 3.1. The above properties of LM are similar to those of PLM and FM except in the use of stains and FM does not employ any stains and FM can employ fluorescent stains as well as fluorescently labelled antibodies.

4 SND, MYB AND KNAT TRANSCRIPTION FACTOR FUNCTION DURING WOOD FORMATION IN *EUCALYPTUS*

4.1 INTRODUCTION

Each developmental stage of a tree is governed by complex regulatory networks acting at transcriptional, post-transcriptional, post-translational and epigenetic levels. Among them, the transcriptional regulation is considered as the core regulatory network. Much of what is known about the transcriptional regulation of tree biology comes from studies related to rooting, flowering, acclimatisation and wood formation. Transcriptional regulation is mediated by Transcription Factors (TFs), which are involved in orchestrating biological processes by tightly regulating the expression of structural genes. Therefore, unravelling the transcriptional regulation behind a biological process sheds light on participating TFs and their functional associations with structural genes.

Secondary xylem or wood formation is one such transcriptionally regulated biological process. Wood formation inarguably represents one of the most striking features of secondary growth and holds an ecological and economic significance (Andersson-Gunneras *et al.* 2006; Dejardin *et al.* 2010; Pan *et al.* 2011; Ye and Zhong 2015). It is an intricate and dynamic differentiation process composed of four main sequential stages; cell division and differentiation, xylem cell expansion and elongation, secondary cell wall (SCW) formation and programmed cell death (PCD) (Bossinger and Spokevicius 2018; Zhang *et al.* 2014). Each stage is regulated and fine-tuned by specific sets of TFs, which ultimately control the progression of the entire wood formation process while defining the morphology and chemical composition of wood cells in each stage. Among these stages, SCW formation is particularly significant as the SCWs make xylem cells stronger, rigid and hydrophobic, thereby provide mechanical and structural support, facilitate water and nutrient transport and provide protection from pathogens and tolerance to abiotic stresses (Cano-Delgado *et al.* 2003; Dejardin *et al.* 2010; Doblin *et al.* 2010; Higuchi 1997; Houston *et al.* 2016; Hussey

et al. 2013; Plomion *et al.* 2001). Also, the SCW is a major determinant of the chemical and physical properties of the resulting wood.

However, the formation of SCWs is an irreversible process; therefore, precise spatio-temporal regulation is vital. This regulation is achieved by a dynamic, tightly coordinated, conserved, three-tier (top, middle and bottom) network of TFs (Cassan-Wang *et al.* 2013; Hussey *et al.* 2013; McCahill and Hazen 2019). The top tier is comprised of TFs that act as on/off switches for the entire SCW deposition mechanism. Top tier master regulators include members of the NAC (NAM/ATAF/CUC) TF family, including for example VASCULAR NAC DOMAIN (VND6 and 7), NAC SECONDARY WALL THICKENINGS (NST1 and NST2), and SECONDARY WALL NAC DOMAIN (SND1). The middle and bottom tier TFs are mostly involved in fine-tuning SCW deposition. TFs in the middle tier includes NAC family members such as SND3, XYLEM NAC DOMAIN (XND1) and TFs from R2R3-type MYELOBLASTOSIS subfamily (MYB46, 55, 61, 83, and 103) and are directly regulated by top tier TFs. Bottom tier TFs are directly regulated either by middle-tier or in some instances top tier TFs. TFs in this bottom tier includes members of the NAC family (SND2), MYB subfamily (MYB4, 7, 20, 32, 52, 54, 58, 63, 69, 75, 79 and 85), KNOTTED-LIKE HOMEODOMAIN (KNAT7), and C3H-TYPE ZINC FINGER (C3H14) TFs. All the members of the bottom tier and some members of top and middle tiers can directly regulate the expression of genes associated with biosynthesis of SCW biopolymers (cellulose, lignin and hemicellulose), signalling and PCD (Cassan-Wang *et al.* 2013; Hussey *et al.* 2013; Hussey *et al.* 2011; Wang *et al.* 2013; Zhong and Ye 2015).

Among these TFs, KNAT, MYB and SND are considered as the central transcriptional regulators involved in orchestrating SCW deposition. Different members of the SND subfamily can be found in all three tiers of the TF network underlying SCW deposition. Unlike SND, the members of the MYB subfamily can be found only in the middle and bottom tiers of the network, whereas the members of KNAT subfamily can be found only in the bottom tier. Several studies have been conducted to characterise the expression and function of certain *Arabidopsis* KNAT, MYB and SND TFs which are associated with SCW formation. Since transcriptional network orchestrating SCW

deposition is reasonably conserved between *Arabidopsis* and woody genera such as *Eucalyptus* and *Populus*, the KNAT, MYB and SND TFs are presumably involved in wood formation in woody genera as well (Goicoechea *et al.* 2005; Legay *et al.* 2010; Soler *et al.* 2016; Zhong *et al.* 2010). In addition, certain members of these TF families and subfamilies show differential expression during tension wood (TW) formation, suggesting their potential involvement in a tree's response to gravitropic stress. However, to date, only a small number of studies have been conducted to characterise the expression and function of orthologs of such TFs in *Eucalyptus* and *Populus*. Therefore, elucidating the expression and function of such uncharacterised TFs during wood formation will help to understand how they influence wood cell morphology and chemical composition, which will shed some light towards unravelling their position in the transcriptional network behind wood and TW formation.

Based on current evidence in the literature, some KNAT, MYB and SND TFs act as activators during the biosynthesis of different cell wall polymers while some act as repressors. Via this activation or repression, these TFs can alter the deposition and composition of cellulose, hemicellulose and lignin, which may cause changes in wood cell morphology and cell wall chemical composition. However, to date, there is no information on how the above properties are influenced by *Eucalyptus* KNAT, MYB and SND TFs nor whether they can affect other stages of wood formation such as cell division, expansion and elongation. Therefore, the main objective of the present chapter is to address some of those knowledge gaps by characterising the expression and function of four previously uncharacterised *Eucalyptus grandis* TFs, *EgKNAT7* (accession number Eucgr.D01935), *EgMYB103* (accession number Eucgr.D01819), *EgSND2* (accession number Eucgr.K01061) and *EgSND3* (accession number Eucgr.E03226), during wood formation. This chapter describes the *in vivo* transformation experiments that were conducted to elucidate their spatio-temporal expression (using promoter constructs) and function (using up- and down-regulating gene constructs) during wood formation.

4.2 METHODOLOGY

Trial I, II and III took place in summer, under identical glasshouse conditions but in subsequent years (2017, 2018 and 2019).

4.2.1 Preparation of plant material

Eucalyptus globulus seedlings were kindly provided by ArborGen nursery (Colac, Victoria) and *Eucalyptus grandis* × *camaldulensis* clones (Saltgrow) were purchased from Yuruga nursery (Queensland). These plants were potted and kept in the glasshouse until required. *Populus alba* ‘*pyramidalis*’ L. clonal material was sourced from trees growing at the University of Melbourne, Creswick Campus, Victoria. Cuttings were taken from *Populus* clonal material and treated with plant cutting powder (Yates, Australia) for rapid root formation in cutting beds. Subsequently, rooted cuttings were transferred to pots and were kept in the glasshouse until required. Plants aged between five to six months were used for the inoculation experiment. During the trial, plants were kept at controlled glasshouse conditions where a 16-hour photoperiod (by supplementary lighting when necessary) and optimum day and night temperatures (between 21-25°C and 14-17°C, respectively) were maintained. All plants were irrigated regularly with tap water and were fertilised with slow-release fertiliser (Osmocote, Yates, Australia).

4.2.2 Creation of vector constructs and transgenic *Agrobacteria*

Synthetic constructs containing the respective promoter and coding sequences (CDS) of *Eucalyptus grandis* TFs were kindly provided by Dr Steven Hussey (Hussey *et al.* 2019). Promoter expression analysis was performed using pMDC162 vector, where the promoter of interest was fused to the β -glucuronidase (*GUS*) reporter gene (Creux *et al.* 2013). Gene functional analyses were conducted using the pCAMBIA 1305.1 vectors modified with an overexpression (pCAMBIA 1305.1 GW+) or RNAi knockdown (pCAMBIA 1305.1 GW-) Gateway enabled cassettes (Spokevicius *et al.* 2007a). The promoter sequences and CDSs in entry vectors were then cloned into respective destination vectors (where necessary) using Gateway LR Clonase™ II Enzyme mix (Invitrogen) according to the manufacturer’s instructions. The integrity

of all constructs was confirmed by sequencing (Macrogen, Korea). A pMDC162 vector containing *CAULIFLOWER MOSAIC VIRUS 35S (CaMV 35S)* promoter (35S) and an empty (promoterless) pMDC162 vector (:GUS) were used in the promoter expression analysis as the positive and negative control, respectively. Empty (without CDS) pCAMBIA 1305.1 GW+ (EVC+) and empty (without CDS) pCAMBIA 1305.1 GW- (EVC-) were used as controls for the functional analysis. The sequence-validated constructs were introduced to *Agrobacterium tumefaciens* AGL1 by electroporation using *E. coli* pulsar (BIO-RAD Laboratories, Gladesville, NSW, Australia), according to the standard protocol (Sambrook and Russell 2001).

4.2.3 Preparation of inoculation media

Agrobacterium for ISSA transformations was prepared as described in Spokevicius *et al.* (2016). Briefly, this involved culturing of all bacteria in Luria-Bertani (LB) liquid media supplemented with Kanamycin (50 µg ml⁻¹) and Rifampicin (25 µg ml⁻¹), for 48 hours at 28°C and 200 rpm. At this point, 1 ml was added to 20 ml of fresh LB media (supplemented with the same antibiotics) and further cultured under the same conditions for four to six hours after which bacteria were centrifuged (4°C, 15mins, 1000g) and then suspended in 1 ml of cooled Murashige and Skoog (MS) media (Murashige and Skoog 1962). The resulting culture, referred to as ‘Inoculation Media’ was stored on ice until required.

4.2.4 Inoculation of plant stems with transgenic *Agrobacteria*

Plant stem inoculations were performed at the beginning of summer (December) when cambial transformation efficiency was highest (Spokevicius *et al.* 2016) using *Eucalyptus globulus*, *Eucalyptus grandis* × *camaldulensis*, and *Populus alba* ‘pyramidalis’ L. Prior to inoculations, plant height and stem diameter at 10 cm from the base were recorded. Plant stems were inoculated as described in Spokevicius *et al.* (2016). In brief, cambial windows (1 cm²) were created at a 90° offset and 1 cm apart on a straight stem section, and 10 µl of inoculation medium was applied on to the exposed cambium (one construct per window). Cambial windows were inoculated ensuring that each construct was randomly distributed along the stem (to exclude any

positional effects) and that the respective control construct/s were represented on each stem.

4.2.5 Harvesting and identification of transgenic tissue

After inoculation, plants were grown under optimum conditions (section 4.2.1), and the stem diameters were intermittently measured at 10 cm from the base. When a sufficient radial growth (> 5 mm) was achieved (usually in about three months), plants were harvested. On the day of harvesting, measurements were taken for plant height and stem diameter at 10 cm from the base. The cambial windows were harvested and stained, as described in Spokevicius *et al.* (2016). Briefly, it involved excising the cambial windows from the stems and removing non-inoculated halves of each window. Cambial windows of promoter expression study were processed by slicing transversely into 0.5-1 mm half-discs, and those of gene function study were processed by removing the bark. These processed cambial windows were rinsed with phosphate buffer (pH 7) and were subjected to GUS staining as described in Spokevicius *et al.* (2016).

4.2.6 Assessment of GUS staining patterns

4.2.6.1 Gene function analysis

Stained cambial windows from gene function analysis were placed on a petri dish using forceps and observed under a stereomicroscope (SZ-PT Olympus, Japan). Each GUS-stained somatic sector will have originated from a single transformed transgenic cell, and therefore, each somatic sector stained in blue (after this referred to as transgenic sector) represents an independent transformation event. Transgenic sectors on the developing xylem/exposed surface (referred to as cambial sectors hereafter), were counted and their number was recorded for each cambial window. In addition, in each cambial window, the new radial growth across the cambial window (the distance from the cambium to the wound site near the old/previously formed xylem) was measured and recorded.

4.2.6.2 Promoter expression analysis

Stained cambial discs from promoter expression analysis were observed as described above, and transgenic sectors were classified according to the classification systems mentioned in Creux *et al.* (2013). The somatic sector classification system was used to classify the somatic sector types observed on existing and newly formed wood (Fig. 4.1a and Table 4.2). The cambial sectors were further subcategorised into seven different staining patterns (Fig. 4.1b) based on the presence of GUS staining in the phloem (P), developing xylem (X1) and mature xylem (X2) (referred to as cambial sector types), to better understand the type of the initially transformed cell (phloem mother cell, xylem mother cell or cambial initial) and the spatio-temporal expression patterns of the promoter of interest. The somatic sector type and the cambial staining/expression pattern (for cambial sectors) were recorded for each transgenic sector observed on each cambial slice. Often, when slicing the window, a sector could be transversely cut into two halves. Therefore, the correct sector type and staining pattern were identified by paying attention to sector size, transparency of wood and the intensity of GUS staining and further serial sectioning was performed where necessary (Creux *et al.* 2013).

4.2.7 Calculation of average transformation efficiency and transformation success

The average transformation efficiency (Average Number of Transformation Events per 1 cm² of Cambium Inoculated (ATScm⁻²)) was calculated for each construct, as described in Spokevicius *et al.* (2016). Similarly, the transformation success, which denotes the percentage of cambial windows that contains at least one transgenic sector, was also calculated for each construct.

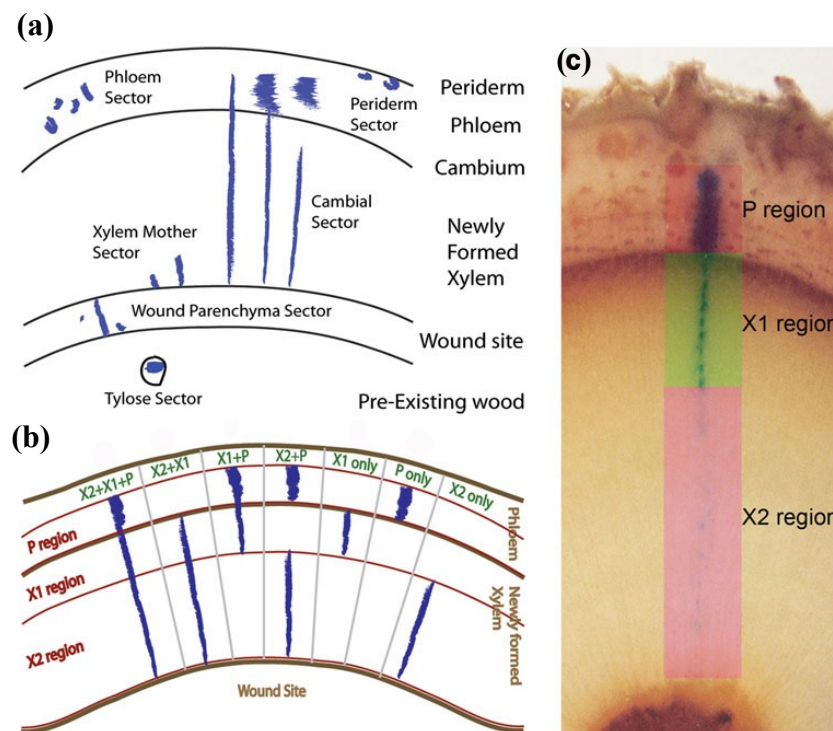


Figure 4.1 Representative diagrams of different somatic sector types and a classification system for ISSA-based promoter expression analysis. (a) a schematic representation of different somatic sector types that can be observed on a cambial slice, (b) a general classification system for different cambial staining patterns that can be observed in cambial sectors based on xylem and/or phloem specificity, (c) a cambial sector from a *Populus* stem demonstrating X2+X1+P cambial staining pattern, where P denotes phloem, X1 denotes developing xylem and X2 denotes mature xylem. Adapted with permission from Creux *et al.* (2013).

Table 4.1 Different types of somatic sectors observed in promoter expression analysis

Sector type	Definition
Periderm	A transgenic sector resulting from an initial transformation of a cell near the cut surface of the cambial window
Wound parenchyma	A transgenic sector resulting from an initial transformation of a parenchyma cell produced during wound response
Tylose	A transgenic sector resulting from an initial transformation of a pre-existing xylem cell
Phloem	A transgenic sector resulting from the initial transformation of a phloem mother cell

Cambial	A transgenic sector resulting from the initial transformation of an undifferentiated cambial initial. One of the seven different types of staining/expression patterns (X1+X2+P, X1+X2, X1+P, X2+P, X1-only, X2-only and P-only) can be observed in a cambial sector (Fig. 4.1b)
Xylem mother	A transgenic sector resulting from the initial transformation of a xylem mother cell that has either eventually replaced by an untransformed mother cell or terminally differentiated into a non-mother cell

4.2.8 Examination of cell and tissue morphology of transgenic cells

Only transgenic sectors obtained from the gene function analysis were used to determine the effects of selected TFs on shaping wood cell morphology. A scanning electron microscopic assessment was done as described in Spokevicius *et al.* (2016). Briefly, the prepared samples were observed under the scanning electron microscope (SEM) (FEI Teneo Volume Scope; low vacuum mode, acceleration voltage of either 5kV or 10kV) and micrographs were acquired at 350× (for the entire sample, Fig. 4.2a) and at 2500× (for a group of transgenic cells located between 100-200 µm from the cambial surface, Fig. 4.2b). A similar micrograph was acquired for a group of non-transgenic cells outside the transgenic area (non-transgenic tissue), located at a similar distance from the cambial surface, ensuring that cells in both transgenic and adjacent non-transgenic sectors were at a similar developmental stage. Micrographs for about 20-30 transgenic sectors and adjacent non-transgenic tissue were acquired for each construct in each genetic background. In these micrographs, five xylem fibres from each transgenic sector and its adjacent non-transgenic tissue were randomly selected (here onwards collectively referred to as a sector pair), and their measurements were taken for cell wall thickness (five measurements from different places on the cell wall), lumen area and cross-sectional area, using ImageJ software (<https://imagej.nih.gov/ij/index.html>). The cell wall area was calculated as the difference between the cross-sectional area and lumen area.

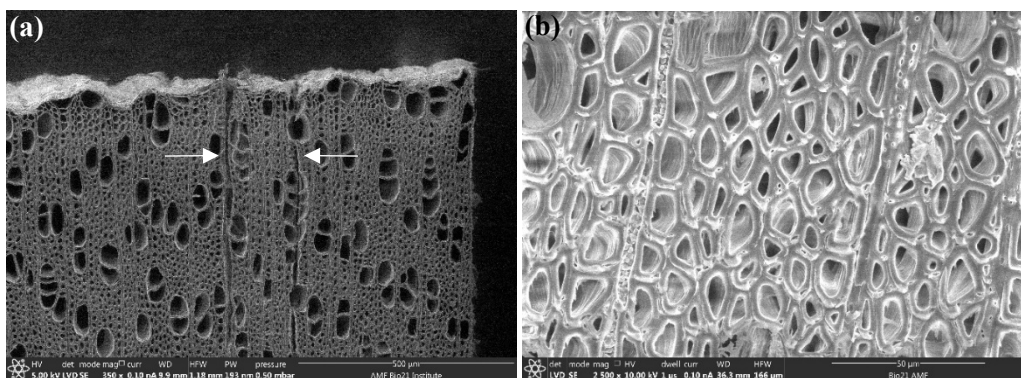


Figure 4.2 Scanning electron micrographs of transgenic sectors and cells. (a) scanning electron micrograph (350 $\times$) of *Populus* transverse stem section containing a transgenic sector (delineated by two radial markings on either side of the sector), (b) scanning electron micrograph (2500 $\times$) of *Populus* transgenic cells in a cross-section of the transgenic sector.

4.2.9 Data analysis

Even though the three trials (I, II and III) took place in summer, under identical glasshouse conditions, since they took place in subsequent years, each trial was analysed separately.

4.2.9.1 Promoter expression analysis

The X1, X2 and P count data obtained for each treatment in promoter expression analysis, were used for chi-squared tests using Minitab (Minitab Inc., State College, PA, USA) to compare overall cambial staining patterns produced by each promoter construct. The cambial and somatic counts were also plotted in graphs to visualise frequencies of different staining patterns produced by each promoter construct (Creux *et al.* 2013).

4.2.9.2 Statistical analysis for overexpression and downregulation experiments

About 15-29 sector pairs (Table 4.2) were used for the following statistical analysis. The statistical analyses were conducted by using the median and ‘difference’ values to perform unpaired ($\alpha = 0.05$) *t*-tests as described in Spokevicius *et al.* (2016). These ‘difference’ values are obtained by comparing the transgenic and adjacent non-transgenic cells in a sector pair. A sector pair is in the same tree, at the same developmental stage and experience the same environmental conditions. Therefore,

the ‘difference’ between the transgenic and adjacent non-transgenic cells in a sector pair, is essentially a representation of the net change that has taken place due to the transformation with a particular construct. Therefore, the use of these ‘difference’ values lowers the variability that may engender when collectively analysing the results generated from separate trees (Hussey *et al.* 2011; MacMillan *et al.* 2015; Spokevicius *et al.* 2016; Spokevicius *et al.* 2007a; Van Beveren *et al.* 2006). The unpaired approach compares these ‘difference’ values of all sector pairs of a TF of interest with those of the respective control construct (EVC+ or EVC-). In addition, these ‘difference’ values were used to plot 95% confidence intervals which allow to visualise and compare the spread/patterns of data produced by each construct against its respective EVC. In these graphs, if the error bar of a construct does not overlap the mean value of the respective EVC, it denotes that the construct influences the trait of interest.

Table 4.2 Number of sector pairs used for the overexpression and knockdown analyses

Analysis	Construct	Symbol	Plant species	Number of sector pairs (n)	Total number of fibres
Overexpression	EgKNAT7	KNAT7+(II)	<i>Egr</i> × <i>Eca</i>	29	290
	EgMYB103	MYB103+(I)	<i>E. globulus</i>	17	170
	EgSND2	SND2+(I)	<i>E. globulus</i>	18	180
	EgSND3	SND3+(I)	<i>E. globulus</i>	20	200
	EVC+	EVC+(I)	<i>E. globulus</i>	15	150
	EVC+	EVC+(II)	<i>Egr</i> × <i>Eca</i>	27	270
	EgKNAT7	KNAT7+(II)	<i>P. alba</i>	20	200
	EgMYB103	MYB103+(I)	<i>P. alba</i>	17	170
	EVC+	EVC+(I)	<i>P. alba</i>	15	150
	EVC+	EVC+(II)	<i>P. alba</i>	17	170
Knockdown	EgKNAT7	KNAT7-(III)	<i>Egr</i> × <i>Eca</i>	25	250
	EgMYB103	MYB103-(III)	<i>Egr</i> × <i>Eca</i>	0	0
	EVC-	EVC-(III)	<i>Egr</i> × <i>Eca</i>	18	180

(I), (II) and (III) denotes the trial numbers. + and – denotes overexpression and knockdown constructs, respectively. *Egr* × *Eca* denotes *E. grandis* × *camaldulensis*.

