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Manipulation of Genes Involved in Secondary Cell Wall Development During Wood Formation in Poplar

Kavitha Satish Kumar

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MANIPULATION OF GENES INVOLVED IN SECONDARY CELL WALL DEVELOPMENT DURING WOOD FORMATION IN POPLAR

By

Kavitha Satish Kumar

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In Biological Sciences

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This dissertation has been approved in partial fulfillment of the requirements for the Degree of DOCTOR OF PHILOSOPHY in Biological Sciences.

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Dedication

To my family

Avinash Satish Kumar, Usha Satish Kumar, and Satish Kumar

With me, my family graduates!
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Preface

Chapters included in this dissertation have been written and formatted with the purpose of publishing them in peer-reviewed journals.

Chapter 2: Study was designed by Kavitha Satish Kumar along with Dr. Chandrashekhar P. Joshi and conducted by Kavitha Satish Kumar with help from Surattana Boonsai and Dane Wouri.

Chapter 3: Study was designed by Kavitha Satish Kumar along with Dr. Chandrashekhar P. Joshi and conducted by Kavitha Satish Kumar with help from Surattana Boonsai and Dane Wouri.

Chapter 4: Study was designed by Kavitha Satish Kumar along with Dr. Chandrashekhar P. Joshi and Dr. Guiliang Tang and conducted by Kavitha Satish Kumar with help from Surattana Boonsai and Dane Wouri.

None of the above chapters have been published or submitted for publication yet.
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Jeswin, Anurag, Kri Nga Do family, Prasanna (ma little one), Avinash, Kaushik, Roshini, Aishwarya and other wonderful friends who are my home away from home. My extended family in Kerala and Singapore, thank you for all your love. Muralee, thank you for being there through all the struggles, good and bad times, to be there and reinstate my confidence in my potentials each time I seemed to lose faith in myself.

I fail to express my gratitude for my family without their unconditional love and support I would not have been able to live my dreams. The struggle was ours and thank you for believing in me!
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<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PRX</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>LAC</td>
<td>Lacasse</td>
</tr>
<tr>
<td>PCW</td>
<td>Primary cell wall</td>
</tr>
<tr>
<td>SCW</td>
<td>Secondary cell wall</td>
</tr>
<tr>
<td>PAL</td>
<td>phenylalanine ammonia-lyase</td>
</tr>
<tr>
<td>C4H</td>
<td>cinnamate 4-hydroxylase</td>
</tr>
<tr>
<td>C3H</td>
<td>4-hydroxycinnamate 3-hydroxylase</td>
</tr>
<tr>
<td>CCoAOMT</td>
<td>caffeoyl CoA 3-O-methyltransferase</td>
</tr>
<tr>
<td>COMT</td>
<td>caffeic acid 3-O-methyltransferase</td>
</tr>
<tr>
<td>F5H</td>
<td>ferulate 5-hydroxylase</td>
</tr>
<tr>
<td>4CL</td>
<td>hydroxycinnamate CoA ligase</td>
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<tr>
<td>CCR</td>
<td>hydroxycinnamoyl CoA:NADPH oxidoreductase</td>
</tr>
<tr>
<td>CAD</td>
<td>hydroxycinnamonyl alcohol dehydrogenase</td>
</tr>
<tr>
<td>SDX</td>
<td>Stem-differentiating xylem</td>
</tr>
<tr>
<td>miRNA</td>
<td>micro-ribonucleic acid</td>
</tr>
<tr>
<td>NREL</td>
<td>National Renewable Energy Laboratory</td>
</tr>
<tr>
<td>STTM</td>
<td>Short Tandem Target Mimic</td>
</tr>
<tr>
<td>H\textsubscript{2}O\textsubscript{2}</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HRP-C</td>
<td>Horseradish peroxidase isoenzyme C</td>
</tr>
<tr>
<td>CWPO-C</td>
<td>Cationic cell wall peroxidase</td>
</tr>
<tr>
<td>Tyr\textsubscript{g}</td>
<td>tyrosine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>AIR</td>
<td>Alcohol insoluble residue</td>
</tr>
<tr>
<td>S/G</td>
<td>Syringyl/guaiaeryl</td>
</tr>
<tr>
<td>HD-ZIPIII</td>
<td>Class III HOMEODOMAIN-LEUCINE ZIPPER</td>
</tr>
<tr>
<td>HB</td>
<td>Homeobox</td>
</tr>
</tbody>
</table>
Abstract