4.3 RESULTS

4.3.1 All plants actively grow under glasshouse conditions

Among the eucalypts, the *E. grandis* × *camaldulensis* plants that were used in trial III grew taller than others (Table 4.3). However, the average daily xylem increment was similar between all the eucalypts. Among the poplars, the plants used in trial II grew slightly taller than those in trial I. The poplars demonstrated similar average daily xylem tissue increments.

Table 4.3 Growth data of the plants used for the ISSA experiments

Growth data	<i>E. globulus</i> (I)	<i>P. alba</i> (I)	<i>Egr</i> × <i>Eca</i> (II)	<i>P. alba</i> (II)	<i>Egr</i> × <i>Eca</i> (III)
Number of plants	30	30	35	36	12
Growth period (days)	97	98	73	76	85
Average daily height increment (cm/day)	1.02 (0.036)	1.01 (0.043)	0.80 (0.049)	1.18 (0.060)	1.33 (0.091)
Average daily diameter increment (mm/day)	0.08 (0.003)	0.09 (0.003)	0.04 (0.002)	0.08 (0.005)	0.03 (0.006)
Average daily xylem increment (mm/day)	0.02 (0.001)	0.03 (0.001)	0.02 (0.001)	0.03 (0.001)	0.02 (0.001)

Egr × *Eca* denotes *E. grandis* × *camaldulensis*. I, II and III denote separate ISSA trials that took place in different years. The **bold** values represent respective **standard error** values. The measurements for diameter growth were taken at 10 cm stem height. The xylem increment in each xylem window was measured as the total radial growth of new xylem tissue as measured from the wound site.

4.3.2 *Eucalyptus* is more efficiently and successfully transformed compared to *Populus*

For all the constructs, the ATScm⁻² and transformation success were separately calculated in *Eucalyptus* and *Populus* (Table 4.4). A total stem tissue area of 145, 118 and 126 cm² were inoculated and harvested from *Eucalyptus* for promoter expression, gene overexpression and gene knockdown analyses, respectively. A total stem tissue area of 160 and 168 cm² were inoculated and harvested from *Populus* for promoter expression and gene overexpression analyses, respectively. In general, the ATScm⁻² and transformation success reported from all the constructs were significantly higher in *Eucalyptus* compared to *Populus*, except for KNAT7 promoter construct. Among the promoter constructs, the highest ATScm⁻² in *Eucalyptus* was reported from 35S. Among the overexpression constructs, MYB103+ demonstrated the highest ATScm⁻².

However, among the knockdown constructs, both KNAT7- and EVC- had similar ATScm⁻² values while MYB103- did not produce any transgenic sectors.

Table 4.4 The transformation efficiency and transformation success of different vectors in *Eucalyptus* and *Populus*

Construct (trial)	Transformation success (%)		ATScm ⁻²	
	Euc	Pop	Euc	Pop
KNAT7	78.57	95.00	3.07 (0.46)	6.93 (1.14)
MYB103	83.33	8.33	4.58 (1.92)	0.08 (0.06)
SND2	75.00	16.67	12.08 (3.82)	0.21 (0.10)
SND3	41.67	25.00	1.08 (0.50)	0.29 (0.11)
35S	91.67	29.17	29.67 (5.38)	0.67 (0.29)
KNAT7+(II)	100.00	91.67	22.71 (2.89)	6.72 (0.98)
EVC+(II)	94.29	94.44	13.74 (1.82)	6.22 (0.91)
MYB103+(I)	100.00	94.12	119.25 (13.94)	3.41 (0.62)
SND2+(I)	100.00	94.12	45.00 (7.44)	-
SND3+(I)	100.00	100.00	67.92 (10.49)	-
EVC+(I)	100.00	100.00	90.75 (16.18)	6.06 (1.86)
KNAT7-(III)	97.62	-	11.90 (1.38)	-
MYB103-(III)	0	0	-	-
EVC-(III)	92.86	-	13.48 (2.13)	-

Euc indicates *Eucalyptus* and Pop indicates *Populus*. I, II and III denote separate ISSA trials that took place in different years. The + and - denotes overexpression and knockdown constructs, respectively. **Standard error** values are written in **bold** within brackets. The constructs that were not tested in *P. alba* are indicated by a hyphen (-).

4.3.3 Cambial sectors are the most frequently observed somatic sector type

In *Eucalyptus*, different promoter constructs produced different combinations and frequencies of somatic sectors (Fig. 4.1 and Table 4.1); although all five somatic sector types (wound parenchyma, cambial, tylose, periderm and phloem) were only observed for 35S (at different frequencies) (Fig. 4.3a). Cambial sectors were the most frequently observed somatic sector type for all promoter constructs. However, no expression was observed in both wound parenchyma and periderm tissues for *MYB103*, *SND2* and *SND3* promoters. Similarly, expression was not observed in phloem tissue (phloem only sector types) for *KNAT7* and *SND2* promoters and in the pre-existing xylem tissue

(tylose sectors) for *SND3* promoter. In *Populus*, the total numbers of transgenic sectors obtained for *MYB103*, *SND2*, *SND3* and *35S* were two, five, seven and 16, respectively and therefore deemed too low to be considered for meaningful analysis.

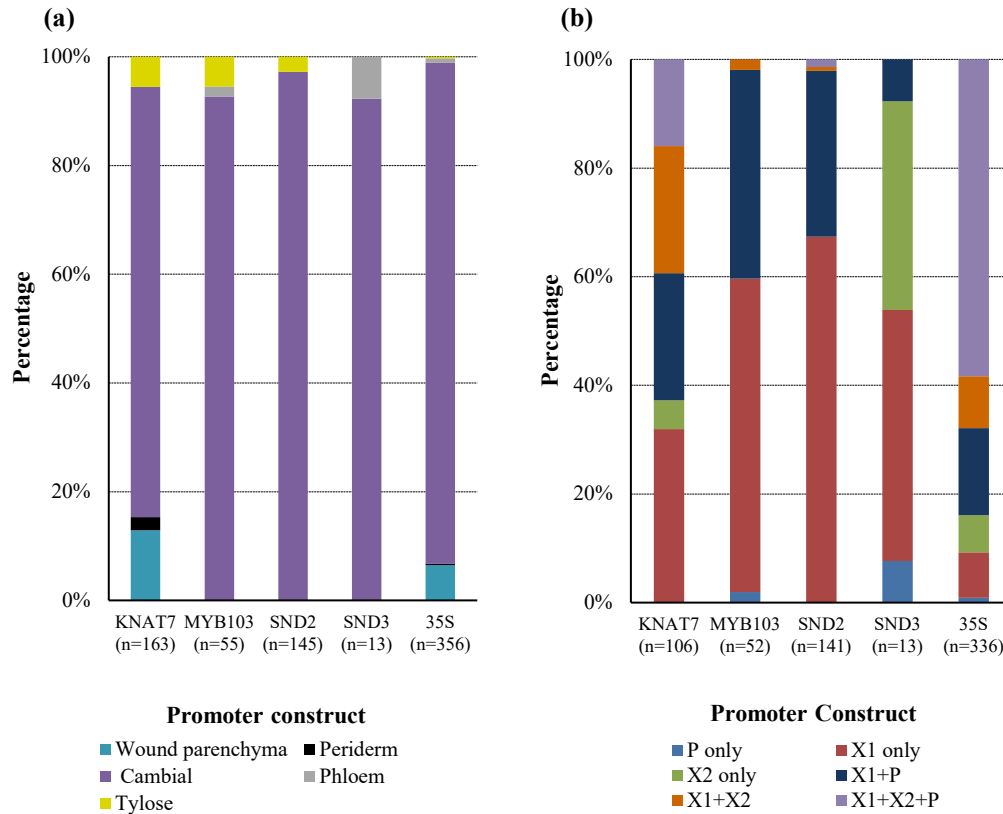


Figure 4.3 Different sector types and different cambial staining patterns observed in *Eucalyptus* stem tissue: (a) frequencies of somatic transgenic sector types and (b) frequencies of cambial staining patterns. Y-axis indicates the percentage of frequency values. The total number of sector types obtained for each promoter construct is denoted by ‘n’. P denotes phloem, X1 denotes developing xylem and X2 denotes mature xylem. Since the promoterless negative control (:GUS) did not yield any GUS-positive sectors, it was not included in above graphs.

4.3.4 *KNAT7*, *MYB103*, *SND2* and *SND3* promoters are preferentially expressed in developing xylem zone

The 35S was used as the control to compare the activities of different promoter constructs across X1, X2 and P regions (Fig. 4.3b and Fig. 4.4). The types of cambial staining patterns and their frequencies varied depending on the promoter construct, except for X2+P, which was not observed for any promoter. Six cambial staining patterns (X1+X2+P, X1+X2, X1+P, X1-only, X2-only and P-only) were observed for

35S promoter (at different frequencies). The most frequently observed cambial staining pattern for *EgKNAT7*, *EgMYB103*, *EgSND2* and *EgSND3* promoters was X1-only, suggesting that these promoters are mostly expressed in the developing xylem (Fig. 4.3b and Fig. 4.4). The most frequently observed cambial staining pattern for 35S was X1+X2+P, presumably due to the constitutive nature of its expression.

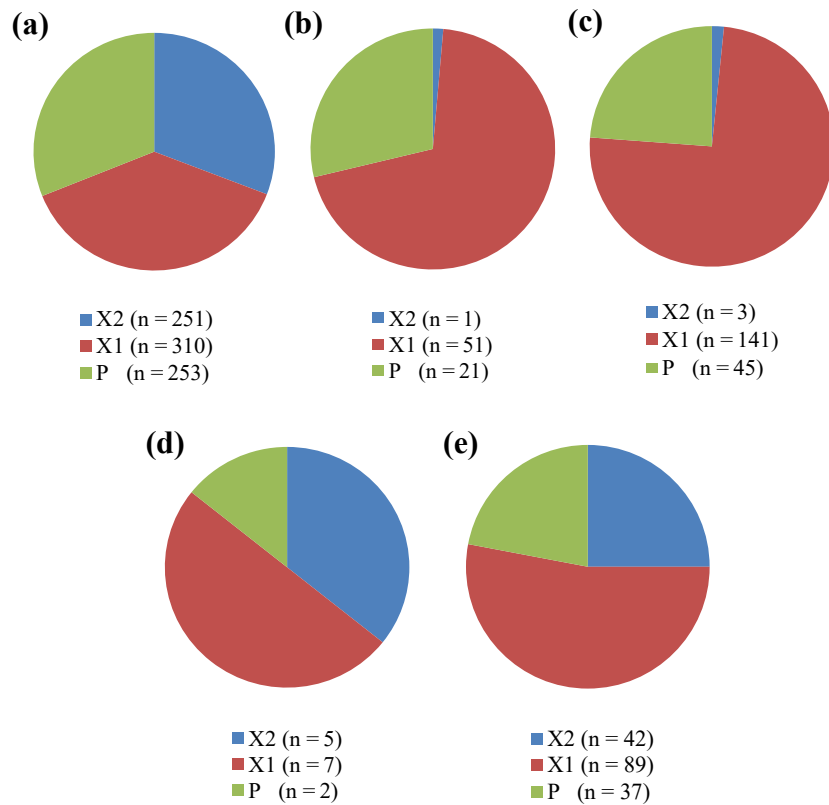


Figure 4.4 Frequencies of the X1, X2 and P cambial staining patterns produced by different promoters in *Eucalyptus* stem: (a) 35S, (b) *EgMYB103*, (c) *EgSND2*, (d) *EgSND3* and (e) *EgKNAT7*. P denotes phloem, X1 denotes developing xylem and X2 denotes mature xylem. The total number (n) of X1, X2 and P sectors observed for each promoter, are mentioned below the respective pie chart.

In addition, chi-square tests were performed to determine similarities/differences between different promoters with respect to their expression in developing xylem, mature xylem and phloem tissues (Table 4.5). The *EgKNAT7*, *EgMYB103* and *EgSND2* promoters demonstrated significantly different ($p < 0.05$) expression patterns compared to 35S, whereas, the expression pattern of *EgSND3* promoter was not significantly different from 35S. In addition, there was no significant difference ($p > 0.05$) between the cambial staining patterns observed for *EgMYB103* and *EgSND2*

promoters, where the highest, second highest and lowest activities for both were observed in the developing xylem, phloem and mature xylem, respectively (Table 4.5, Fig. 4.4b and c). Similarly, there was no significant difference ($p > 0.05$) between the cambial staining patterns observed for *EgKNAT7* and *EgSND3* promoter constructs, where the highest, second highest and the lowest activities for these promoters were observed in the developing xylem, mature xylem and phloem, respectively (Table 4.5, Fig. 4.4d and e).

Table 4.5 Chi-squared tests comparing expression frequencies of different promoters across X1, X2 and P in *Eucalyptus*

Chi-squared values	<i>KNAT7</i>	<i>MYB103</i>	<i>SND2</i>	<i>SND3</i>	<i>35S</i>
<i>KNAT7</i>	-	19.39 (0.00)*	45.26 (0.00)*	0.96 (0.62)	13.06 (0.00)*
<i>MYB103</i>	-	-	0.69 (0.71)	21.72 (0.00)*	37.21 (0.00)*
<i>SND2</i>	-	-	-	40.11 (0.00)*	100.04 (0.00)*
<i>SND3</i>	-	-	-	-	1.88 (0.39)

Chi-squared values were obtained by conducting pair-wise comparisons using GUS expression frequencies given by different promoter constructs, across X1, X2 and P regions. Degrees of freedom was 2 for all Chi-squared values. *P* values are indicated within brackets ($\alpha = 0.05$). * denotes $p < 0.05$.

As mentioned above, *EgKNAT7*, *EgMYB103*, *EgSND2* and *EgSND3* promoters were preferentially expressed in secondary xylem tissue, providing further evidence for their possible involvement in wood formation. To better understand how they influence wood formation and wood cell (xylem fibre) morphology, ISSA experiments were conducted using overexpression and knockdown constructs.

4.3.5 *EgKNAT7* significantly alters *Eucalyptus* xylem fibre morphology

Although *KNAT7* is involved in regulating SCW deposition in *Arabidopsis* and *Populus*, to date, the function of this TF has not been investigated in *Eucalyptus*. In the present study, we observed that transformation with *KNAT7+* or *KNAT-* altered xylem fibre morphology, in terms of cell wall thickness, cell wall area, lumen area and cross-sectional area (Fig. 4.5). Compared to *EVC+*, xylem fibres transformed with *KNAT7+* demonstrated average increments in cell wall thickness by 0.11 μm , cell wall

area by $5.89 \mu\text{m}^2$ ($p = 0.03$), lumen area by $3.70 \mu\text{m}^2$ and cross-sectional area by $8.62 \mu\text{m}^2$. In contrast, compared to EVC-, the xylem fibres transformed with KNAT7- demonstrated average reductions in cell wall thickness by $0.08 \mu\text{m}$, cell wall area by $3.37 \mu\text{m}^2$, lumen area by $5.14 \mu\text{m}^2$ and cross-sectional area by $9.15 \mu\text{m}^2$. Based on the observed contrasting phenotypes, KNAT7 in *Eucalyptus* is presumably a positive regulator of xylem fibre development. However, *Populus* xylem fibres transformed with the same KNAT7+ did not demonstrate any changes in fibre morphology with respect to the above four xylem fibre properties (Fig. 4.6).

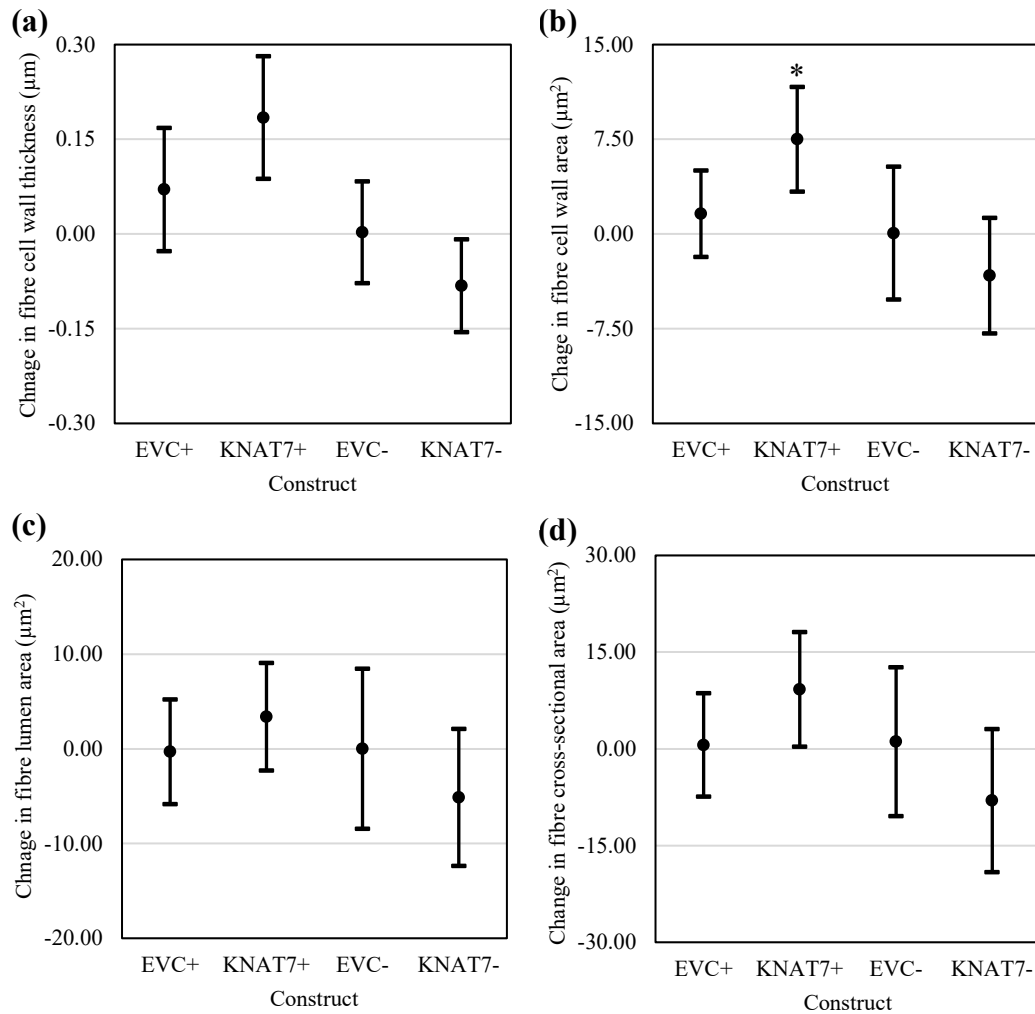


Figure 4.5 Comparison of average changes in (a) cell wall thickness, (b) cell wall area, (c) lumen area and (d) cross-sectional area of *Eucalyptus* xylem fibres transformed with overexpression (+) and knockdown (-) constructs of EgKNAT7. The black dots represent the mean value. Error bars represent 95% confidence intervals. * denotes $p \leq 0.05$

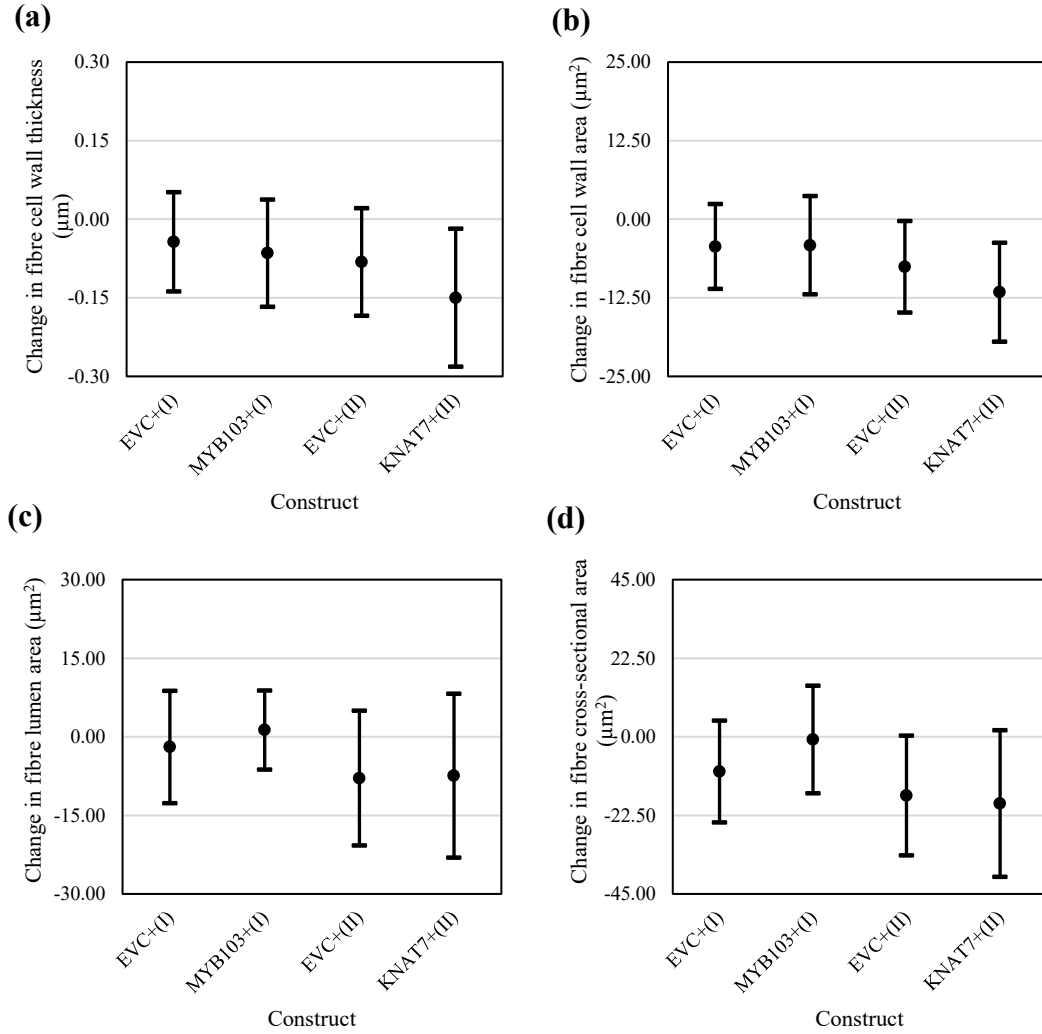


Figure 4.6 Comparison of average changes in (a) cell wall thickness, (b) cell wall area, (c) lumen area and (d) cross-sectional area of *Populus* xylem fibres transformed with overexpression (+) constructs of KNAT7 and MYB103. I and II denote the trial number. Since the two experiments were conducted in two years, the respective EVCs from each experiment were used. The black dots represent the mean value. Error bars represent 95% confidence intervals.

4.3.6 EgMYB103+ significantly alters *Eucalyptus* xylem fibre morphology

Similar to KNAT7, in *Arabidopsis*, MYB103, SND2 and SND3 TFs function as regulators of SCW deposition (Cassan-Wang *et al.* 2013; Hussey *et al.* 2013; Zhong *et al.* 2008). However, their *Eucalyptus* homologs have not been functionally investigated during wood formation, and therefore, we employed overexpression and knockdown (where necessary) to elucidate the functions of these eucalypt TFs. These studies revealed that SND2+ and SND3+ do not alter *Eucalyptus* xylem fibre morphology in terms of cell wall thickness, cell wall area, lumen area and cross-

sectional area (Fig. 4.7). However, transformation with MYB103+ altered *Eucalyptus* xylem fibre morphology. Compared to EVC+ the xylem fibres transformed with MYB103+ demonstrated average increments in cell wall thickness by $0.07 \mu\text{m}$, cell wall area by $5.29 \mu\text{m}^2$, lumen area by $10.99 \mu\text{m}^2$ ($p = 0.03$) and cross-sectional area by $13.87 \mu\text{m}^2$ ($p = 0.04$). However, transformation with the same MYB103+ did not alter *Populus* xylem fibre morphology in terms of cell wall thickness, cell wall area, lumen area and cross-sectional area (Fig. 4.6). In addition, a knockdown experiment was also conducted in *Eucalyptus*; however, no transgenic sectors were observed from the transformation of *Eucalyptus* cambial initials with MYB103-.

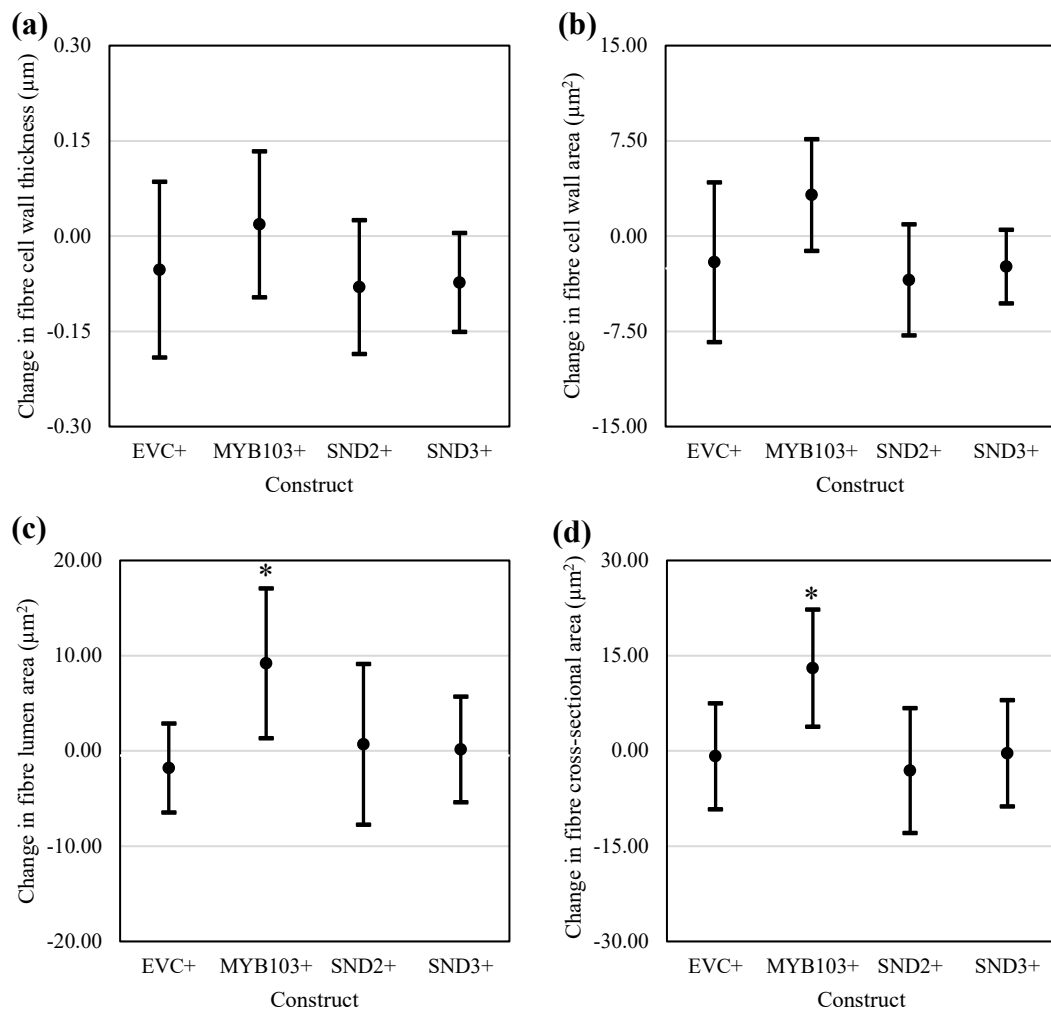


Figure 4.7 Comparison of average changes in (a) cell wall thickness, (b) cell wall area, (c) lumen area and (d) cross-sectional area of *Eucalyptus* xylem fibres transformed overexpression constructs; MYB103+, SND2+ and SND3+. The black dots represent the mean value. Error bars represent 95% confidence intervals. * denotes $p \leq 0.05$

4.4 DISCUSSION

4.4.1 In most of the trials, *Eucalyptus* grew faster than *Populus*

In all trials, plants were grown under controlled glasshouse conditions, providing them with identical temperature, photoperiod, and watering conditions and by allowing ample space for growth. These conditions provided for the active growth of plants over the duration of each experiment. On average the growth rates of *Populus* (in terms of height, diameter and woody tissue increment) were higher than *Eucalyptus* in the trial I and II. However, during all three trials the plants sufficiently grew and yielded adequate transgenic tissue sectors for the downstream analysis.

4.4.2 Transformation efficiency depends on the plant species and promoter/gene construct

Agrobacterium-mediated transformation successfully transferred overexpression, knockdown and promoter constructs, into the cambial cells in growing stems of *Eucalyptus* and *Populus*. The ATScm⁻² of the promoter constructs were higher in *Eucalyptus* compared to *Populus* (except KNAT7 promoter construct). However, the observed low ATScm⁻² and transformation successes of MYB103, SND2, SND3 and 35S promoter constructs could be a result of the possible onset of dormancy in the *Populus* plants that were inoculated by these constructs, which deems our results in this regard inconclusive. In contrast, the gene overexpression and knockdown constructs had higher ATScm⁻² values, all of which were significantly higher in *Eucalyptus* compared to *Populus*. Previous studies also have reported variations in ATScm⁻² between different genera such as *Eucalyptus*, *Populus* and *Pinus*, demonstrating that the genetic background can influence the transformation efficiency (Van Beveren *et al.* 2006). Therefore, the transformation efficiency in ISSA does not solely depend on the vector construct. It also depends on the genetic background as well as on the composition of the woody stems, as the woody stems are composed of different types of tissues and cells, all of which might have different levels of susceptibility for *Agrobacterium* transformation.

4.4.3 *KNAT7*, *MYB103*, *SND2* and *SND3* promoters are preferentially expressed in SCW forming tissues

A transgenic sector in ISSA-based promoter analysis corresponds to a small subgroup of cells, tissue types or growth stages in which the respective promoter is expressed. Based on these facts, the presence or absence of a particular sector type with respect to a specific promoter construct could be attributed to the tissue-specificity of the respective promoter. Therefore, the observed predominant staining patterns in cambial tissues, especially in the developing xylem, suggest preferential expression of *EgKNAT7*, *EgMYB103*, *EgSND2* and *EgSND3* during wood formation. Furthermore, since the spatio-temporal expression patterns of *EgSND2/EgMYB103* pair and *EgSND3/EgKNAT7* pair were not significantly different from each other ($p > 0.05$), presumably the two TFs in these pairs are involved in regulating same or similar processes during SCW formation. As an example, several previous studies in *Arabidopsis* have suggested *AtMYB103* as one of the downstream targets of *AtSND2* (Hussey *et al.* 2013; Hussey *et al.* 2011; Ohman *et al.* 2013; Zhong *et al.* 2008). Although these are contradictory in terms of their hierarchical position, this evidence suggests that *AtMYB103* and *AtSND2* are potentially involved in the same regulatory processes such as activation of biosynthesis of cellulose, lignin and hemicellulose. However, in addition to cambial sectors, a few tylose sectors (by *EgMYB103* and *EgSND2* promoters), wound parenchyma sectors (by *EgKNAT7* promoter) and phloem sectors (by *EgMYB103*, *EgSND2* and *EgSND3* promoters) were observed. These sectors suggest the likely involvement of these TFs in tylose formation (a response to wounding), in wound response and phloem development, respectively (Grant *et al.* 2010). Alternatively, these non-xylem sector types might be an indication that the promoter regions used in our constructs (1.5-2 kb upstream) might lead to ectopic expression (Botha *et al.* 2011). Nevertheless, the observed xylem specific expression patterns of *EgMYB103*, *EgKNAT7*, *EgSND2* and *EgSND3* are in agreement with the xylem or developing fibre specific expression of their *Arabidopsis* homologs.

In *Arabidopsis*, *AtKNAT7*, *AtMYB103*, *AtSND2* and *AtSND3* are preferentially expressed during SCW formation in the xylem and in interfascicular fibres. Li *et al.* (2012) demonstrated that in *Arabidopsis*, *AtKNAT7* is active in cells undergoing SCW deposition as well as in cells which are nearby the fibres undergoing SCW deposition.

Also, *AtKNAT7* is expressed in the stem (metaxylem and vascular bundles) and in the vascular tissues of hypocotyl, roots and cotyledons (Li *et al.* 2012a). Similar results have also been reported by some other studies which confirmed that *AtKNAT7* is active during the deposition of SCWs in interfascicular fibres (Li *et al.* 2011a; Wang *et al.* 2019b; Zhong *et al.* 2008). In addition, previous studies demonstrate that *AtSND2* is preferentially expressed in the xylem and interfascicular fibres in *Arabidopsis* stem (Zhong *et al.* 2008). Similarly, its *Populus* homolog, *PtrNAC154* also demonstrated a tissue preferential expression where its expression was significantly elevated in the growing stems and xylem (Grant *et al.* 2010; Wang *et al.* 2013). Furthermore, the highest *AtSND3* and *AtMYB103* expression levels have been observed in the *Arabidopsis* stems where these TFs were preferentially expressed in the xylem and interfascicular fibres (Zhong *et al.* 2008). Since the eucalypt homologs of these TFs exhibit similar expression patterns during wood formation, these eucalypt TFs are presumably involved in regulating SCW formation.

To further explore this, transgenic tissue sectors were created on *Eucalyptus* and *Populus* stems using overexpression constructs of these *Eucalyptus* TFs. The tissue sectors transformed with these overexpression constructs were used to assess the effects of candidate TFs in shaping xylem fibre morphology. Here, we primarily focused on the morphology of xylem fibres as the morphology, and chemical composition of xylem fibres can significantly influence the quality of resulting wood, and its end uses such as pulp (for paper production) or structural timber. Furthermore, since the overexpression of a native gene can lead to Homology Dependent Gene Silencing (HDGS), transgenic tissue sectors were created on *Eucalyptus* stems using the RNAi knockdown constructs of the TFs that altered xylem fibre morphology during overexpression study, to compare the phenotypes generated during overexpression and knockdown experiments and thereby to elucidate whether the observed phenotypes are due to overexpression or HDGS.

In ISSA, several independent transformation events are taken into consideration when determining the overall effect of the transgene/TF. Several transgenic studies have reported that the expression levels of a transgene in different independent transformation events can vary significantly since the transgene might have inserted at

different locations within the genome, causing positional effects. Some studies have reported that even between two similar integration events, the level of transgene expression can be different (Cooley *et al.* 1995; Kumpatla and Hall 1999; Twell 1992). Such reports provide possible explanations for variations between sector pairs and for the observed subtlety in phenotypes (discussed below) from TFs from which a significant impact was expected.

4.4.4 EgKNAT7 alters *Eucalyptus* xylem fibre morphology

The KNAT7⁺ and KNAT7⁻ significantly alter *Eucalyptus* xylem fibre morphology. Transformation with KNAT7⁺ lead to an increase in cell wall thickness, cell wall area and cross-sectional area. In contrast, transformation with KNAT7⁻ demonstrated a decreasing pattern of these fibre properties. These SCW-related phenotypes in our study confirm the hypothesised regulatory role of EgKNAT7 in SCW deposition, which agrees with the previous studies that showcased the regulatory role of *Populus* and *Arabidopsis* KNAT7 in xylem fibre SCW deposition. However, the results of the present study also demonstrate changes in xylem fibre cross-sectional area, providing very first insights into a potential function of EgKNAT7 in xylem fibre expansion. Therefore, our results suggest that the function of EgKNAT7 is not limited to SCW deposition and that it could also be involved in regulating other aspects of *Eucalyptus* xylem fibre development such as radial growth (expansion).

The homologs of EgKNAT7 in other species also act as regulators of SCW deposition. Although KNAT7 is a regulator of SCW deposition, the activator (positive) or repressor (negative) nature of this regulation is an open question (Schuetz *et al.* 2013). A couple of previous studies conducted in *Arabidopsis* have reported AtKNAT7 as a positive regulator (He *et al.* 2018; Zhong *et al.* 2008). According to He *et al.* (2018), AtKNAT7 acts as a positive regulator of xylan biosynthesis during SCW formation and according to Zhong *et al.* (2008), dominant repression of AtKNAT7 reduces SCW thickness in *Arabidopsis* xylary fibres. In contrast, several other studies in *Arabidopsis* have suggested a repressor role for AtKNAT7 during SCW formation (Bhargava *et al.* 2013; Li *et al.* 2012a; Liu *et al.* 2014b). Additionally, a recent study in *Oryza sativa* demonstrated OsKNAT7 as a repressor of wall thickening and expansion of rice grains

(Wang *et al.* 2019c). Likewise, the current information on the regulatory role of KNAT7 is contradictory.

One possible explanation for these conflicting observations could be the different interacting partners of KNAT7, as KNAT7 is known to mediate its regulation via protein-protein interactions with other TFs (Hussey *et al.* 2013; Li *et al.* 2012a; Schuetz *et al.* 2013). Li *et al.* (2012) suggested that the cell or the tissue-specific positive or negative regulatory role of KNAT7 depend on the composition and the abundance of its interacting partners in these cells or tissues of interest. Although to date, the interacting partners of EgKNAT7 in *Eucalyptus* remains unexplored, a few interaction partners of AtKNAT7 in *Arabidopsis* have been identified. As an example, AtKNAT7 interacts with AtMYB61, which is known to modulate carbon allocation during processes such as formation of SCWs, seed coats and xylem (Romano *et al.* 2012). The *atmyb61* loss of function mutants had thinner cell walls indicating a decrease in SCW production, whereas the gain of function mutants (*35S:MYB61*) exhibited an ectopic lignification indicating an increase in SCW production. These observations put AtMYB61 in the light of a positive regulator of SCW formation. Therefore, we speculate that the observed positive regulatory role of EgKNAT7 could be a result of its potential interaction with the *Eucalyptus* homolog of AtMYB61 (Eucgr.B02197).

In *Arabidopsis*, AtKNAT7 also interacts with some repressors. It interacts with OVATE FAMILY PROTEIN1 (OFP1) and OFP4, which are transcriptional repressors (Li *et al.* 2011a). In addition, AtKNAT7 interacts with AtMYB75, which is also a repressor of SCW biosynthesis, especially lignin (Bhargava *et al.* 2013; Bhargava *et al.* 2010). BELL1-LIKE HOMEODOMAIN6 (BLH6) is another interaction partner of KNAT7, where the interaction between these two proteins is known to enhance the repressive effects of KNAT7 (Liu *et al.* 2014b). However, based on our observations we speculate that during xylem formation in *Eucalyptus* EgKNAT7 potentially interacts with interacting partner/s with an activator (positive) nature or at least that these interactions are stronger compared to the interactions with repressors.

EgKNAT7 is also a potential regulator of TW formation. During TW formation in *Eucalyptus*, *EgKNAT7*, the homolog of *AtMYB52* (Eucgr.B02197) and Eucgr.B02197 are upregulated, suggesting a possible co-regulation of these TFs (Mizrachi *et al.* 2015). Therefore, presumably, both EgKNAT7-Eucgr.B02197 (repressor) and EgKNAT7-Eucgr.B02197 (activator) functional modules exist in *Eucalyptus*. Furthermore, these modules could be involved in regulating SCW specific TW features which are generated as a result of repression (reductions in lignin deposition and microfibril angle (MFA)) and activation (promotion in cellulose deposition and S:G ratio) of biosynthesis of SCW biopolymers including cellulose and lignin.

4.4.5 EgMYB103 increases xylem fibre size

Transformation with MYB103+ significantly affected xylem fibre morphology in *Eucalyptus*. *Eucalyptus* xylem fibres containing MYB103+ showed an increasing pattern for cell wall thickness and cell wall area. In addition, they also demonstrated a significant increase ($p = 0.05$) in both fibre cross-sectional area and lumen area. Up to now, MYB103 has been functionally investigated only in a couple of studies in *Arabidopsis*. One study revealed that *myb103* mutants demonstrate a significant decrease in S lignin which is associated with a decrease in the transcript abundance of one of the key lignin biosynthetic genes (*FERULATE-5-HYDROXYLASE*) and a marginal decrease in cellulose content (Ohman *et al.* 2013). According to the second study, AtMYB103 is a possible positive regulator of cellulose biosynthesis, thereby it positively regulates SCW thickening in xylary fibres and interfascicular fibres (Zhong *et al.* 2008). Therefore, MYB103 is as a positive regulator of SCW formation in *Arabidopsis*. As we observed an increasing pattern of cell wall thickness and cell wall area in MYB103+ transformed xylem fibres, our results agree with the positive regulatory role of MYB103 during SCW formation. Therefore, we speculate Eucgr.D01819 as a functional homolog of AtMYB103 (AT1G63910).

In addition, our results suggest that the function of EgMYB103 is not exclusive to SCW formation. Since no transgenic sectors were produced by MYB103-, the knockdown of *EgMYB103* might be lethal. Therefore, although not yet proven, EgMYB103 alone or EgMYB103 as a member of a protein complex could be involved

in mediating *Eucalyptus* xylem formation. In addition, the potential involvement of EgMYB103 in regulating xylem fibre development has been demonstrated by the increased radial growth of xylem fibre. Therefore, our results also provide the very first insights to other potential regulatory aspects of EgMYB103, including regulation of xylem formation and/or xylem fibre development.

The absence of a phenotype in *Populus* xylem fibres transformed with MYB103+ could be due to one or many reasons that influence the expression of a transgene in a heterologous genetic background. Some of these reasons include post-translational modifications, quality control procedures and incorrect localisation of the protein (Ghag *et al.* 2016; Kohli *et al.* 2006; Vyacheslavova *et al.* 2012). In addition, although the upregulation of *MYB103* during TW formation in *Populus* (Chen *et al.* 2015; Zinkgraf *et al.* 2018) suggests it as a potential regulator of TW formation, in the present study an apparent TW feature was not observed.

4.4.6 EgSND2 and EgSND3 do not affect xylem fibre morphology

Several previous studies have demonstrated that orthologs of EgSND2 and EgSND3 in other species act as positive regulators of xylem fibre development and SCW formation. As an example, in *Arabidopsis* dominant repression of *AtSND2* severely reduced xylem fibre SCW thickness, whereas overexpression of *AtSND2* increased SCW thickness. Similar results have been observed from overexpression and dominant repression of *AtSND3* (Zhong *et al.* 2008). Also, according to Hussey *et al.* (2011), overexpression of *AtSND2* in *Eucalyptus* increase xylem fibre area, and it also influences fibre SCW thickness. Similarly, overexpression of *OsSND2* in rice increased cellulose content and upregulated SCW synthesis related genes and as expected knockdown of *OsSND2* produced opposite results (Ye *et al.* 2018). In addition, overexpression of a chimeric repressor of *PtSND2* in *Populus* drastically reduced wood formation and SCW thickness along with cellulose and lignin contents (Wang *et al.* 2013). Therefore, the literature suggests positive regulatory roles for SND2 and SND3 during wood formation, more specifically during xylem fibre development. However, according to our results, transformation with SND2+ and SND3+ did not produce any phenotypes in *Eucalyptus* xylem fibre morphology in

terms of fibre cell wall thickness, cell wall area, fibre cross-sectional area and lumen area.

This absence of a phenotype could be due to the combined effect of HDGS and gene redundancy. HDGS explains the instances where the high degree of homology between the endogenous (native) gene and transgene leads to silencing of both native gene and transgene. This phenomenon has been observed in various transgenic experiments including ISSA (Baucher *et al.* 1996; Moller *et al.* 2003; Spokevicius *et al.* 2007a; Tsai *et al.* 1998). If the native gene has been silenced (as a result of HDGS), then functional redundancy may rescue the resulting HDGS related phenotype, leading to no apparent phenotypes. Functional redundancy has been frequently observed between genes involved in plant development. Some examples include SCW associated genes such as *MYB83-MYB46* and *NST1-NST3* (*NST3* is also referred to as *SND1*), *VND* (*VND6* and *VND7*), *RECEPTOR FOR ACTIVATED C KINASE 1* (*RACK1A*, *RACK1B* and *RACK1C*) and *SAWTOOTH* (*SAW1* and *SAW2*) (Guo and Chen 2008; Kumar *et al.* 2007; McCarthy *et al.* 2009; Mitsuda and Ohme-Takagi 2008; Yamaguchi *et al.* 2010; Yamaguchi *et al.* 2008; Zhong *et al.* 2007). Therefore, our results could have been influenced by HDGS and gene redundancy.

In addition, it is also possible that even though no apparent changes were observed with respect to the four xylem fibre features analysed in this study, there can be some other aspects of plant growth that are regulated by EgSND2 and EgSND3. One such example is PopNAC154; one of the SND2 orthologs in poplar. When *PopNAC154* was overexpressed, no changes were observed in SCW thickness; however, *PopNAC154* overexpression decreased overall plant height and increased phloem to xylem ratio (Grant *et al.* 2010). Similarly, EgSND2 and EgSND3 could be affecting some other features such as bark to xylem ratio and SCW chemical composition, in terms of cellulose, hemicellulose and lignin composition (Grant *et al.* 2010; Hussey *et al.* 2011; Ye *et al.* 2018; Zhong *et al.* 2008). Therefore, although no apparent phenotypes were observed for EgSND2 and EgSND3, in terms of xylem fibre morphology, their potential involvement in the transcriptional regulation of wood formation cannot be ruled out.