Lignins are second most abundant components of vascular plant cell walls. They provide plants with structural rigidity and are polymers of monolignols. Lignin polymerization is catalyzed by peroxidases and/or laccases. These enzymes are suggested to share functional overlap and mechanism by which they coordinate this process is not clearly understood. There are about 100 peroxidases and 50 laccase genes known in poplar genome out of which some stem differentiating xylem-specific (SDX) enzymes were selected for our study. The main objective was to genetically manipulate genes expressed in the SDX region in the cell wall to see the effects on lignin content in the wood without affecting yield of the transgenic poplar trees. We also aimed to understand the role these enzymes play during lignin polymerization. We also adopted the short tandem target mimic (STTM) strategy to manipulate miRNAs that might be involved in wood development of poplar trees. Our results indicate that peroxidases, laccases and miRNAs under investigation play some specific roles in secondary cell wall biosynthesis. Therefore, manipulation of expression of these genes may prove beneficial towards future genetic engineering of the poplar trees for improved downstream applications.
Chapter 1

Introduction

Plant cell walls are classified into primary and secondary cell walls that provide cellular elasticity and structural rigidity, respectively. The primary cell walls (PCW) are synthesized during cytokinesis, separated by a pectin-rich layer that forms the middle lamella and remodeled during cell expansion. Programmed cell death of specialized cells such as xylem and sclerenchyma that are rich in lignin and hydroxycinnamic acids, gives rise to the secondary cell wall (SCW). Polymers such as polysaccharides and polyphenols are deposited in the secondary cell wall in the form of cellulose, hemicellulose and lignin (Mellerowicz and Sundberg 2008) (Zhong and Ye 2014) (Bashline et al. 2014).

Cellulose are the most abundant plant biomass. They are polymers of β (1, 4)-linked d-glucopyranoses comprising of 15-30% and 40-50% of the primary and secondary cell wall, respectively, making it the first choice for biomass conversion to biofuel such as bioethanol. There have been a lot of studies to genetically manipulate to quantitatively improve and increase the cellulose content in the wood or biomass (Harris, Stork, and Debolt 2009) (Sahoo et al. 2013) and reduce the lignin content owing to its recalcitrant nature to biological degradation (Chen and Dixon 2007). Cellulose is polymerized by cellulose synthase (CESA) enzyme and alteration in this enzyme has shown enhanced saccharification for fermentable sugars (Joshi and Mansfield 2007). Saccharification involves physical, chemical or biological pre-treatment of lignocellulosic material that results in delignified pulp. This pulp is subjected to cellulytic enzymes that hydrolyze
sugar polymers and release simple sugars (monomers) which are further fermented to obtain bioethanol.

Lignins are second most abundant components of vascular plant cell walls after cellulose. Lignins constitute a colossal reservoir of fixed carbon in land plants. Lignins are synthesized from the oxidative coupling of three major monolignols – p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. They provide structural stability, rigidity and hydrophobicity to vascular plants. The proportions of these monomers differ between varied plant species or even within the same species in different organs and cells. This variation is easily observed when comparing gymnosperms and angiosperms. The lignin in gymnosperms comprises of guaiacyl lignin (G units) and p-hydroxyphenyl lignin (H units) polymerized from coniferyl alcohol and p-coumaryl alcohol. The lignin in angiosperms comprises of guaiacyl lignin and syringyl lignin (S units) polymerized from coniferyl alcohol and sinapyl alcohol. Lignin also functions to provide resistance to pathogen infection, biotic and abiotic stresses which pose an obstruction to microbial degradation to convert plant cell wall biomass to biofuel or other bioproducts (also known as recalcitrance). The importance of understanding more about lignin and its biosynthesis increased with the desire to generate transgenic plants with less or more easily digestible lignin for reducing the processing cost involved in producing biofuels (Boudet et al. 2003) (Chen and Dixon 2007) (Nookaraju et al. 2013) (Barros et al. 2015).