4.5 CONCLUSIONS, PERSPECTIVES AND FUTURE WORK

- I. *Eucalyptus* is an extensively grown commercial crop which is a significant contributor of global lignocellulosic feedstock. This lignocellulosic feedstock provides a renewable source for pulp, paper and energy production (Rockwood *et al.* 2008). The digestibility of this feedstock, its quality and pulp yield greatly depend on wood features such as density, chemical composition and xylem fibre and vessel properties such as length, cell wall thickness and diameter (Ona *et al.* 2001; Ramirez *et al.* 2009). Since the central regulator of wood formation is a transcriptional network, manipulation of participating TFs offers a mean of improving xylem fibre properties to enhance the quality and pulp yield of the resulting lignocellulosic biomass. In our study, compared to EVC+, on average EgKNAT7 increased xylem fibre thickness, fibre diameter and lumen diameter by about 0.11 μm ($\sim$ three-fold increase), 2.55 μm ($\sim$ four-fold increase) and 1.47 μm ($\sim$ three-fold increase), respectively. Similarly, compared to EVC+, on average EgMYB103 increased xylem fibre thickness, fibre diameter and lumen diameter by about 0.07 μm ($\sim$ two-fold increase), 3.03 μm ($\sim$ four-fold increase) and 1.90 μm ($\sim$ two-fold increase), respectively. Since EgKNAT7 and EgMYB103 can alter these important xylem fibre properties including cell wall thickness and diameter, they could be potential candidates for genetical manipulation of xylem fibre properties to improve the quality of the lignocellulosic feedstock.
- II. The EgSND2+ and EgSND3+ did not alter *Eucalyptus* xylem fibre morphology in terms of cross-sectional area, lumen area, cell wall thickness and cell wall area. However, given the possible influence of HDGS and gene redundancy on these results, further investigations are required for clarification. One way of further investigating their function is to create transgenic tissue sectors using RNAi knockdown constructs of EgSND2 (SND2-) and EgSND3 (SND3-). If the absence of phenotypes in SND2+ and SND3+ were due to HDGS, then the xylem tissue sectors transformed with SND2- and SND3- may exhibit changes in either SCW thickness or SCW area or both since the orthologs of EgSND2 and EgSND3 in other species are known to regulate SCW deposition.

III. Further insights into the regulatory roles of EgKNAT7, EgMYB103, EgSND2 and EgSND3 concerning SCW formation can be gained by analysing the cell wall polymers in the ISSA-derived transgenic tissue. However, ISSA technique generates only microgram quantities of transgenic tissue, which makes it challenging to conduct chemical analyses (Spokevicius *et al.* 2016). As a part of this project, we have identified a suite of phenotyping techniques that can chemotype microgram quantities of secondary xylem tissue (Chapter 3). Therefore, chemotyping transgenic sectors generated in this study following one of these chemotyping techniques such as pyrolysis GC/MS has the potential to reveal more information on whether or how these TFs influence biosynthesis of cellulose, lignin and hemicellulose during wood formation in *Eucalyptus*.

5 FUNCTIONAL CHARACTERISATION OF POTENTIAL NOVEL REGULATORS OF WOOD AND TENSION WOOD FORMATION

5.1 INTRODUCTION

Trees are frequently challenged by their ecosystem. These challenges are imposed either by biotic factors such as other plants and animals or by abiotic factors such as water, wind, sunlight, and gravity. For example, trees are constantly required to maintain their upright growth and branch angles in the face of changing directionality of gravitational forces caused by wind, erosion, snow, load and/or slope. However, the highly dynamic nature of trees allows them to develop various mechanisms to face these challenges successfully. For instance, trees respond to the gravitational stress by forming reaction wood (RW), which generates the force required for reorientation. RW in angiosperms and gymnosperms are known as tension wood (TW) and compression wood (CW), respectively. Both TW and CW differ from normal wood (NW) and from each other in terms of their location, cell/tissue morphology and cell wall chemical composition. As an example, TW forms on the upper side of leaning stem/branches and pulls the stem/branches upwards by applying tensile forces. In contrast, CW, which forms on the lower (ground facing) side, pushes the stem/branches upwards by applying compressive forces (Zinkgraf *et al.* 2018). RW maintains tree posture by reinforcing the stem to maintain its position, maintaining branch angles to keep them in place and by reorienting branches to replace lost leaders (Du and Yamamoto 2007; Groover 2016).

In addition, TW and CW have ecological and economic significance. The formation of TW or CW has significant importance in ecology (Chave *et al.* 2009), as these RWs enable trees to adapt to different environmental conditions and serve as a sign of ecosystem disturbances such as landslides and storms (Groover 2016). RW also has an economic significance. For instance, in the timber industry RW is undesirable as it leads to warpage, has low dimensional stability and shows greater shrinkage during

drying (Plomion *et al.* 2001; Wimmer and Johansson 2014). Similarly, for pulping purposes, CW is undesirable although TW is desirable as it can be easily converted to pulp. However, the specific characteristics of the paper resulting from either TW or CW, are different from those obtained from NW (Parham *et al.* 1977). In addition, as TW is a cellulose-rich material with decreased lignin content, it serves as a good substrate for liquid biofuel production (Brereton *et al.* 2012). These biological, economic, and ecological roles are supported by the distinct morphological features and cell wall chemical compositions of RW cells/tissue.

TW exemplifies the distinctive features of RW. These features assist TW cells/tissue in generating the tensile forces required for the maintenance of stems and branches. For instance, the accelerated cell division during TW formation produces more tissue on the TW side, leading to stem/branch eccentricity, which helps in maintaining stem or branch position (Gritsch *et al.* 2015). In addition, TW can be further characterised by a reduced number of vessels, which are longer and less porous, and an increased number of more elongated fibres, with thicker secondary cell walls (SCWs) and smaller lumens (Jourez *et al.* 2001; Ruelle *et al.* 2006). In some genera like *Populus*, TW fibres are known as G fibres due to the presence of a characteristic ‘Gelatinous layer’ (G layer) which entirely or partially replaces other SCW layers (Gritsch *et al.* 2015; Nishikubo *et al.* 2007). G layers in TW are enriched with cellulose, and while xyloglucan, pectin and mannan contribute less than 10%, only trace amounts of lignin are observed (Mellerowicz and Gorshkova 2012; Nishikubo *et al.* 2007). Furthermore, TW cellulose is characterised by a higher degree of crystallinity, higher α -cellulose content and TW lignin has an altered monomer composition where the syringyl (S lignin) to guaiacyl (G lignin) subunit ratio is increased (Al-Haddad *et al.* 2013; Ma *et al.* 2013b). In contrast, opposite wood (OW) cell walls contain less cellulose, more lignin and larger microfibril angles (MFA)s compared to TW and NW (Ma *et al.* 2013b; Qiu *et al.* 2008; Ruelle *et al.* 2006). These characteristic features make TW a unique type of woody tissue.

TW is often used as a model system to study the molecular regulation of wood formation and to understand how environmental signals integrate with wood development (Almeras and Clair 2016; Mellerowicz and Gorshkova 2012; Mizrachi

et al. 2015). This molecular regulation is mainly driven by molecular controllers such as phytohormones, transcription factors (TFs) and the structural genes regulated by them (Abel *et al.* 1995; Chen *et al.* 2015; Felten *et al.* 2018; Gerttula *et al.* 2015; Seyfferth *et al.* 2018; Seyfferth *et al.* 2019). Transcriptomic studies between TW and OW and/or NW in different genera such as *Eucalyptus*, *Populus* and *Betula*, demonstrate differential expression of many TFs and some members of phytohormonal signalling pathways, which suggest their involvement in TW formation (Andersson-Gunneras *et al.* 2006; Chen *et al.* 2015; Mizrachi *et al.* 2015). Therefore, TW serves as a model to dissect into the phytohormonal and transcriptional regulation of wood formation.

Although many TFs are differentially expressed during TW formation, only some of them have been studied in some detail. Consequently, our knowledge remains sparse for most of these TFs with respect to their specific roles in regulating wood or TW formation and in defining wood or TW cell morphology and chemical composition. Many examples of such TFs are discussed in detail in Chapter 2. In the present chapter, five members representing four of such understudied TF families and/or subfamilies (INDOLE-3-ACETIC ACID INDUCIBLE (Aux/IAA or IAA), BELL1-LIKE HOMEODOMAIN (BLH), WRKY and GATA type zinc finger family WLIM) have been selected for functional investigations. These selected TF genes include *EgIAA13* (Eucgr.H02914), *EgWRKY2* (Eucgr.F01981), *EgBLH6* (Eucgr.I01293) and *EgWLIM1* (hereafter, Eucgr.B00345 will be denoted as *EgWLIM1a* and Eucgr.F02243 will be indicated as *EgWLIM1b*). Since none of these selected *Eucalyptus* TFs nor their homologs in other species have been previously characterised, they present five novel transcriptional regulators of wood and TW formation. Among them, *EgIAA13* is an important member of *Aux/IAA* family, and it exhibits differential expression during both wood (Li *et al.* 2009; Yang *et al.* 2014) and TW formation (Chen *et al.* 2015; Gerttula *et al.* 2015; Mizrachi *et al.* 2015; Yu *et al.* 2015), hinting at its potential regulatory role in xylogenesis and in its responsiveness to gravitational stresses. Similarly, *EgWRKY2*, *EgBLH6* and *EgWLIM1* TFs are also potential regulators of wood and TW formation (Andersson-Gunneras *et al.* 2006; Azri *et al.* 2014; Chen *et al.* 2015; Mizrachi *et al.* 2015; Wang *et al.* 2014). This chapter describes how we have used *in vivo* transformation experiments to unravel the function of these eucalypt TFs

by using up- and down-regulating gene constructs to investigate how they influence wood fibre and vessel morphology in *Eucalyptus* and *Populus*, aiming to identify their specific roles during xylogenesis and TW formation.

5.2 METHODOLOGY

5.2.1 Preparation of plant material, transgenic *Agrobacterium* and obtainment of transgenic tissue sectors

The steps involved in the preparation of *Eucalyptus grandis* × *camaldulensis* and *Populus alba* plant material, creation of overexpression and knockdown constructs (Table 5.1), transformation of *Agrobacterium*, inoculation of plant stems, harvesting cambial windows and detection of transgenic tissue were conducted, as described in sections 4.2.1 through to 4.2.6.

5.2.2 Investigation of xylem cell morphology and cell counts in transgenic tissue

5.2.2.1 Scanning electron microscopy

Sample preparation, imaging and examination of wood cell ultrastructure using scanning electron microscopy were conducted according to the protocol described in section 4.2.8. For the analysis of cell counts, the cell counts of five radial fibre cell lines (where available) were obtained using the same 2500× micrographs. In contrast to the method described in section 4.2.8, when taking measurements for xylem vessel cross-sectional area, the 350× micrographs were used, and an equal number of vessels were selected from each transgenic sector, and its adjacent nontransgenic tissue as some micrographs/sectors contained less than five vessels.

5.2.2.2 Light microscopy

For the light microscopic analysis of xylem fibre length, twenty sector pairs were selected, as described in section 4.2.6.1. These selected sectors were excised and macerated according to the protocol published by Spokevicius *et al.* (2016). The resulting macerated fibres were mounted on a glass slide using Entellan[®] mounting medium (Barotto *et al.* 2017) and observed under an Olympus BH-2 microscope at 10× and micrographs were acquired using a Leica DFC450 microscope camera and

LAS v4 software. From the acquired micrographs, five transgenic and five adjacent nontransgenic fibres were selected per sector pair, and their lengths were measured using ImageJ software (<https://imagej.nih.gov/ij/index.html>).

5.2.3 Statistical analysis

The average transformation efficiency (ATScm⁻²) and transformation success were calculated as described in section 4.2.7. Between 10-27 sector pairs (Table 5.1) were used for the statistical analyses across the vectors, as described in section 4.2.9.2.

Table 5.1 Number of sector pairs used for statistical analysis

Feature	Construct	Symbol	Plant species	Number of sector pairs (n)	Total number of cells/cell files [†]
Fibre area, lumen area, cell wall thickness and cell wall area	EgIAA13	IAA13+	<i>Egr</i> × <i>Eca</i>	21	210
	EgBLH6	BLH6+	<i>Egr</i> × <i>Eca</i>	21	210
	EgWRKY2	WRKY2+	<i>Egr</i> × <i>Eca</i>	18	180
	EgWLIM1a	WLIM1a+	<i>Egr</i> × <i>Eca</i>	13	130
	EgWLIM1b	WLIM1b+	<i>Egr</i> × <i>Eca</i>	18	180
	EVC+	EVC+	<i>Egr</i> × <i>Eca</i>	27	270
	EgIAA13	IAA13+	<i>Populus alba</i>	20	200
	EVC+	EVC+	<i>Populus alba</i>	17	170
	EgIAA13	IAA13-	<i>Egr</i> × <i>Eca</i>	20	200
	EVC-	EVC-	<i>Egr</i> × <i>Eca</i>	24	240
Vessel area	EgIAA13	IAA13+	<i>Egr</i> × <i>Eca</i>	20	90
	EVC+	EVC+	<i>Egr</i> × <i>Eca</i>	20	84
	EgIAA13	IAA13-	<i>Egr</i> × <i>Eca</i>	21	78
	EVC-	EVC-	<i>Egr</i> × <i>Eca</i>	24	80
Fibre length	EgIAA13	IAA13+	<i>Egr</i> × <i>Eca</i>	20	200
	EVC+	EVC+	<i>Egr</i> × <i>Eca</i>	10	100
Cell counts	EgIAA13	IAA13+	<i>Egr</i> × <i>Eca</i>	21	196 [†]
	EVC+	EVC+	<i>Egr</i> × <i>Eca</i>	15	122 [†]
	EgIAA13	IAA13-	<i>Egr</i> × <i>Eca</i>	25	212 [†]
	EVC-	EVC-	<i>Egr</i> × <i>Eca</i>	20	171 [†]

+ and - denotes overexpression and knockdown constructs, respectively. [†] denotes the number of cell files that were counted for the cell count analysis. *Egr* × *Eca* denotes *E. grandis* × *camaldulensis*.

5.3 RESULTS

5.3.1 Glasshouse conditions allowed for active plant growth

The highest average daily growth rates were observed in *Populus* (Table 5.2). Compared to *Eucalyptus*, the average daily increment of diameter in *Populus* was more than double. Similarly, the average daily increment of height was also higher in *Populus*. However, both *Eucalyptus* and *Populus* demonstrated similar xylem growth.

Table 5.2 Plant growth data for *E. grandis* × *camaldulensis* and *P. alba*

Growth data	<i>E. grandis</i> × <i>camaldulensis</i>	<i>P. alba</i>
Number of plants	35	36
Growth period (days)	75	74
Average daily height increment (cm/day)	0.78 (0.048)	1.21 (0.061)
Average daily diameter increment (mm/day)	0.04 (0.002)	0.09 (0.005)
Average daily xylem increment (mm/day)	0.02 (0.001)	0.03 (0.001)

Bold values represent respective **standard error** values. The measurements for diameter growth were taken at 10 cm stem height. The xylem increment in each xylem window was measured as the total radial growth of xylem tissue, measured from the wound site.

5.3.2 Transformation efficiency is higher in *Eucalyptus* compared to *Populus*

Transformation success rates and ATScm⁻² varied depending on construct and species. For the overexpression analysis, a total stem tissue area of 210 cm² (210 cambial windows) and 72 cm² (72 cambial windows) were inoculated and harvested from *Eucalyptus* and *Populus*, respectively. For the knockdown analysis, a total stem tissue area of 70 cm² (70 cambial windows) was inoculated and harvested from *Eucalyptus*. In addition, the transformation success (the percentage of windows with at least one transgenic sector) and ATScm⁻² values were separately calculated for each construct. A similar transformation success (about 94%) was observed for EVC+ in both species; however, the transformation efficiency (ATScm⁻²) differed as it was higher (more than double) in *Eucalyptus* compared to *Populus* (Table 5.3). Therefore, the transformation success of all the constructs was high in both species; however, *Eucalyptus* was more efficiently transformed compared to *Populus*.

Table 5.3 The ATScm⁻² and transformation success of different vectors in *Eucalyptus* and *Populus*

Transformation information	Construct	<i>E. grandis</i> × <i>camaldulensis</i>	<i>P. alba</i>
Transformation success	EVC+	94.29%	94.44%
	IAA13+	100%	88.89%
	BLH6+	100%	-
	WRKY2+	100%	-
	WLIMa+	100%	-
	WLIMb+	100%	-
	EVC-	97.14%	-
	IAA13-	97.14%	-
ATScm ⁻²	EVC+	13.74 (1.82)	6.22 (0.91)
	IAA13+	17.66 (2.03)	8.78 (1.62)
	BLH6+	18.74 (2.34)	-
	WRKY2+	21.77 (2.87)	-
	WLIMa+	22.40 (1.81)	-
	WLIMb+	23.11 (2.34)	-
	EVC-	15.46 (2.17)	-
	IAA13-	13.83 (2.03)	-

+ and - denotes overexpression and knockdown constructs, respectively. **Standard error** values are written in **bold** within brackets. The constructs that were not tested in *P. alba* are indicated by a hyphen (-).

5.3.3 EgIAA13 alters *Eucalyptus* xylem cell morphology

To date, only limited evidence links *EgIAA13*, *EgBLH6*, *EgWRKY2*, *EgWLIM1(a)* or *EgWLIM1(b)* to wood and TW formation based on their differential expression in TW and OW/NW, respectively (Andersson-Gunneras *et al.* 2006; Azri *et al.* 2014; Chen *et al.* 2015; Mizrachi *et al.* 2015; Wang *et al.* 2014; Yu *et al.* 2015). To determine their potential function in these processes, we conducted *in vivo* transformation experiments (ISSA; Spokevicius *et al.* (2016)) using overexpression constructs of these TFs to determine how they affect xylogenesis and xylem cell morphology in woody plants.

Six overexpression constructs (IAA13+, BLH6+, WRKY2+, WLIM1(a)+ and WLIM1(b)+) were transformed into stems of *Eucalyptus*. The subsequent analysis of transformed xylem fibres revealed that BLH6+, WRKY2+, WLIM1(a)+ and WLIM1(b)+ did not reveal any significant alterations in xylem fibre cross-sectional area, lumen area, cell wall thickness and cell wall area (Fig. 5.4). In contrast, IAA13+ demonstrated significant changes ($p < 0.05$) in transformed *Eucalyptus* xylem fibres and vessels (Fig. 5.1). Compared to the EVC+, the fibres transformed with IAA13+ were smaller, where cell wall thickness, cell wall area, lumen area, fibre length and cross-sectional area were reduced. Relative to the EVC+, xylem fibres transformed with IAA13+ demonstrated average reductions in cell wall thickness by $0.18\ \mu\text{m}$ ($p = 0.02$), cell wall area by $9.25\ \mu\text{m}^2$ ($p = 0.01$), lumen area by $14.72\ \mu\text{m}^2$ ($p = 0.003$), fibre length by $76.78\ \mu\text{m}$ ($p = 0.05$) and cross-sectional area by $21.99\ \mu\text{m}^2$ ($p = 0.002$) (Fig. 5.1a-d and f). However, no changes were observed in the cross-sectional area of the xylem vessels transformed with IAA13+ (Fig. 5.e). As expected, transformation with EVC+ did not change xylem fibre and vessel morphology.

In certain instances, silencing of the transgene and the native gene has been observed, as a result of the high sequence homology between the transgene and the native gene. This phenomenon known as Homology Dependent Gene Silencing (HDGS) was first reported in petunia when transformation with an overexpression construct containing *CHALCONE SYNTHASE* gene resulted in a decrease in the expression of both introduced transgene and the native gene (Napoli *et al.* 1990; van der Krol *et al.* 1990). Later on, this phenomenon has been reported in various studies in plants, including previous ISSA experiments (Baucher *et al.* 1996; Moller *et al.* 2003; Spokevicius *et al.* 2007a; Tsai *et al.* 1998). Therefore, we conducted a knockdown experiment using IAA13- in eucalypts to test whether the observed *EgIAA13* phenotypes were indeed related to overexpression or HDGS.

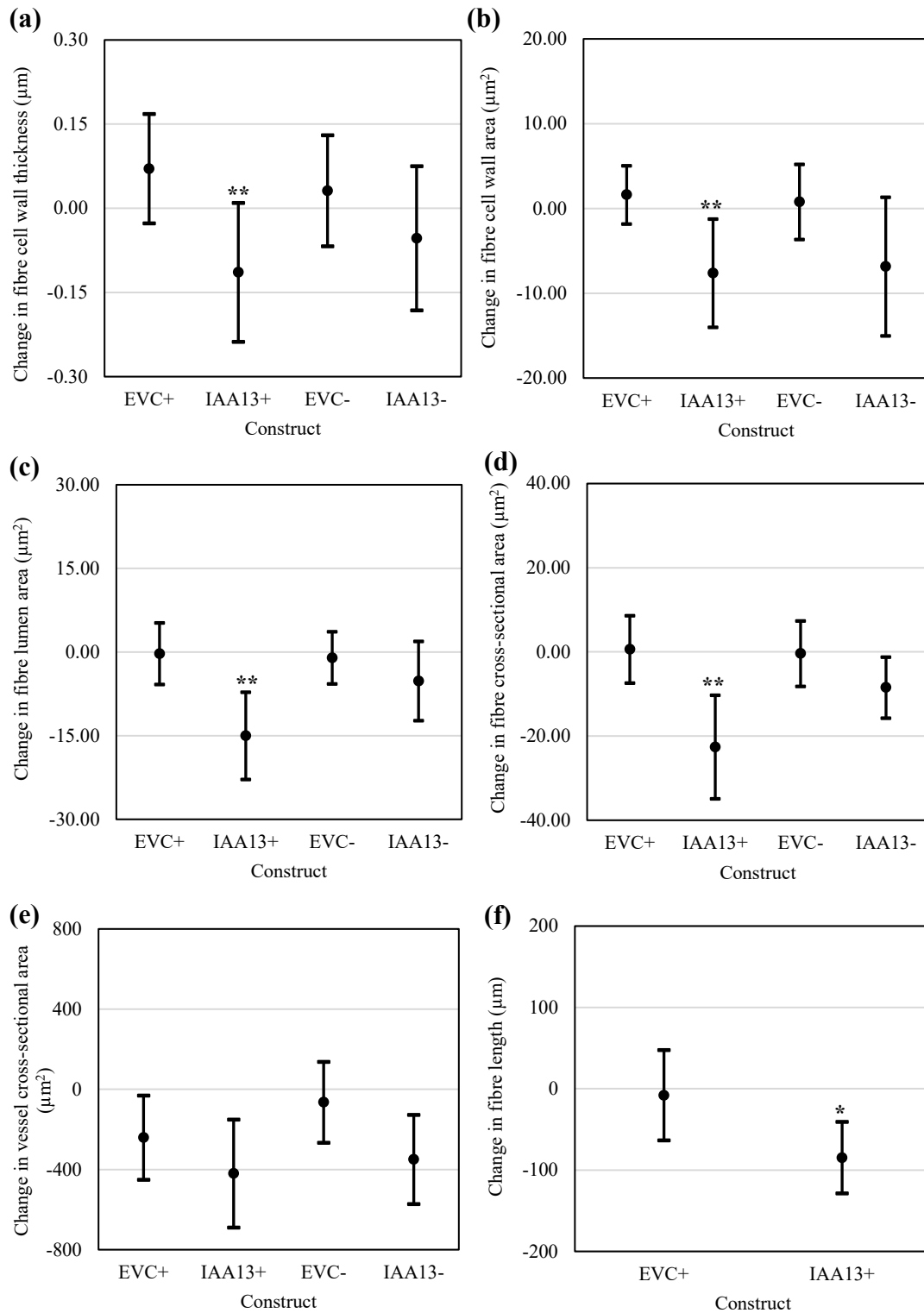


Figure 5.1 Comparison of average changes in xylem (a) fibre cell wall thickness, (b) fibre cell wall area, (c) fibre lumen area, (d) fibre cross-sectional area, (e) vessel cross-sectional area and (f) fibre length of *E. grandis* $\times$ *camaldulensis* xylem cells transformed with overexpression (+) and knockdown (-) constructs of EgIAA13. The black dots represent the mean value. Error bars represent 95% confidence intervals. ** denotes $p \leq 0.005$, * denotes $p \leq 0.05$

Compared to EVC-, the xylem fibres and vessels transformed with IAA13- displayed near-significant average reductions in fibre cell wall area by $7.62 \mu\text{m}^2$, fibre cross-sectional area by $8.06 \mu\text{m}^2$ and vessel cross-sectional area by $284.99 \mu\text{m}^2$ ($p = 0.06$) (Fig. 5.1a,d and e). No changes were observed in the morphology of xylem fibres and vessels transformed with EVC-. Since these observations are similar to those observed by IAA13+, presumably the phenotypes observed for IAA13+ are related to HDGS rather than to overexpression.

5.3.4 EgIAA13 potentially influence *Eucalyptus* xylem cell division

Previously, Xu *et al.* (2019) reported that overexpression of a stabilised mutant form of *PtoIAA9* decreases the number of xylem cell layers, fibre area and vessel area in *Populus*. They speculated these alterations as the results of *PtoIAA9* mediated repression of xylem division and expansion stages. Since this study demonstrated how Aux/IAA TFs could influence multiple stages involved in wood formation, we presumed EgIAA13 also might be involved in regulating multiple stages. In addition, the observed smaller xylem fibre and vessel sizes could be attributed to an altered cell division at the vascular cambium. To examine whether EgIAA13 can affect xylem division, we conducted an analysis to compare the number of cells within a $110 \mu\text{m}$ distance (an area of $166 \times 110 \mu\text{m}^2$), in IAA13+ or IAA13- transformed tissue. This analysis revealed that the average number of fibres in IAA13+ transgenic tissue was 0.8 cells greater ($p = 0.06$) compared to EVC+ (Fig. 5.2). Similarly, the average number of fibres in IAA13- transgenic tissue was 0.1 cells greater compared EVC- (Fig. 5.2). Since the average new xylem growth in *Eucalyptus* during the entire period of the experiment was 1.5 mm (Table 5.1), the changes in the number of cells per cell file in the new xylem could be approximated to about seven cells for IAA13+ and to about three cells for IAA13-. Therefore, transformation with IAA13+ or IAA13- increases the rate of xylem cell division at the vascular cambium, as demonstrated by the presence of more cells in a given area with xylem fibres transformed with IAA13+ or IAA13-.

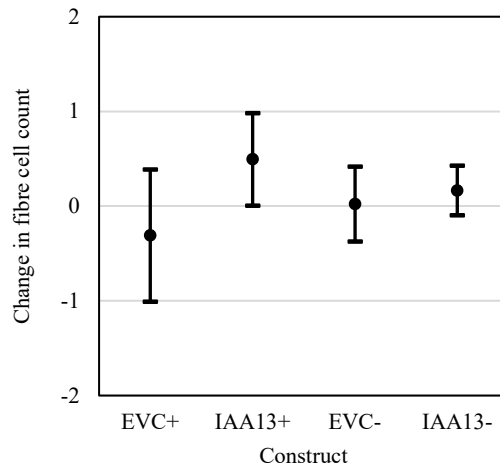


Figure 5.2 Comparison of average changes in the number of fibre cells in a radial cell file of *E. grandis* × *camaldulensis* xylem transformed with IAA13+ and IAA13-. The black dots represent the mean value. Error bars represent 95% confidence intervals.

5.3.5 EgIAA13+ does not alter *Populus* xylem fibre morphology

Similarly, the IAA13+ vector was transformed into stems of *Populus alba*. In contrast to what was observed in *Eucalyptus*, transformation with IAA13+ did not change *Populus* xylem fibre morphology in terms of cell wall thickness, cell wall area, cross-sectional area, and lumen area (Fig. 5.3). This observation leads to the conclusion that transformation with IAA13+ influences xylem fibre morphology in *Eucalyptus* but not in *Populus*. Also, similar to the overexpression analysis in *Eucalyptus*, the EVC+ did not alter *Populus* xylem fibre morphology.

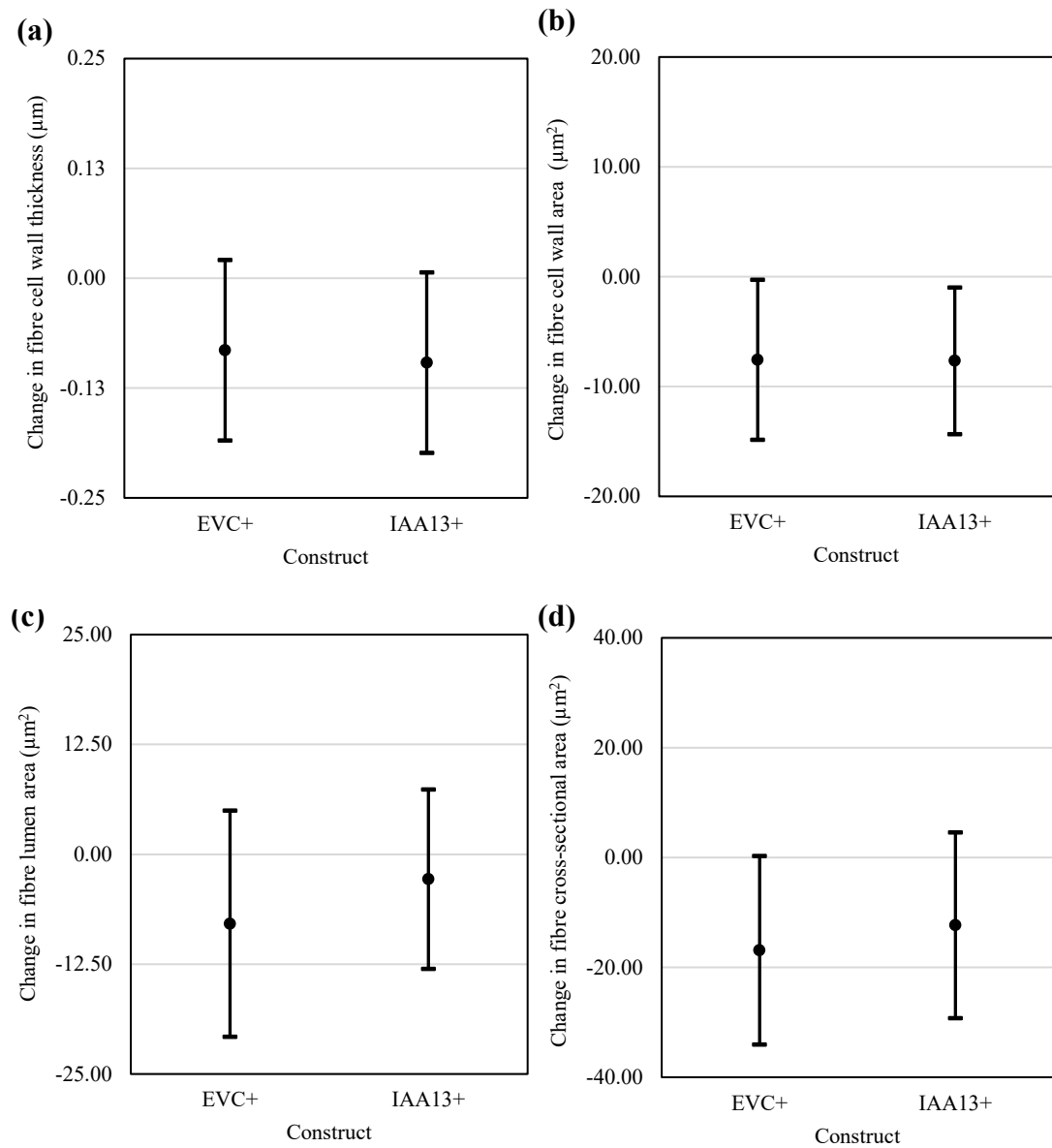


Figure 5.3 Comparison of average changes in (a) cell wall thickness, (b) cell wall area, (c) lumen area and (d) cross-sectional area of *Populus alba* xylem fibres transformed with overexpression construct of EgIAA13. The black dots represent the mean value. Error bars represent 95% confidence intervals

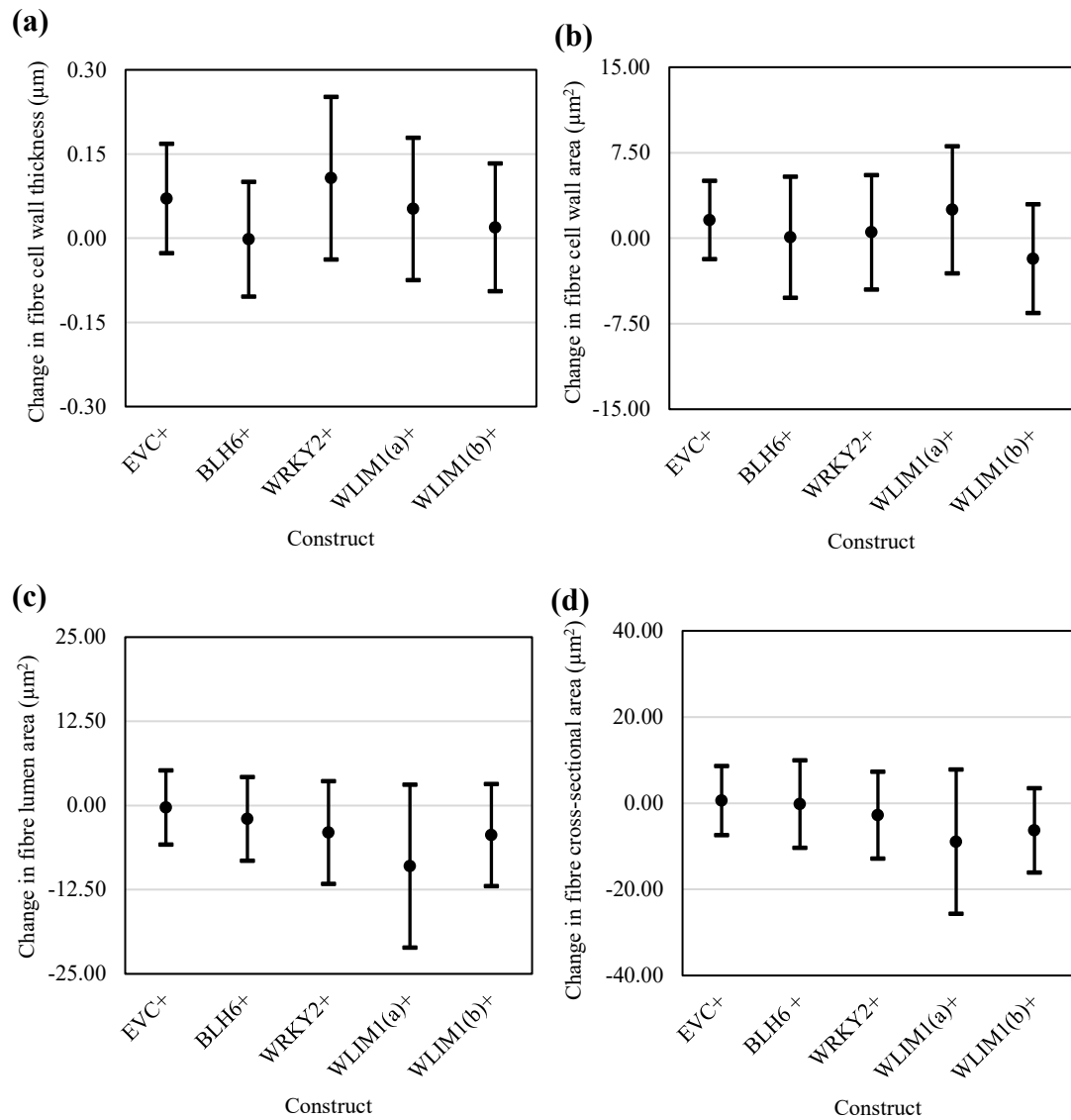


Figure 5.4 Comparison of average changes (a) cell wall thickness, (b) cell wall area, (c) lumen area and (d) cross-sectional area of *E. grandis* $\times$ *camaldulensis* xylem fibres transformed with BLH6+, WRKY2+, WLIM1(a)+ or EgWLIM1(b)+. The black dots represent the mean value. Error bars represent 95% confidence intervals.

5.4 DISCUSSION

5.4.1 EgIAA13 influences xylem fibre expansion and elongation

EgIAA13 undergoes significant changes in its expression during xylem development and in response to stem bending. As an example, in *Arabidopsis*, *AtIAA13* is highly expressed in stems (Abel *et al.* 1995). In *Eucalyptus*, *EgIAA13* is preferentially and highly expressed in vascular tissue, and its expression in juvenile xylem is higher compared to mature xylem implying its involvement in the development of xylem tissue (Yu *et al.* 2015). In addition, transcript levels of *EgIAA13* are lower in *Eucalyptus* TW compared to NW (NW > OW > TW) highlighting the importance of adjusting its expression levels to facilitate TW formation (Mizrachi *et al.* 2015; Yu *et al.* 2015). Similarly, transcript levels of the *Populus IAA13* homolog (POPTR_0008s17220) were lower in TW compared to NW (Chen *et al.* 2015). Another *IAA13* homolog in *Populus* (Potri.010G065200) was found to be downregulated in wildtype *Populus tremula* × *tremuloides* and in *ethylene receptor1* mutant line upon application of exogenous ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) suggesting a possible response in auxin signalling to changing levels of ACC (Felten *et al.* 2018). Although these authors did not link changing transcript levels of *IAA13* to ethylene signalling, *IAA13* could be a possible mediator in the crosstalk between auxin and ethylene. Therefore, *EgIAA13* is an important candidate for further studies into auxin-mediated regulation of wood and TW formation.

Our results demonstrate that *EgIAA13* influence xylem fibre and vessel development in *Eucalyptus*. Transformation with *IAA13+* substantially reduced *Eucalyptus* xylem fibre expansion, elongation and SCW deposition. The *Eucalyptus* xylem cells transformed with *IAA13-* also showed a reduction in both xylem fibre and vessel expansion, similar to *IAA13+*. Therefore, we speculate that *EgIAA13* is potentially involved in regulating the expansion and elongation stages of wood formation. Furthermore, smaller xylem fibres are one of the characteristic features of TW, and since previous studies have reported lower transcript levels of *EgIAA13* in TW (Chen *et al.* 2015; Mizrachi *et al.* 2015; Yu *et al.* 2015), we hypothesise a connection between

EgIAA13 and this characteristic TW feature. Therefore, EgIAA13 is a potential regulator of both wood and RW formation in *Eucalyptus*.

ISSA studies that use overexpression vectors in a native genetic background are prone to co-suppression events like HDGS (Spokevicius *et al.* 2007a). Although RNA-based analysis is the most common approach of determining the expression levels of a transgene, in ISSA, it is challenging due to the destructive preparation of samples for GUS staining. In the present study, we addressed this issue by comparing the phenotypes generated from IAA13⁺ and IAA13⁻. Since similar phenotypes were observed for both constructs, this approach could validate that the phenotypes generated by IAA13⁺ are most likely to be resulting from a co-suppression event like HDGS.

5.4.2 EgIAA13 influences xylem cell division

Our results also demonstrate that EgIAA13 influence xylem fibre division/density in *Eucalyptus*. In *Eucalyptus* transformation with IAA13⁺ substantially increased the number of xylem fibres and similarly, the xylem fibres transformed with IAA13⁻ also showed an increase in fibre count. Therefore, EgIAA13 is potentially involved in the regulation of xylem fibre division/density. However, it is also plausible that this increased cell count is a result of another cambial mechanism/signalling, which may function as a downstream feedback loop to compensate for the smaller cells by producing more cells. In ISSA, either side of a transgenic sector contains normal xylem tissue (non-transformed) in which the xylem cell sizes are not altered. Therefore, when the transgenic sector produces smaller cells (in the case of IAA13), it is plausible that the vascular cambium generates more cells to maintain its uniformity. Furthermore, this increased cell divisions/density is another characteristic feature of TW. Since previous studies report a downregulation of *EgIAA13* during TW formation, we hypothesise another potential connection between EgIAA13 and TW where EgIAA13 could be involved in regulating the increased xylem division during TW formation. Therefore, we speculate EgIAA13 as a potential regulator of xylem cell division during both wood and RW formation in *Eucalyptus*.

To date, only a couple of *Eucalyptus* and *Populus* Aux/IAAs have been functionally characterised during wood formation. One of these Aux/IAAs is EgIAA4 where the overexpression of a stabilised version of *EgIAA4* in *Arabidopsis*, lead to lateral root appearance, gravitropism, inhibition of primary root elongation, delayed lignification of interfascicular fibres and inhibited lignification in xylary fibres (Yu *et al.* 2015). The second Aux/IAAs is PtoIAA9. Xu *et al.* (2019) reported that the overexpression of a stabilised version of *PtoIAA9* in *Populus* curbs secondary xylem development. This study also demonstrated a dual role of PtoIAA9 where the overexpression of *PtoIAA9* inhibited both xylem cell division and expansion in *Populus* (Xu *et al.* 2019). Therefore, in addition to presenting very first insights into how EgIAA13 alters xylem development in *Eucalyptus*, our study also supports the idea presented by Xu *et al.* (2019) by demonstrating that another Aux/IAA potentially influences wood formation at two different stages (xylem division and expansion/elongation).

During xylem cell development, auxin signalling influences cell division and the duration of expansion, both of which are critical determinants of xylem cell density and final xylem cell size (Denne and Dodd 1981; Majda and Robert 2018; Mellerowicz *et al.* 2001). Therefore, EgIAA13 mediated regulation of wood and RW formation in *Eucalyptus* presumably occurs via attenuation of auxin signalling. In auxin signalling pathway, Aux/IAAs mediate their downstream regulation via heterodimerisation with ARFs (Hamann *et al.* 2002; Krogan *et al.* 2014; Xu *et al.* 2019; Yamauchi *et al.* 2019). Therefore, specific EgIAA13-EgARF module/s could be responsible for mediating xylem cell division and expansion stages (further discussed in Chapter 6).

According to our EgIAA13 study in *Eucalyptus*, the smaller xylem cell and increased cell division phenotypes are caused by the downregulation of *EgIAA13*. Molecular studies have shown that Aux/IAAs form heterodimers with ARFs and that resulting Aux/IAA-ARF modules are responsible for auxin-mediated transcriptional regulation of auxin-responsive genes (Luo *et al.* 2018). Some ARFs function as transcriptional activators while the others function as transcriptional repressors and therefore, the dimerisation between Aux/IAA and ARF prevents ARF from activating/repressing the transcription of auxin-responsive genes. However, at high auxin concentrations Aux/IAAs are ubiquitinated by TRANSPORT INHIBITOR RESPONSE 1/AUXIN

SIGNALLING F-BOX (TIR1/AFB) receptors and are subsequently subjected to 26S proteasome mediated degradation, releasing ARFs, which can then regulate the expression of auxin-responsive genes (Parry *et al.* 2009; Weijers *et al.* 2005). If our EgIAA13 phenotypes are indeed mediated by specific EgIAA13-EgARF module/s, then the downregulation of *EgIAA13* would result in more free EgARF/s in the system, which can then either activate or repress the downstream target gene/s depending on the activator or repressor nature of the respective EgARF. Furthermore, if all or most of the corresponding EgARF proteins are already bound by native EgIAA13, then overexpression of *EgIAA13* may not cause a change in the system. Thus, the overexpression of *EgIAA13* may not produce a phenotype, which could be one possible explanation for not observing a phenotype from *Populus* given that the intended *EgIAA13* overexpression has presumably occurred in *Populus* since HDGS is unlikely to occur in a heterologous genetic background. However, it is also possible that other reasons such as incorrect assembly of the protein, post-translational modifications, quality control procedures and incorrect localisation into sub-cellular compartments might have influenced the heterologous gene expression (Ghag *et al.* 2016; Kohli *et al.* 2006; Vyacheslavova *et al.* 2012).

5.4.3 BLH6+, WRKY2+ and WLIM1+ do not alter *Eucalyptus* xylem fibre morphology

Since most of the members of BLH, WRKY and WLIM TF families/subfamilies have not been investigated in any woody genera with respect to their function in wood and TW formation, we conducted functional investigations for four TFs from these families/subfamilies: EgBLH6, EgWRKY2, EgWLIM1(a) and EgWLIM1(b). Some members of these TF subfamilies are differentially expressed during wood and TW formation. For instance, the *Populus* ortholog of *EgBLH6* (POPTR_0004s16660, AT4G34610) is downregulated in TW (Chen *et al.* 2015) and therefore it could be a potential regulator of TW formation (Gerttula *et al.* 2015). In addition, in *Populus*, the ortholog of *EgWRKY2* (AT5G56270) was found to be upregulated in TW (Andersson-Gunneras *et al.* 2006). In contrast, according to Gerttula *et al.* (2015), *WRKY2* expression is lower in TW. Similarly, certain members of the *WRKY* TF family, show preferential upregulation in *Arabidopsis* and *Vitis vinifera* xylem where they regulate processes such as lignification and xylem formation (Guillaumie *et al.* 2010; Ko *et al.*

2004). Furthermore, in *Populus*, the ortholog of *EgWLIM1* (AT1G10200, Potri.009G087200) is upregulated in TW (Andersson-Gunneras *et al.* 2006; Mauriat *et al.* 2015). Likewise, the other members of the *WLIM* TF family also show preferential expression in secondary xylem and TW suggesting a potential role in wood formation and in its responsiveness to mechanical stresses (Chen *et al.* 2015). Since, all these TFs are understudied in woody genera, they present potential novel regulators to further investigate the transcriptional regulation of wood and TW formation.