Studies have been performed to understand the difference in lignin composition and content by developing a network of reactions involved in the phenylpropanoid metabolism. The core enzymes involved in the biosynthesis of lignin from phenylalanine are
phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-hydroxycinnamate 3-hydroxylase (C3H); caffeoyl CoA 3-O-methyltransferase (CCoAOMT); ferulate 5-hydroxylase (F5H) and caffeic acid 3-O-methyltransferase (COMT), hydroxycinnamate CoA ligase (4CL), hydroxycinnamoyl CoA:NADPH oxidoreductase (CCR) and hydroxycinnamonyl alcohol dehydrogenase (CAD). These enzymes have been functionally characterized and act as catalysts for monolignol production from phenylalanine via a series of intermediary reactions. Downregulation or upregulation of synthesis of one or more of these enzymes in different plants has been attempted to understand the role they play in the biosynthesis process. Results suggest that the regulatory metabolic network for the cell wall components is linked and may play a concerted role in cell wall biosynthesis (Boudet 1998). These modifications in the lignin biosynthesis pathway have also shown to improve saccharification efficiency in the several plant species (Nookaraju et al. 2013). Intensive research has been carried out to understand the regulatory mechanism involved in lignin biosynthesis to bring about a change in the lignin content/composition (Liang, Davis, et al. 2006) (Wang et al. 2008) (Marjamaa, Kukkola, and Fagerstedt 2009) (Li and Chapple 2010) (Wang et al. 2013) (Trumbo, Zhang, and Stewart 2015). This understanding will help generate transgenic trees with altered lignin that can be used for improved biofuel production via saccharification.
Figure 1.1 Schematic representation of intermediary reactions involved in lignin biosynthesis and polymerization pathway

After synthesis, the monolignols are suggested to be transported from the cytosol through the plasma membrane to the cell wall. Co-expression analysis and transcript profiling suggest that ATP-binding cassette (ABC) transporters could be involved in transport of monolignols for free radical based oxidative polymerization catalyzed by laccase and/or peroxidase (Li and Chapple 2010). Polymerization event creates two topographic zones of lignifying cell walls based on the amounts of polymerizing enzyme and substrates. The PCW of xylem parenchyma cells is categorized by increased levels of peroxidase enzyme and substrates such as monolignols and hydrogen peroxide (H$_2$O$_2$). The second zone created by the SCW thickening of xylem vessels is categorized by lower levels of peroxidase and unaltered substrates. This gave rise to two types of polymerization event.
namely, rapid “bulk polymerization” and gradual “end-wise polymerization”. As the name suggests, the former is a fast process and facilitates the C-C coupling of monolignols. This forms highly branched and cross-linked polymers rich in p-coumaryl residues with β-5, β -1, β - β, 5-5 and 5-O-4 inter-unit bonds. The latter is a relatively slow process facilitates β-O-4 coupling of monolignols at pH 5.5 forming linear polymers of lignin in the SCW (Grabber, Hatfield, and Ralph 2003) (Barceló et al. 2004) (Grabber 2005).

The two gene families supposed to be involved in this final step of polymerization of lignin monomers are peroxidases (PRX) and/or laccases (LAC). Monolignol radicals formed by oxidation of monomers are catalyzed by these enzymes. They are large gene families which are said to exhibit overlapping functions. PRXs uses H2O2 whereas LAC uses O2 as electron acceptor to form these radicals that bind with each other/oligomers to form the large lignin macromolecule. Several studies have been performed on a wide range of plants – Arabidopsis, Nicotiana, switchgrass, poplar, and others, to understand the molecular mechanism behind lignin polymerization from monolignol units. Some studies suggest that these enzymes share a functional overlap while others suggest their functions are non-redundant with each other (Zhao et al. 2013b).
However, the mechanism by which plants regulate the polymerization process is not clearly understood and leaves these open questions:

1. Which enzyme drives lignin polymerization? Is it PRX or LAC or a combinatorial action of both these enzymes?
2. Do they play separate non-redundant functions in lignification?
3. What are the effects of PRX and/or LAC genetic manipulations on lignin content in transgenic wood and changes in saccharification efficiency?

Recent studies suggest regulatory role of miRNAs in SCW and wood development and it seemed interesting to look at certain miRNA that will help provide insights to our understanding of cell wall synthesis. Plant microRNAs (miRNAs) are 21nt non-coding RNAs that play a modulatory role in gene expression at the post-transcriptional and translational stages of development. Two miRNAs – miR397a and miR166, stood out to be interesting candidates to study these functions. (Lu et al. 2013) (Trumbo, Zhang, and Stewart 2015). The overexpression of miR397a has been shown to negatively regulate laccases thereby affecting the lignin content in poplar. This led to a coordinated repression of specific laccases expressed in the xylem area and also an upregulation of PRXs in these plants. The miR166 regulates a class of transcription factors called the class III HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIP III) thereby regulating the expression of homeobox (HB) genes that are involved in cambial and wood development. These findings involving the regulation exerted by these two miRNAs in cell wall biosynthesis led to these questions:
1. Can regulation of miRNA gene expression help to understand the role of its target
genes?