As suggested by their tissue-specific expression patterns, the homologs of *EgBLH6*, *EgWRKY2*, *EgWLIM1(a)* and *EgWLIM1(b)* TFs in other plants have demonstrated a commitment towards the biosynthesis of SCW biopolymers. As an example, BLH6 is known as a regulator of cell wall biosynthesis (Laubscher *et al.* 2018). According to Cassan-Wang *et al.* (2013), in *Arabidopsis*, 60% of the 50 genes that are co-expressed with *AtBLH6*, are related to cellulose, xylan and lignin biosynthesis and they speculate *AtBLH6* as an activator of the biosynthesis of all three SCW biopolymers. In contrast, Liu *et al.* (2014) suggest that *AtBLH6* function as a repressor of SCW deposition, where its interaction with *AtKNAT7* further enhances this repressor role. Although *EgBLH6* is a potential regulator of SCW deposition, based on the studies mentioned above, it is difficult to predict whether *EgBLH6* is an activator or a repressor explicitly. However, depending on its activator or repressor role, transformation with *BLH6+* would presumably result in either an increase or decrease in cell wall thickness, respectively. In addition, in *Vitis vinifera*, overexpression of *VvWRKY2* decreased S:G lignin ratio, and therefore, it is a potential regulator of lignin biosynthesis and lignin composition (Guillaumie *et al.* 2010). In *Populus*, *PtrWRKY19* is known to function as a negative regulator of lignin biosynthesis (Yang *et al.* 2016). Although WRKY TFs are understudied with respect to wood and TW formation, based on available information WRKY2 is a potential negative regulator of lignin composition or lignin biosynthesis or both. Therefore, presumably, the transformations with *WRKY2+* would result in a decrease in cell wall thickness or a decrease in S:G ratio or both.

Similarly, *WLIM* TFs are also known to regulate lignin biosynthesis (Chen *et al.* 2015; Dharmawardhana *et al.* 2010; Kawaoka *et al.* 2000; Rogers and Campbell 2004). During TW formation in *Populus*, *WLIM* TFs are often co-expressed with lignin

biosynthetic genes such as *PHENYLALANINE AMMONIA-LYASE* (*PAL*), *4-COUMARATE:COA LIGASE* (*4CL*), *CINNAMATE 4-HYDROXYLASE* (*C4H*) *HYDROXYCINNAMOYL-COA*, *CINNAMOYL-COA REDUCTASE* (*CCR*) and *SHIKIMATE/QUINATEHYDROXYCINNAMOYLTRANSFERASE* (*HCT*), showing the potential regulatory role of *WLIM* TFs during lignin biosynthesis (Chen *et al.* 2015). According to Chen *et al.* (2015), although lignin biosynthetic genes such as *Pt-PAL1* to *Pt-PAL4*, *Pt-HCT1*, *Pt-4CL3* and *Pt-4CL5* are positively regulated by POPTR_0002s11880 (Pt-LIM1), they are negatively regulated by POPTR_0014s01590 (Pt-LIM2). The *WLIM* TFs that were used in our study, EgWLIM1(a) and EgWLIM1(b) share equal levels of sequence similarities with both Pt-LIM1 and Pt-LIM2. Therefore, it is difficult to precisely predict activator or repressor roles for EgWLIM(a) and EgWLIM1(b) with respect to lignin biosynthesis, however, both of these TFs are likely to influence lignin biosynthesis either positively or negatively. Therefore, transformations with WLIM(a)+ and WLIM(b)+ would presumably alter cell wall thickness depending on their positive or negative regulatory role in lignin biosynthesis. However, in the present study, xylem fibres transformed with BLH6+, WRKY2+, WLIM1(a)+ or WLIM1(b)+ did not demonstrate any changes in their morphology in terms of cell wall thickness, cell wall area, cross-sectional area and lumen area.

The lack of phenotypes from transformation with BLH6+, WRKY2+, WLIM1(a)+ and WLIM1(b)+ could be due to several reasons. Although it is possible that the *Eucalyptus* gene accessions used in this study, do not alter above fibre properties, it is also possible that HDGS and gene redundancy might have influenced our results. Although HDGS is more likely to explain a phenotype which is opposite to the expected phenotype, the combinatorial effect of HDGS and gene redundancy may result in no changes in the phenotype of interest as the function of the TF which has been silenced by HDGS could be complemented by one of its functional homologs (gene redundancy). This combinatorial effect of HDGS and gene redundancy could lead to a lack of an apparent phenotype either from a positive regulator (activator) or a negative regulator (repressor). A similar case has been reported in a previous study where downregulation of *AtSND1* (by RNAi knockdown) did not demonstrate the expected change in cell wall thickness since the decrease in *AtSND1* expression was

compensated by its functional homolog *AtNST1* (Zhong *et al.* 2007). However, as expected, the simultaneous knockdown of both *AtSND1* and *AtNST1* impaired SCW formation in fibres, corroborating that these two TFs are functionally redundant in regulating SCW formation in xylem fibres. A similar simultaneous knockdown approach of our candidate TFs and their potential homologs would, therefore, be useful to decipher whether gene redundancy has influenced our results. In addition, the overexpression of a TF may not always lead to activation or repression of its target genes due to reasons including lack of interaction partners (like the dependency of Aux/IAA on ARFs to mediate specific downstream target genes) or potential saturation of interaction partners (if the overexpression is too strong). Therefore, if the function of EgBLH6, EgWRKY2, EgWLIM1(a) or EgWLIM1(b), depends on such interaction partners in the system, then depending on the nature of such interactions, the expected phenotypes may not be observed. To date these TFs have not been studied in detail in terms of their interactions with other TFs; therefore, although speculations on such interactions cannot be made with certainty, given the complex nature of transcriptional regulation such possibilities cannot be negated.

It is also plausible that these TFs influence cell wall chemical composition in terms of S:G lignin ratio or the ratio between lignin, cellulose, and hemicellulose polymers or both. Such changes in the composition may not always be reflected as an overall change in the cell wall thickness. Therefore, semi quantification and/or analysis of polymer distribution on cell walls could be useful to unravel whether or how these TFs affect cell wall chemical composition. However, this has not been investigated in the present study since an established cell wall chemotyping platform does not currently exist for ISSA derived transgenic tissue as it is only generated in microgram quantities (micro-samples). Nevertheless, a suit of microanalytical chemotyping techniques (Chapter 3) including pyrolysis GC/MS, could be investigated for their suitability to chemotype ISSA derived micro-samples, to gain insights into the role of these TFs in determining SCW polymer composition.

5.5 CONCLUSIONS, PERSPECTIVES AND FUTURE WORK

- I. Downregulation of *EgIAA13* alters the morphology of *Eucalyptus* xylem vessels and both morphology and density of xylem fibres. Therefore, *EgIAA13* is a potential regulator of xylem cell division, SCW deposition, expansion, and fibre elongation. Wood density, cell wall thickness, fibre and vessel dimensions are of significant importance to solid wood properties as well as to pulp and paper industries (Chen and Evans 2005; Evans *et al.* 2000; Kibblewhite *et al.* 2004; Li *et al.* 2012b). Wood density determines the biomass of tree stems, their capacity for carbon storage and the stiffness of resulting wood (Dungey *et al.* 2006; Evans and Ilic 2001; Ketterings *et al.* 2001). In addition, wood properties such as wood density, cell wall chemical composition, cell wall thickness, cross-sectional area and length strongly influence the pulp yield, digestibility and quality (Ona *et al.* 2001; Ramirez *et al.* 2009). Since xylem fibres are the central structure of paper, fibre properties determine the potential for strength development, opacity, density, and porosity of the resulting paper. As an example, fibre length affects the properties related to paper strength (Ek *et al.* 2009; Mansfield and Weineisen 2007) as increasing fibre length positively influences fibre flexibility (Via *et al.* 2004). Generally, the length and width of typical hardwood fibres range between 0.7-1.6 mm and 20-40 μm , respectively (Atchison 1987). However, compared to other hardwood species, *Eucalyptus* fibres are generally smaller, and their length and width range between 0.6-0.8 mm and 15-17 μm , respectively (Clarke *et al.* 2008). In the present study, the observed changes in fibre cell wall thickness, diameter and length correspond to about two-fold, six-fold, and eleven-fold reductions, respectively. Therefore, since *EgIAA13* can alter xylem cell density, cell size and fibre length, it is a potential candidate for genetically manipulating xylem fibre properties to improve the quality of wood and to make better suited raw materials for pulp and paper industries.
- II. Further investigations can be conducted to verify that the overexpression of *EgIAA13* resulted in HDGS. One potential ISSA-based approach is to use an overexpression construct of *EgIAA13* which is tagged with a nuclear-targeted

fluorescent protein. The absence of a fluorescent signal in transformed tissue sectors can be considered as an indication of HDGS which impedes transgenic EgIAA13 from being expressed.

- III. Overexpression constructs of EgWRKY2, EgBLH6, EgWLIM(a) and EgWLIM(b) in *Eucalyptus* did not alter xylem fibre morphology in terms of fibre cross-sectional area, lumen area, cell wall thickness and cell wall area. However, given the possible interference from HDGS and gene redundancy, further investigations are required to clarify the involvement of these TFs in wood formation. One approach for further investigating is the transformation of *Populus* cambial initials with these overexpression constructs of *Eucalyptus* TFs. Since HDGS and gene redundancy is unlikely to influence the expression of a gene in a heterologous genetic system, the *Populus* xylem cells transformed with these overexpression constructs may demonstrate changes in SCW thickness/area, and they may also exhibit TW features since orthologs of WRKY2, BLH6 and WLIM in other species are known to regulate the deposition of SCW biopolymers and also to be differentially expressed during TW formation. Another approach is the generation of transgenic tissue sectors using RNAi knockdown constructs of EgWRKY2 (WRKY2-), EgBLH6 (BLH6-), EgWLIM(a) (WLIM(a)-) and EgWLIM(b) (WLIM(b)-). If the absence of phenotypes in WRKY2+, BLH6+, WLIM(a)+ and WLIM(b)+ were due to HDGS, then the xylem tissue sectors transformed with WRKY2-, BLH6- and WLIM- may exhibit SCW phenotypes or TW features or both.
- IV. Chemotyping the ISSA-derived transgenic wood samples would be useful to elucidate whether EgIAA13, EgWRKY2, EgBLH6, EgWLIM(a) and EgWLIM(b) TFs regulate the biosynthesis of SCW biopolymers including cellulose, hemicellulose, and lignin.

6 THE ROLE OF EgIAA13 IN AUXIN SIGNALLING DURING WOOD AND REACTION WOOD FORMATION

6.1 INTRODUCTION

Auxin is a crucial phytohormonal regulator of plant growth, development, and adaptation. Discovery of auxin marks the discovery of one of the first plant phytohormones, which dates to the mid to late nineteenth century when Charles and Francis Darwin discovered a mobile signal that can mediate plant tropism (Darwin 1880). Later, this mobile signal was termed auxin, which is a derivative of the Greek word auxein meaning ‘to grow’ or ‘to expand’. Only several decades later, auxin was chemically recognised as Indole-3-Acetic Acid (Went and Thimann 1937). Auxin functions as a regulator of a variety of biological processes including embryogenesis, root development, fruit development, tropic responses and differentiation and development of vascular tissue (Yu *et al.* 2015; Yu *et al.* 2014).

Auxin concentration plays a central role in maintaining vascular cambium activity, development of secondary xylem and its responsiveness to bending. Involvement of auxin in promoting cambial activity and development of secondary xylem has been exemplified by an auxin concentration gradient across the cambium and developing secondary xylem, in which auxin concentration peaks at the cambial cell division/differentiation region and gradually decreases towards the region where secondary cell wall (SCW) deposition takes place (Bhalerao and Bennett 2003; Nilsson *et al.* 2008). This auxin gradient supports cambial division and differentiation, as well as the duration of xylem cell expansion (Brackmann *et al.* 2018; Mellerowicz *et al.* 2001; Tuominen *et al.* 1997). Similarly, in response to gravitropic stress in angiosperm trees, a surge of auxin is applied on to the upper (tension) side of the cambium demonstrating the involvement of auxin in the induction of tension wood (TW) formation (Gerttula *et al.* 2015). Consequently, auxin flux between cambial and vascular tissues/cells is vital to ensure the active state of the vascular cambium and the proper development of secondary xylem and TW.

Auxin flux between plant tissues, cells and cellular organelles involves different pathways and transporters. The majority of the auxin produced at the stem apex is transported to sink tissues such as the vascular cambium via the phloem (Petrasek and Friml 2009). Also, short distance, directional auxin transport, as an example transport of auxin from phloem to the vascular cambium and transport of auxin between xylem cells occurs via polar auxin transport, facilitated by auxin influx and efflux transporters (Goldsmith 1977). As an example, AUXIN TRANSPORTER PROTEIN 1 (AUX1) regulates the cellular auxin influx while PIN-FORMED1 (PIN1), P-GLYCOPROTEIN1 (PGP1) and PGP19 regulate cellular auxin efflux (Geisler *et al.* 2005; Krecek *et al.* 2009; Paciorek and Friml 2006; Ragni and Greb 2018). In addition, PIN5 and PIN8 regulate auxin influx and efflux from the endoplasmic reticulum (ER), respectively (Middleton *et al.* 2018). Although the nucleus takes up some auxin via diffusion through nuclear pores, the major subcellular pathway for nuclear auxin uptake is the ER-to-nucleus flux (Middleton *et al.* 2018). Therefore, irrespective of the developmental process (maintaining cambial activity or development of wood or TW), auxin signalling ultimately results in auxin perception by the nucleus (either in cambial cells or in xylem cells).

Inside the nucleus, the perceived auxin signal triggers auxin-mediated cellular transcriptional responses. Auxin that enters the nucleus binds to the TRANSPORT INHIBITOR RESPONSE 1 (TIR1)/F-BOX PROTEINS (AFB) auxin receptor (Dharmasiri *et al.* 2005; Parry *et al.* 2009). The resulting auxin-TIR1/AFB receptor binds INDOLE-3-ACETIC ACID INDUCIBLE (Aux/IAA) proteins which leads to ubiquitination and subsequent proteasome-mediated degradation of Aux/IAA, resulting in the release of AUXIN RESPONSE FACTOR (ARF) proteins which are now free to interact either with other ARF proteins or with the Auxin Responsive Element (AuxRE) on the promoter of target genes. These interactions lead to either activation or repression of target genes depending on the activator or repressor nature of the interacting ARF (Tiwari *et al.* 2003; Yu *et al.* 2014). As an example, in *Populus*, PtoIAA9 proteins bind to PtoARF5; however, in the absence of PtoIAA9, PtoARF5 can bind to AuxREs in the promoters and activate *HOMEODOMAIN (HB)* genes such as *CLASS III HOMEODOMAIN LEUCINE ZIPPER (HD-ZIP III)* genes. Therefore, the PtoIAA9/PtoARF5 module regulates wood formation in *Populus* via regulating the

expression of *HD-ZIPs*, which are well known to induce differentiation of cambial cells into the xylem (Xu *et al.* 2019). Likewise, cellular auxin signalling in the vascular cambium or in xylem is ultimately converted into transcriptional responses via Aux/IAA-ARF modules.

Therefore, in addition to changes in auxin concentration, the auxin-mediated regulation of vascular cambial activity, development of secondary xylem and TW also involve changes in the auxin-related transcriptome, including up- or downregulation of members of the auxin signalling pathway (*Aux/IAA* and *ARF*) and their downstream target genes (Esmon *et al.* 2006; Schrader *et al.* 2004; Uggla *et al.* 2001; Uggla *et al.* 1998). Besides maintaining cambial activity, auxin is also responsible for its inactivation, since auxin regulates cambial dormancy which is exclusively driven via changes in the auxin-related transcriptome (Baba *et al.* 2011). Furthermore, *Aux/IAAs* and *ARFs* demonstrate tissue-specific expression patterns suggesting their potential involvement in imparting auxin-mediated regulation. As an example, *EgIAA13*, *EgIAA4*, *PoptrIAA3.1*, *PoptrIAA3.2*, *PoptrIAA3.2-like*, *PoptrARF7.3*, *PttIAA8* and *PtoIAA9* are preferentially expressed in wood forming tissues, and *PoptrARF7.3*, *EgIAA3*, *EgIAA13*, *EgIAA31*, *EgARF3*, *EgARF4*, *EgARF6A*, *EgARF9A* and *EgLAX2* are differentially expressed in TW, opposite wood (OW) and/or normal wood (NW) (Andersson-Gunneras *et al.* 2006; Kalluri *et al.* 2007; Mizrachi *et al.* 2015; Moyle *et al.* 2002; Paux *et al.* 2005; Xu *et al.* 2019; Yu *et al.* 2015; Yu *et al.* 2014). Therefore, although auxin has been previously proposed as a morphogen, recent evidence suggests that auxin-mediated regulation of vascular tissues is more likely to be exerted through the changes taking place in the auxin-related transcriptome (Nilsson *et al.* 2008; Sundell *et al.* 2017). Even though auxin is the principal phytohormonal regulator of cambial activity, wood and TW formation, our understanding of auxin-related transcriptomes behind these processes remain sketchy (Smetana *et al.* 2019; Yu *et al.* 2014).

To date, specific functions have been determined for only a few Aux/IAAs and ARFs. In *Arabidopsis*, cambial activity is promoted by ARF3 and ARF4, whereas it is suppressed by ARF5 (Brackmann *et al.* 2018). In *Populus*, PttIAA3 diminishes secondary xylem development by inhibiting periclinal divisions of the vascular

cambium. In contrast, it promotes cambial maintenance by stimulating its anticlinal divisions, demonstrating that Aux/IAs could mediate multiple processes such as cambial maintenance and development of secondary xylem, which presumably occurs via interactions with distinct ARFs (Nilsson *et al.* 2008). Similarly, the PtoIAA9 mentioned above mediates multiple processes during wood formation, including xylem cell differentiation, expansion and formation of xylem vessels, via its interactions with PtoARF5 (Xu *et al.* 2019). In addition, EgIAA4 can reduce cambial activity and delay SCW deposition in *Populus*, demonstrating its involvement in auxin-mediated regulation of xylem fibre development and SCW deposition (Yu *et al.* 2015). To the best of our knowledge, only three Aux/IAs have been previously investigated for their roles in wood formation of woody species such as *Eucalyptus* and *Populus*. Therefore, the role of Aux/IAs in mediating auxin-related transcriptional responses, remain poorly understood for most Aux/IAs.

In Chapter 5, we identified EgIAA13 as a potential novel regulator of cambial dynamics and development of wood and reaction wood (RW) as it influenced xylem fibre division, elongation, and expansion as well as xylem vessel expansion. However, EgIAA13 has not been previously investigated in detail and its interactions with EgARFs or its role in mediating auxin-related transcriptional responses remain obscure. The present chapter therefore aims to elucidate interactions of EgIAA13 with EgARFs to shed some light on EgIAA13-mediated transcriptional responses during wood and RW formation.

6.2 METHODOLOGY

6.2.1 Analysis of *IAA13* expression patterns

Two *in silico* analyses were conducted to determine the expression patterns of *EgIAA13* (Eucgr.H02914) in the whole plant (using EucGenIE version, 1.0, <https://eucgenie.org/>) (Hefer *et al.* 2011) and the expression pattern of its aspen ortholog during different stages of wood formation in *Populus tremula* (using AspWood version 3.0, <http://aspwood.popgenie.org/aspwood-v3.0/>) (Sundell *et al.* 2017). Similarly, using AspWood, the expression patterns of the aspen orthologs (identified as the *Populus* best hit from BLASTP, Phytozome v12,

<https://phytozome.jgi.doe.gov/pz/portal.html>) of *EgIAA3B*, *EgIAA4*, *EgIAA9A*, *EgIAA11*, *EgIAA15A*, *EgIAA20*, *EgIAA29*, *EgIAA31* and *EgIAA33A* were compared with those of *EgIAA13* as all have been reported to be preferentially expressed in vascular tissues (Yu *et al.* 2015). In addition, the expression patterns of aspen orthologs of *EgARFs* (identified using BLASTP in Phytozome as mentioned above) during various stages of wood formation were also analysed using AspWood.

6.2.2 Construction of interaction networks of AtIAA13 and EgIAA13

In silico analyses were conducted to identify candidate ARFs and Aux/IAs, which are most likely to interact with IAA13. The first *in silico* analysis was performed on the BioGRID database (version 3.5.184, <https://thebiogrid.org/>) to predict the potential interacting partners of *Arabidopsis* AtIAA13 (Chatr-Aryamontri *et al.* 2017; Stark *et al.* 2006). The interaction network was generated for interactions that were supported by at least three lines of evidence. The second *in silico* analysis was performed using the STRING database (version 11.0, <https://string-db.org>) (Szklarczyk *et al.* 2017). For this analysis, the amino acid sequence of EgIAA13 was used as query sequence, and *Eucalyptus* was selected as model organism. This search was conducted in the confidence mode where the required minimum interaction score was set to 0.9 (highest score). All seven active interaction sources (text mining, experiments, database, co-expression, neighbourhood, gene fusion and co-occurrence) were selected, and the remaining parameters were kept at default values (Song *et al.* 2019). These interaction networks and the *ARF* expression data from previous TW studies were analysed to identify the most suitable candidate ARFs for the following protein-protein interaction studies.

6.2.3 *In vivo* protein-protein interaction assays

The EgARFs which were identified from the above analysis (EgARF2, EgARF4, EgARF5, EgARF6 and EgARF19) were used for the following protein-protein interaction studies.

6.2.3.1 Generation of vector constructs

The synthetic constructs containing *EgARF4*, *EgARF5* and *EgARF19* coding sequences (CDSs) were kindly provided by Prof Jacqueline Grima-Pettenati and Assoc. Prof Hua Cassan-Wang (CNRS University, Toulouse, France). The CDSs of *EgARF2* and *EgARF6* were amplified from cDNA using CDS specific primers. The *AtIAA12*, *AtIAA13*, *AtARF5* and *AtARF10* CDSs were kindly provided by the *Arabidopsis* Biological Resource Centre (ABRC). For yeast-2-hybrid assays, the cloning method described in section 4.2.2 was followed to Gateway clone *Aux/IAA* and *ARF* CDSs into pGBKT7 and pGADT7, respectively. The pGBKT7 (bait or binding domain (BD)), and pGADT7 (prey or activation domain (AD)) vectors were obtained from TaKaRa. For the bimolecular fluorescence complementation (BiFC) assays, two different vector systems were used. These vector systems, pDEST-VYNE(R)^{GW}/pDEST-VYCE(R)^{GW} and pJG1047/pJG1048 were kindly provided by Dr Ebert Berit (Gehl *et al.* 2009) and Dr John Golz, respectively (both University of Melbourne, Australia). The constructs for the above-mentioned first BiFC system were generated by Gateway cloning (section 4.2.2) *Aux/IAA* and *ARF* CDSs into respective pDEST-VYNE(R)^{GW} or pDEST-VYCE(R)^{GW} vector. The constructs for the latter BiFC system were generated by following seamless cloning protocol (NEBuilder[®] HiFi DNA Assembly Cloning) to insert respective *Aux/IAA* and *ARF* CDSs into the respective vector (pJG1047 or pJG1048). After cloning, all constructs were verified by sequencing.

6.2.3.2 Yeast-2-hybrid assay

The sequence-verified pGBKT7 and pGADT7 constructs were used as pairs to co-transform freshly prepared competent yeast cells (*Saccharomyces cerevisiae* strain AH109) following polyethylene glycol/Lithium acetate mediated yeast transformation protocol (Clontech). The transformed yeast was grown on several Synthetic Defined (SD) dropout media; double dropout SD media (SD/-Trp/-Leu) which lacks tryptophan (Trp) and leucine (Leu), quadruple dropout SD media (SD/-Trp/-Leu/-His/-Ade) which lacks Trp, Leu, histidine (His) and adenine (Ade) and quadruple dropout media supplemented with 2 mM 3-amino-1,2,4-triazole (SD/-Trp/-Leu/-His/-Ade + 3AT).

Three replicates were plated for each interaction, and the plates were incubated at 30°C for five days.

6.2.3.3 Bimolecular fluorescence complementation (BiFC) assay

The sequence-verified BiFC constructs were electroporated into *Agrobacterium tumefaciens* AGL1 to generate BiFC partner strains. The 48 hours old transgenic *Agrobacterium* cultures were centrifuged, and the cell pellets were resuspended in the infiltration buffer (10 mM MES at pH 5.5, 10 mM MgSO₄ and 100 µM acetosyringone). The resulting infiltration solutions of the respective BiFC partner strains and p19 helper plasmid were mixed to achieve the desired OD. Several OD values (0.05, 0.1, 0.3, 0.5, 0.75 and 1.0) were tested to identify the optimum OD with respect to each BiFC vector pair. In addition, the nuclear localising vector ERL322, containing cyan fluorescent protein (CFP) was used as the marker to confirm leaf infiltrations (Kaplan-Levy *et al.* 2014). Finally, 1 ml from the final infiltration solution (containing BiFC partner strains and p19) was used to infiltrate three to six-week-old *Nicotiana benthamiana* leaves where three leaves were infiltrated per interaction. The fluorescence on the lower epidermis of leaf discs was daily observed from the third to the fifth day following the leaf infiltrations, using a spinning disk microscope (Inverted Nikon Eclipse Ti-E with optical autofocus system).

6.3 RESULTS

Since *IAA13* has not been previously studied in any plant genera, analysing its expression patterns within a tree and within wood formation tissue have the potential to provide more insights into its regulatory role.

6.3.1 *EgIAA13* is preferentially expressed in vascular tissues analogous to the expression pattern of *EgIAA4*

EgIAA13 is preferentially expressed in *Eucalyptus* vascular tissue, in stem and roots (Fig. 6.1). In stem vascular tissue, the normalised expression (given in Variance-Stabilizing Transformation (VST) units) of *EgIAA13* in the immature xylem (VST = 4.88) is lower than that in the phloem (VST = 5.15) while the expression level of

EgIAA13 in the TW xylem (VST = 4.43) is lower than that in upright control xylem (VST = 5.67). The lowest expression levels of *EgIAA13* were observed in leaves and shoot tips.

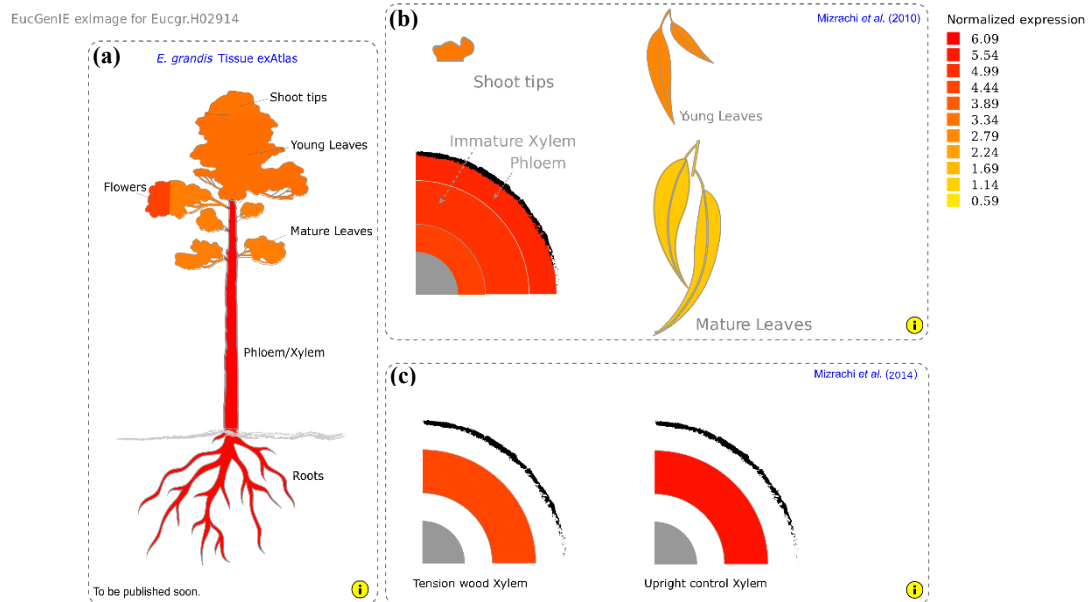


Figure 6.1 The relative expression of *EgIAA13* in *E. grandis* (a) whole tree, (b) xylem, leaves and shoot tips and (c) TW xylem and upright control xylem (revised from figures obtained from EucGenIE database).

The fine-scale gene expression profile of the aspen ortholog of *EgIAA13* across the stem (Fig. 6.2), demonstrates a sharp rise in *EgIAA13* transcript levels in the cambial zone where xylem cell division and differentiation take place. Subsequent xylem cell expansion commences under this elevated *EgIAA13* transcript level; however, the transcript level gradually decreases towards the end of the xylem expansion zone. Although the transcript level continues to decline during the initial phase of the subsequent SCW deposition stage, the transcript levels then gradually increase.

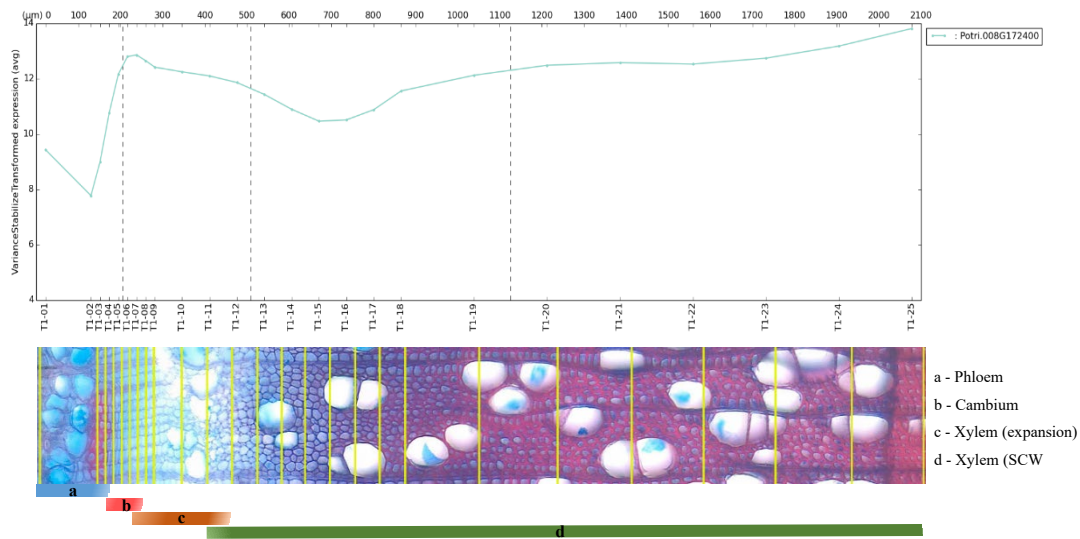


Figure 6.2 Fine-scale gene expression profile of Potri.008G172400 (*EgIAA13* ortholog) during different stages of wood formation in *P. tremula* (revised from the figure adapted with permission from AspWood - Sundell *et al.* 2017).

Furthermore, the expression patterns of the aspen orthologs of *EgIAAs*, which are preferentially expressed in the vascular tissues (Yu *et al.* 2015) were compared to those of the aspen ortholog of *EgIAA13* (Fig. 6.3). Although AspWood provides the expression patterns of the *Populus* orthologs of *EgIAAs*, to make the analysis convenient, these expression patterns are presented and discussed in the following sections by referring to the respective *Eucalyptus* orthologs. Among the *EgIAAs* that have been reported to be preferentially expressed in vascular tissues, the expression pattern of *EgIAA4* (pink line in Fig. 6.3) was similar to that of *EgIAA13* (purple) across the cambial division, differentiation, xylem expansion and SCW deposition regions. In addition, the expression patterns of *EgIAA3B* (orange), *EgIAA20* (light green) and *EgIAA29* (blue) were similar to that of *EgIAA13* in the cambial division and differentiation regions. Similarly, the expression pattern of *EgIAA9A* (brown) is similar to that of *EgIAA13* in xylem expansion and SCW deposition regions.

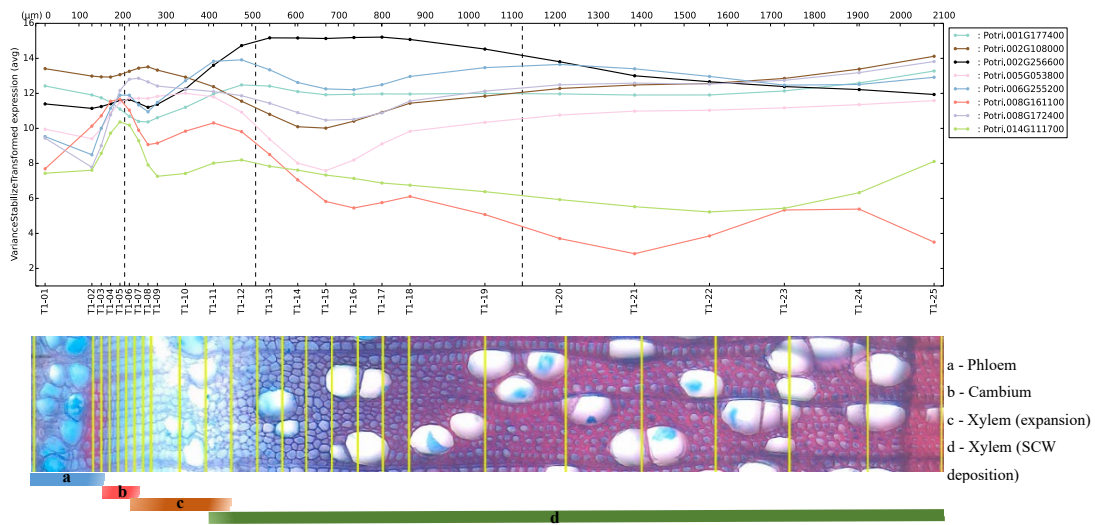


Figure 6.3 Fine-scale gene expression profile of aspen orthologs of vascular tissue preferential *EgIAAs* during different stages of wood formation in *P. tremula* (revised from the figure adapted with permission from AspWood - Sundell *et al.* 2017). The respective *Eucalyptus* orthologs of the above *Populus* gene accessions are as follows; Potri.001G177400 (*EgIAA15*), Potri.002G108000 (*EgIAA9A*), Potri.002G256600 (*EgIAA11*), Potri.005G053800 (*EgIAA4*), Potri.006G255200 (*EgIAA29*), Potri.008G161100 (*EgIAA3B*), Potri.008G172400 (*EgIAA13*) and Potri.014G111700 (*EgIAA31*).

Since Aux/IAA mediates its regulation via their interactions with ARFs, we conducted an *in silico* analysis to assess the expression patterns of *ARFs* in vascular tissues. Also, more *in silico* analyses were performed to construct protein-protein interaction networks for IAA13 in *Arabidopsis* and *Eucalyptus*, aiming to select candidate EgARFs for *in vivo* protein-protein interaction assays.

6.3.2 Certain ARFs are more likely to interact with IAA13

The aspen orthologs of *EgARFs* are expressed in vascular tissues (Fig. 6.4). Among these aspen orthologs, the expression patterns of those of *EgARF5* and *EgARF10* were similar to that of *EgIAA13*. In contrast, the expression patterns of the aspen orthologs of *EgARF2B*, *EgARF9A*, and *EgARF16A* were opposite to that of the aspen ortholog of *EgIAA13*. However, since all these aspen orthologs of *EgARFs* (except the ortholog of *EgARF24*) are expressed across all stages of xylem development, selection of EgARFs for *in vivo* protein-protein interaction studies could not be made based on these *ARF* expression patterns alone.

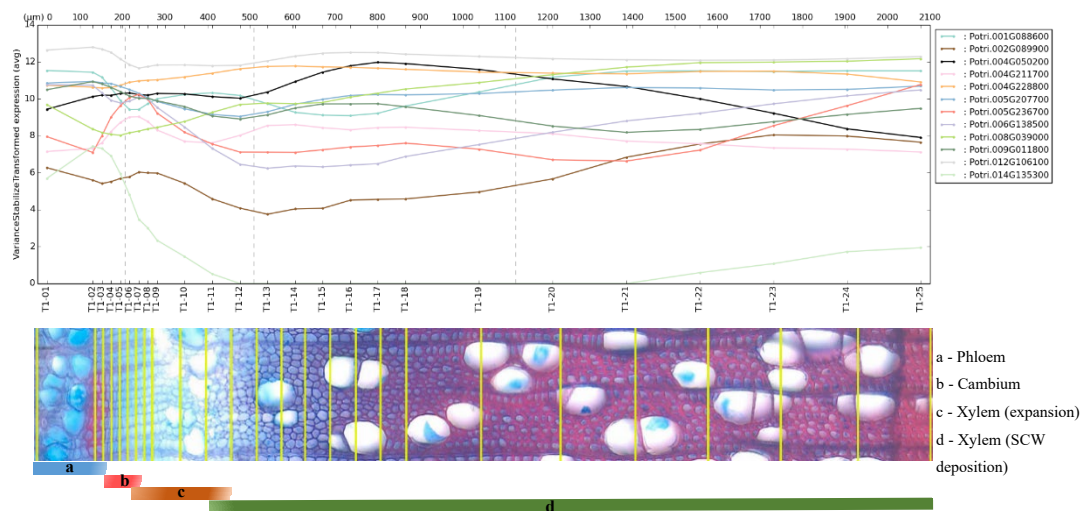


Figure 6.4 Fine-scale gene expression profile of aspen orthologs of *EgARFs* during different stages of wood formation in *P. tremula* (revised from the figure adapted with permission from AspWood - Sundell *et al.* 2017). The respective *Eucalyptus* orthologs of the above *Populus* gene accessions are as follows; Potri.001G088600 (*EgARF9*), Potri.002G089900 (*EgARF17*), Potri.004G050200 (*EgARF3*), Potri.004G211700 (*EgARF10*), Potri.004G228800 (*EgARF1*), Potri.005G207700 (*EgARF6*), Potri.005G236700 (*EgARF5*), Potri.006G138500 (*EgARF19*), Potri.008G039000 (*EgARF16*), Potri.009G011800 (*EgARF4*), Potri.012G106100 (*EgARF2*) and Potri.014G135300 (*EgARF24*).

Therefore, the following *in silico* interaction networks were generated to identify the best candidate *EgARFs*. The interaction network generated by the BioGRID database is based on *Arabidopsis*, and it demonstrates several potential interactions of AtIAA13 with some other AtIAAs and AtARFs (Fig. 6.5). According to this network, AtARF2, AtARF4, AtARF5, AtARF6, AtARF7, AtARF8, AtARF9 and AtARF19 are most likely to interact with AtIAA13. Similarly, the interaction network generated by the STRING database predicts *EgIAA13* to potentially interact with two *EgARFs* (three proteins; however, two of them correspond to *EgARF19*) in *Eucalyptus* (Fig. 6.6). Both interaction networks highlight ARF5 and ARF19 as potential interaction partners for IAA13, and therefore, *EgARF5* and *EgARF19* were selected as main candidates for *in vivo* protein-protein interaction studies. In addition, *EgARF2*, *EgARF4* and *EgARF6* were also selected, since their genes are differentially expressed during TW formation (similar to *EgIAA13*) and because their *Arabidopsis* orthologs interact with AtIAA13 (Li *et al.* 2011b; Piya *et al.* 2014; Tatematsu *et al.* 2004; Vernoux *et al.* 2011; Yu *et al.* 2015).

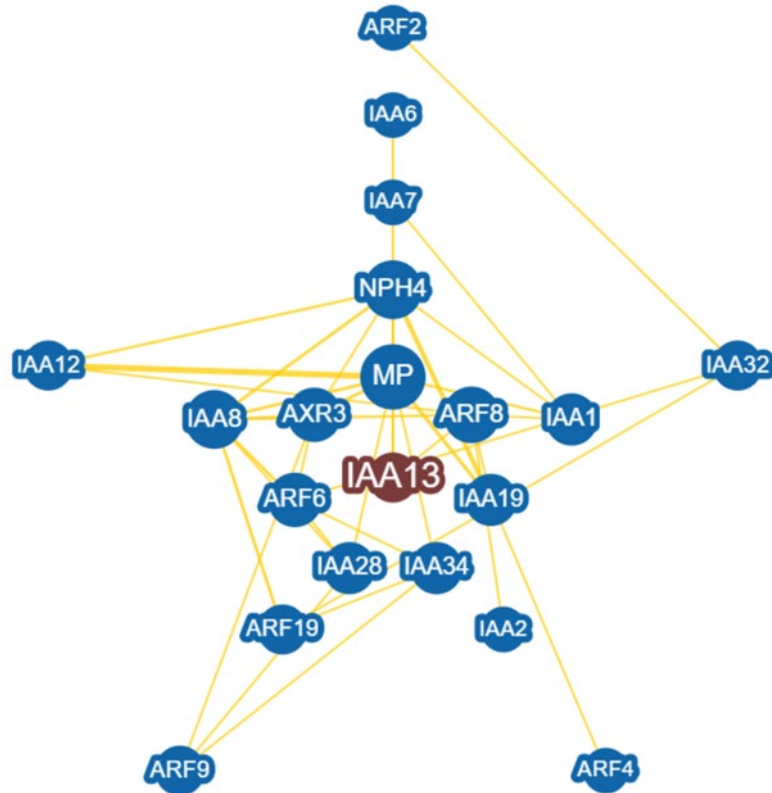


Figure 6.5 Protein-protein interaction network predicted for AtIAA13 by BioGRID database. MP stands for MONOPTEROS (ARF5), NPH4 stands for NON-PHOTOTROPIC HYPOCOTYL (AtARF7), and AXR3 stands for AUXIN RESISTANT3 (AtIAA17).

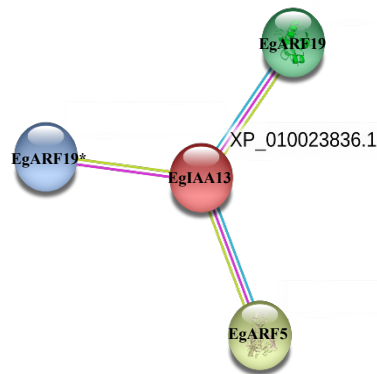


Figure 6.6 Protein-protein interaction network predicted for EgIAA13 (revised from figure produced by STRING database). The corresponding protein accessions for EgARF5, EgARF19 and EgARF19* are XP_010061491.1, XP_010048533.1 and XP_010049370.1, respectively. The edges connecting the nodes are colour coded; pink denotes the predictions based on experimental evidence, blue denotes the predictions based on database evidence and green denotes the predictions based on neighbourhood evidence.

6.3.3 EgIAA13 interacts with EgARF2, EgARF5, EgARF6 and EgARF19

Our yeast-2-hybrid *in vivo* protein-protein interaction study confirmed that EgIAA13 interacts with EgARF2, EgARF5, EgARF6 and EgIAA19 but not with EgARF4 (Fig. 6.7). During these experiments, to ensure that yeast mating was successful, yeast co-transformed with each vector combination was plated on double dropout selective medium. The appearance of yeast colonies for all vector combinations confirmed successful yeast mating. Also, to confirm that there was no self-activation of the bait construct in the absence of an interacting prey, we tested both bait constructs (EgIAA13 and AtIAA13) by co-transforming yeast with EgIAA13 + empty AD and AtIAA13 + empty AD vector combinations and plated them on quadruple medium and quadruple medium supplemented with 3AT. Yeast colonies were not observed from either of these combinations, confirming that there was no self-activation. Therefore, the observed yeast growth from interactions between EgIAA13 and EgARF2, EgARF5, EgARF6 and EgARF19 demonstrates true interactions between these proteins. The growth of yeast in the presence of 3AT is known to reflect the strength of protein-protein interactions. Since EgIAA13-EgARF5 leads to marginally faster growth on quadruple medium supplemented with 3AT, this interaction is presumably stronger than others.

In order to analyse these interactions in an *in-planta* system, we undertook BiFC assays with two different vector systems. When employing BiFC to test Aux/IAA-ARF interactions that take place in the nucleus, it is crucial to use appropriate controls which are also localised in the nucleus. Therefore, prior to testing target interactions, we tested several combinations to select a suitable nuclear localising positive and negative control. The tested combinations for the positive control included AtIAA12 + AtARF5 and AtIAA13 + AtARF5 (Piya *et al.* 2014). As negative controls, in addition to entire CDSs, we also used some mutated versions; AtIAA13 and AtARF5 mutated by removing C terminal domains III and IV (these two domains are known to facilitate interactions between Aux/IAs and ARFs), denoted by AtIAA13m and AtARF5m, respectively. The tested combinations for negative controls included AtIAA13 + AtARF10, AtIAA12 + AtARF10, AtIAA13m + AtARF5m, AtIAA13 + AtARF5m, AtIAA13m + AtARF5, EgIAA13 + EgARF4, EgKNAT7 + EgWRKY2 and EgIAA13 + EgKNAT7. Unexpectedly, a fluorescent signal was observed for all combinations

(not shown). Therefore, presumably self-assembly of the fluorophores takes place when the pDEST-VYNE(R)^{GW}/pDEST-VYCE(R)^{GW} and pJG1047/pJG1048 vector pairs are confined to the nucleus. Planned experiments aimed at investigating this surprising result were initiated but could as yet not be conducted due to COVID-19 pandemic restrictions and so our results of this experiment for now have to remain inconclusive.

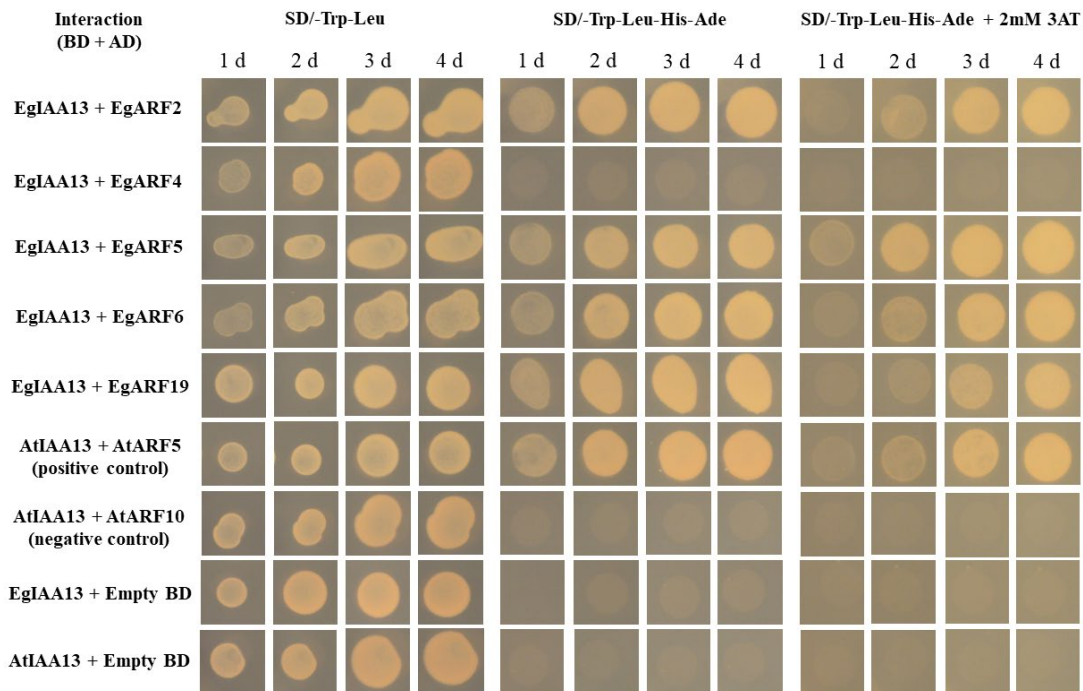


Figure 6.7 Results of the yeast-2-hybrid assays for protein-protein interactions between EgIAA13 and selected EgARFs. The AtIAA13 + AtARF5 combination was used as the positive control, and the AtIAA13 + AtARF10 combination was used as the negative control. EgIAA13 and AtIAA13 with empty BD combinations were used to test for autoactivation. The symbol ‘d’ denotes day. Since there were no differences between the yeast growth on the fourth and fifth days, the above figure only contains observations up to the fourth day. As all three replicates produced similar results, results of only one replicate out of the three are shown in this figure.

6.4 DISCUSSION

EgIAA13 resembles a classic Aux/IAA protein. It contains all four domains (I, II, III and IV), nuclear localising signals and the degron sequence GWPPI (Yu *et al.* 2015). The presence of GWPPI instead of GWPPV might be responsible for the higher auxin tolerance of EgIAA13 which enables it to be active in regions (cambial) where auxin concentration peaks (Calderón Villalobos *et al.* 2012). Also, several previous studies have demonstrated that in *Arabidopsis*, auxin treatment increases *AtIAA13* transcript levels (Abel *et al.* 1995; Liu *et al.* 2015b; Nemhauser *et al.* 2006). Furthermore, in *Arabidopsis*, *AtIAA13* is directly upregulated upon activation of AtARF5, suggesting negative feedback onto AtARF5 function (Krogan *et al.* 2014). These features of *EgIAA13* potentially support its expression patterns across wood forming tissues and thereby its tissue-specific functions which are discussed in detail in the following sections.

6.4.1 Expression of *IAA13* suggests its potential involvement in regulating vascular tissue development

The expression of *IAA13* varies across different tissues, suggesting tissue specific function. As an example, in *Arabidopsis*, *AtIAA13* is preferentially expressed in xylem (Cassan-Wang *et al.* 2013). Similarly, in *Eucalyptus*, *EgIAA13* is highly expressed in developing xylem indicating its possible involvement in the regular progression of secondary xylem formation. In addition, the possible involvement of EgIAA13 in maintaining regular wood formation is also highlighted by the attenuated *EgIAA13* expression level in TW whose cellular/tissue morphology and chemical composition differ from those of normal wood (NW) (Yu *et al.* 2015). Furthermore, the fluctuating expression level of a *Populus* ortholog of *EgIAA13* through different stages of wood formation in *Populus* stems suggests that EgIAA13 might be having distinct roles at each stage of wood formation. Therefore, the spatio-temporal expression of *EgIAA13* could play a vital role in fine-tuning different aspects of wood formation. Based on these expression patterns we propose that

- I. elevated *EgIAA13* expression is essential to maintain regular cambial cell division

- II. elevated *EgIAA13* expression which undergoes a marginal decrease in the expansion zone is vital to ensure proper xylem cell expansion
- III. attenuation of *EgIAA13* expression is essential to facilitate the formation of TW or to generate TW specific features.

These hypotheses are further supported by *EgIAA13* overexpression and knockdown studies (Chapter 5). For example, downregulation of *EgIAA13* (either by RNAi-mediated knockdown or presumably due to silencing of the native *EgIAA13* gene by HDGS) lead to an increase in xylem fibre count which suggests a possible increase in cambial cell division towards the xylem side. Therefore, an elevated *EgIAA13* expression level is potentially crucial to maintain cambial dynamics (hypothesis I). Also, throughout the xylem cell expansion stage in *Populus*, a higher *IAA13* level is maintained although there is a marginal decrease in *IAA13* levels between the onset and cessation of xylem cell expansion. This pattern of expression demonstrates a potential role for IAA13 in regulating the two crucial aspects of xylem cell expansion; the duration and the rate of expansion both of which are known to be influenced by auxin signalling (Dodd and Fox 1990; Mellerowicz *et al.* 2001; Tuominen *et al.* 1997). Furthermore, in Chapter 5 we demonstrated that the downregulation of *EgIAA13* led to smaller xylem fibres and vessels which is consistent with hypothesis II implying the importance of maintaining *EgIAA13* levels to ensure proper xylem cell expansion. Likewise, eccentric radial growth (more wood on the TW side), smaller xylem fibres and downregulation of *EgIAA13* in TW further support the hypothesised importance of *EgIAA13* for the regular progression of wood formation (hypothesis III) (Mizrachi *et al.* 2015). Based on the above hypotheses and supporting evidence, *EgIAA13* is most likely to have a dual role in the wood formation where it acts as a repressor of xylem division and a promoter of xylem cell expansion.

6.4.2 *EgIAA13* mediates transcriptional regulation via *EgIAA13*-*EgARF* modules

Aux/IAs are known to mediate auxin signalling via repression of ARFs. Our yeast-2-hybrid studies confirm *in vivo* interactions of *EgIAA13* with *EgARF2B*, *EgARF5*, *EgARF6A* and *EgARF19A*. Among these ARFs, *EgARF5*, *EgARF6* and *EgARF19* are potential transcriptional activators since they contain a glutamine-rich region

which is a characteristic feature of activator ARFs. In contrast, EgARF2 is a potential transcriptional repressor (Tiwari *et al.* 2003; Yu *et al.* 2014). Similar *in vivo* interactions have been observed between AtIAA13 and *Arabidopsis* orthologs of above ARFs (Li *et al.* 2011b; Piya *et al.* 2014; Tatematsu *et al.* 2004; Vernoux *et al.* 2011) demonstrating some evolutionary conservation between IAA13-ARF interactions.

The hypothesised dual role of EgIAA13 during wood formation is potentially mediated by distinct EgIAA13-EgARF modules. Notably, the EgIAA13-EgARF5 module is important since ARF5 is already known to play a central role in cambial dynamics. Downregulation of *EgIAA13* may result in more free (not bound by EgIAA13) EgARF5 proteins which could subsequently interact with its downstream target genes such as *WOX4* and *HB*; *WOX4* is a central cambium regulator which controls cambial divisions and also imparts auxin responsiveness to the cambium and *HB* promotes xylem differentiation (Brackmann *et al.* 2018; Xu *et al.* 2019). Therefore, EgIAA13 presumably acts as a repressor of EgARF5-mediated xylem division where EgIAA13 either hinders the xylem cell division or maintains the rate of xylem division. Consequently, the accelerated wood growth in TW could result from the downregulation of *EgIAA13* during TW formation. Furthermore, the regulation of wood and root formation by ARF5 in combination with various Aux/IAs have been reported by several previous studies. As an example, a recent study conducted in *Populus* demonstrated the regulation of *PtoHB7/8* by the PtoIAA9-PtoARF5 module, which mediates the regulation of several aspects of wood formation including xylem cell specification, vessel formation and xylem expansion (Xu *et al.* 2019). Similarly, in *Arabidopsis*, AtIAA12 (the functional ortholog of AtIAA13) in conjunction with AtARF5 is known to regulate root initiation (Hamann *et al.* 2002; Hardtke *et al.* 2004; Weijers *et al.* 2005). Also, Brackmann *et al.* (2018) suggest that AtARF5 supports xylem formation by directly activating xylem-associated genes and by repressing *WOX4* which is known to convey auxin signalling to the cambium (Suer *et al.* 2011). Since ARF5 influences cambial dynamics and xylem formation in different genera (Brackmann *et al.* 2018; Xu *et al.* 2019), presumably the EgIAA13-EgARF5 module is involved in regulating cambial dynamics and wood formation in *Eucalyptus*.

Similarly, the EgIAA13-EgARF19 module is potentially involved in EgIAA13-mediated transcriptional regulation of wood formation. Previous studies have reported that the IAA13-ARF19 module is involved in regulating different aspects of plant growth. As an example, *Arabidopsis* root growth and gravitropic responses are mediated via AtIAA13-ARF7 and AtIAA13-ARF19 modules (Weijers *et al.* 2005). Also, the transcriptional regulation of *Arabidopsis* lateral root initiation is mediated through negative regulation of AtARF7 and AtARF19 by AtIAA14 and AtIAA12 (Fukaki *et al.* 2002; Okushima *et al.* 2005). These two ARFs are known to regulate lateral root formation by activating *LATERAL ORGAN BOUNDARIES DOMAIN 16/ASYMMETRIC LEAVES2-LIKE18 (LBD16/ASL18)* and *LBD29/ASL16* (Lee *et al.* 2009a; Lee *et al.* 2009b). During lateral root formation, LBD16 together with LBD18/ASL20 is known to activate *E2Fa*, *EXPANSIN14 (EXPA14)* and *EXPA17*. E2Fa TF is known to control asymmetric cell division, and the EXPAs are responsible for primary cell wall loosening (Berckmans *et al.* 2011; Cosgrove 2015). Similarly, in *Oryza sativa*, the IAA13-ARF19 module presumably regulates lateral root formation via regulation of *LBD1-8* (Yamauchi *et al.* 2019). In *Eucalyptus*, *EgIAA13* and *EgARF19* are expressed throughout wood forming tissues, and since these proteins interact *in vivo*, the EgIAA13-EgARF19 module could be involved in mediating xylem cell expansion via regulating the *EXPAs*. Hence, presumably, the potential regulation of *EXPAs* via the EgIAA13-EgARF19 module restricts radial expansion of xylem fibres and vessels during wood formation (as described in Chapter 5).

The current literature does not hold evidence linking the remaining two EgIAA13-EgARF pairs (EgIAA13-EgARF2 and EgIAA13-EgARF6) to a specific phenotype. However, Yu *et al.* (2014) demonstrate that EgARF6 is downregulated in TW and upregulated in OW compared to an upright control. Also, the *Populus* orthologs of both *EgARF2* and *EgARF6* are expressed in cambial and xylem tissues during wood formation. Therefore, also these TFs could be potentially involved in regulating some aspects related to wood formation; however, without experimental evidence, their potential involvement in generating the observed phenotypes can be neither corroborated nor negated.

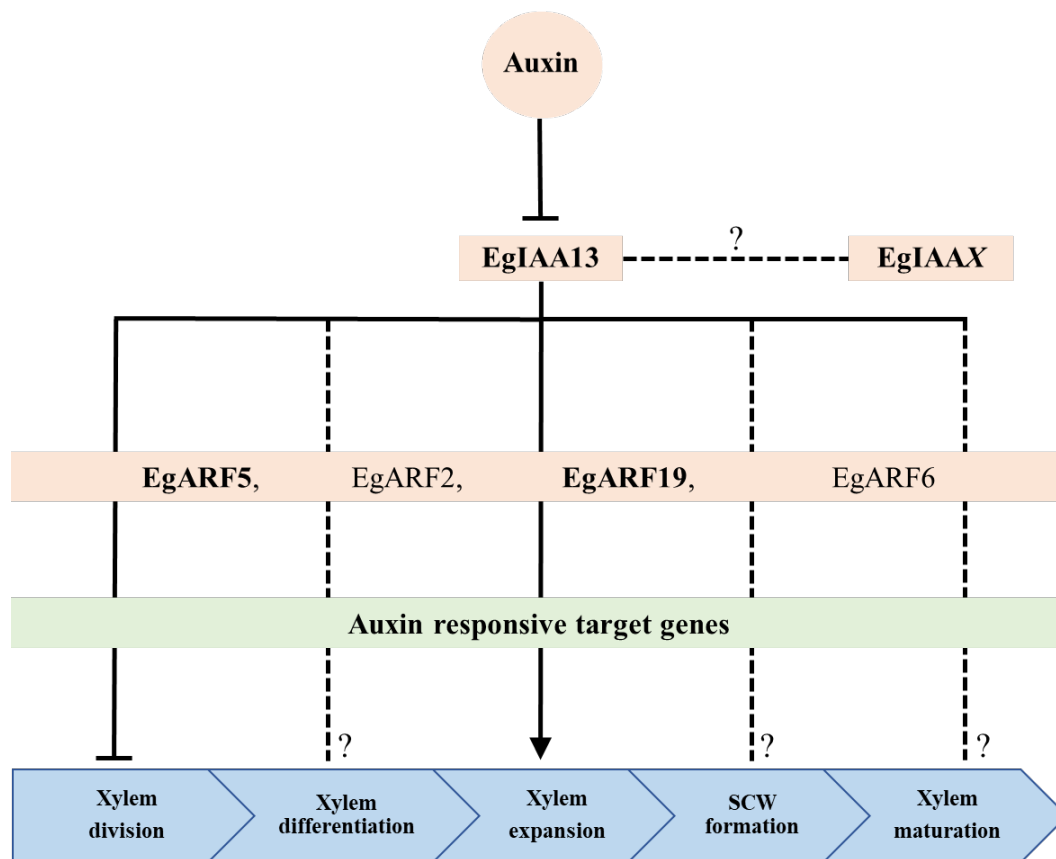


Figure 6.8 Conceptual model for the EgIAA13 dependent regulation of wood formation in *Eucalyptus*. EgIAAX denotes either EgIAA3, EgIAA4, EgIAA9, EgIAA12, EgIAA15, EgIAA19 or EgIAA32. The arrow represents positive regulation, and the line ending with a flat top represents negative regulation. Dotted lines represent unverified interactions/regulations.

To summarise our data and hypotheses, we generated a model (Fig. 6.8) to demonstrate the hypothesised regulatory role of EgIAA13 in mediating *Eucalyptus* wood formation. Auxin triggers proteasome-mediated degradation of Aux/IAs; however, EgIAA13 presumably has a higher auxin tolerance due to the presence of degron sequence GWPPI, allowing EgIAA13 to operate in the cambial division and differentiation regions which feature elevated auxin levels. EgIAA13 can form both homo- and heterodimers with other EgIAs and EgARFs, respectively. Based on *in silico* analysis and on the preferential expression patterns of *EgIAs* in *Eucalyptus* xylem tissue (Yu *et al.* 2015), EgIAA3, EgIAA4, EgIAA9, EgIAA12, EgIAA15, EgIAA19 and EgIAA32 are most likely to interact with EgIAA13. Based on our *in vivo* experiments, EgIAA13 forms heterodimers with EgARF2, EgARF5, EgARF6 and EgARF19 and the resulting EgIAA13-EgARF modules potentially target downstream auxin-responsive genes.

In *Eucalyptus*, several genes involved in the regulation of various aspects of wood formation possess features that make them auxin responsive. As an example, a crucial factor in deciding auxin responsiveness of a downstream target gene is the presence of an AuxRE in its promoter. The canonical AuxRE in plants is the TGTCTC sequence (Roosjen *et al.* 2018). In *Eucalyptus*, certain genes involved in the regulation of various aspects of wood formation, contain this TGTCTC sequence in their promoters. Some examples include *Eucalyptus* orthologs of *AtHB9*, *MYELOBLASTOSIS 4 (AtMYB4)*, *AtMYB20*, *AtMYB42*, *AtMYB46*, *AtMYB91*, *AtMYB103*, *AtMYB124*, *XYLEM NAC DOMAIN 1 (AtXND1)*, *SECONDARY WALL NAC DOMAIN 2 (AtSND2)*, *AtWRKY11*, *AtWRKY41*, *CELLULOSE SYNTHASE A4 (AtCESA4)*, *AtASL11/LBD15*, *AtIAA9*, *AtIAA16* and *AtARF10*. Furthermore, we speculate that presumably the EgIAA13-EgARF5 module regulates downstream *HB* and *WOX* TF genes thereby influencing cambial division, differentiation, and xylem formation. We also hypothesize that the EgIAA13-EgARF19 module is likely to mediate xylem cell expansion and elongation via regulation of auxin-responsive downstream target genes such as *ASL/LBD* and *EXPAs*. In addition, these EgIAA13-EgARF modules could also be involved in mediating other aspects of wood formation such as SCW deposition via regulation of auxin-responsive SCW related TFs and genes such as *MYB*, *SND*, *XND*, *WRKY* and *CesA*. Therefore, we introduce EgIAA13 as a novel regulator which mediates auxin regulation on multiple wood formation related processes including cambial division, differentiation, xylem cell expansion and elongation.

6.5 CONCLUSIONS, PERSPECTIVES AND FUTURE WORK

- I. Auxin signalling plays a vital role in cambium maintenance and wood formation. This auxin signalling is translated into auxin-mediated transcriptional responses by IAA-ARF modules. This chapter elaborated on the roles of EgIAA13 via EgIAA13-EgARF (2, 5, 6 and 19) modules in mediating transcriptional responses in the process of wood formation. Elucidating the regulatory role of EgIAA13, not only demonstrates the importance of EgIAA13 in regulating various stages of wood formation, but it also sheds light onto the complex regulatory roles of auxin signalling during wood formation. Therefore, EgIAA13 presents an excellent candidate for further investigations

into the auxin-related transcriptional regulation of wood formation, while also serving as a potential useful target for genetically manipulating wood properties such as fibre and vessel size and wood density.

- II. The proposed future work for further verification of EgIAA13-EgARF interactions involves BiFC and Co-immunoprecipitation (co-IP). The planned BiFC experiments involve the use of nuclear localising N/C-halves (BiFC vector pair without IAA or ARF) to test whether the nuclear-localised vectors are capable of generating a signal in the absence of interacting proteins. In addition to explaining the unexpected BiFC results obtained in this project, this experiment can also serve as a screening tool to select a suitable pair of BiFC vectors that do not interact in the absence of interacting proteins although the vectors localise to the nucleus. Furthermore, the widely used co-IP assays can be used as an alternative method to test EgIAA13-EgARF interactions.

7 CONCLUDING REMARKS

Attempting to fill some knowledge gaps in our current understanding of the regulatory molecular network behind xylogenesis in perennial woody forest tree species, this thesis laid the groundwork for unravelling the potential transcriptional network underlying reaction wood (RW) formation by reviewing the relevant literature on RW studies, collecting many pieces of evidence and demonstrating the feasibility of employing RW as a model to identify transcriptional regulators of wood formation.

In preparation for the functional characterisation of so identified potential candidate transcription factors (TFs), it assessed the microanalytical techniques that can be employed to phenotype secondary xylem especially when the available sample size is restricted only to few micrograms, which is a common challenge faced by the Induced Somatic Sector Analysis (ISSA) approach which was followed throughout this thesis.

Specifically, this thesis uncovered the regulatory roles of three *Eucalyptus* TFs.

- I. Primarily, it provided the very first insights into the regulatory role of INDOLE-3-ACETIC ACID INDUCIBLE (Aux/IAA) TF; EgIAA13, by demonstrating its involvement in maintaining cambial dynamics and in shaping *Eucalyptus* xylem fibre and vessel morphology during wood and RW formation.
- II. It elucidated the function of KNOTTED-LIKE HOMEODOMAIN (KNAT) TF; EgKNAT7, by demonstrating that it positively regulates secondary cell wall (SCW) deposition and fibre development during xylogenesis in *Eucalyptus*.
- III. It demonstrated that the R2R3-type MYELOBLASTOSIS (MYB) TF; EgMYB103, regulates xylem fibre development in *Eucalyptus*.

The groundwork laid by this thesis can now be used for an exploration into the feasibility of chemotyping platforms such as pyrolysis GC/MS for the analysis of cell wall chemical composition of ISSA-derived transgenic samples. Adaptation of such a

chemotyping platform will be useful to address one of the current limitations in ISSA and to expand examinations of EgIAA13, EgKNAT7 and EgMYB103 in terms of their influence on SCW polymer composition.

In addition, a major caveat in ISSA is the inability to profile gene expression (due to destructive sampling); therefore, further functional characterisation of these TFs should also consider use of approaches including *Eucalyptus* hairy roots (Dai *et al.* 2020) and full transgenic plants in an effort to elucidate their influence on plant development as a whole and to better understand their impact on the underlying transcriptome.

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