2. Can this help to provide further insights into the role of miRNA in wood
development?

Based on these proposed questions for lignin biosynthesis and bioenergy production, the
current study has been designed to understand how the PRX and LAC enzymes, and
miRNAs function with respect to lignin polymerization during SCW formation. This will
help to elucidate whether either of the enzymes, one or none is the rate limiting step in
lignin biosynthesis.

The broad objectives of this study include:

1. Impact of genetic manipulation of PRXs and LACs on lignin content and
   composition of wood from transgenic trees

2. Explore functional redundancy (if any) between the lignin polymerizing enzymes,
   PRXs and LACs in poplar

3. Regulation of specific miRNAs to decipher their role(s) in secondary cell wall
   biosynthesis.

Specific objectives of each chapter to achieve the broader objectives included:

Availability of the entire sequence of the genome, amenable plant transformation
procedures and being a woody tree species made poplar, the model plant to work on for
this project.
Chapter 2: We aimed to manipulate and overexpress specific stem-differentiating xylem (SDX)-specific PRXs in a tissue-specific manner to alter lignin content in poplar trees.

Chapter 3: We adopted the same method as chapter 2, to manipulate and overexpress specific SDX LACs in tissue-specific manner with an aim to alter lignin content in poplar trees. We also aimed at manipulating the expression of miR397a which is supposed to regulate the laccase activity during lignin polymerization and to achieve this, we used the short tandem target mimic (STTM) strategy.

Chapter 4: In this chapter, using STTM strategy, we aimed to understand the regulation exerted by miR166 during cambial and wood development which could be an alternative approach to manipulate wood for bioenergy purposes.
Approaches

Approach 1: There are 93 peroxidases and 49 laccase genes in poplar genome (Lu et al. 2013) (Ren et al. 2014). Based on the experimental and in-silico expression profile in tissues, we selected two SDX PRX and two LAC genes, respectively from these gene families in poplar. We overexpressed the selected genes under developing xylem-specific promoter, DX15 (Ko et al. 2012).

![Diagram of PRX and LAC genes](image)

*Figure 1.2: Graphical representation of selected of PRX and LAC genes for the current study*

Approach 2: We adopted STTM strategy developed by Dr. Guiliang Tang’s group to study loss-of-function of miR397a and miR166 where the expression of these miRNAs was suppressed and the downstream effects on wood development was studied.
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2 Chapter 2

Genetic manipulation of two peroxidase genes in poplars

Abstract

The growing importance of conversion plant biomass into value-added products makes it important to understand in the process of plant cell wall biosynthesis. Over years, significant understanding of lignin biosynthesis has helped pave a way for efficient use of plant biomass for saccharification purposes. But the process of polymerization of lignin still caters to several unanswered questions mainly due to the suggested involvement of two gene families namely, peroxidase (PRX) and laccase (LAC) in this process. Our study aimed at understanding the role PRXs and LACs play in lignin polymerization and to specifically can suggest which of these enzymes are the rate limiting step during this process. For this, we selected and overexpressed two out of six stem differentiating-xylem (SDX)-specific PRX genes – PRX25 and PRX36 using tissue-specific promoter (DX15) to manipulate the expression of PRXs and to study its downstream effects on lignin formation. We transformed our gene constructs into Agrobacterium and performed poplar leaf-disc transformation to generate transgenic poplar plants expressing the PRX genes. The overexpression of PRX25 led to its co-suppression in the PRX25 transgenic lines. The PRX36 gene was overexpressed in PRX36 transgenic lines. We determined that every PRX in SDX has an essential role in lignin polymerization. We were also able to delineate functional characterization of PRXs and LACs in lignin polymerization. Our study could also demonstrate that PRXs and LACs played some specific role in this process and that they did not compensate for each other’s functions. Overall, we observed that manipulation
of PRXs did not significantly change the lignin content but affected the S/G lignin ratio. These alterations brought about structural changes in the plant cell wall leading to improved sugar release but did not affect the plant growth yield. These characteristics are highly desirable for lignocellulosic biomass to be used for downstream bioenergy applications.
2.1 Introduction

Peroxidases are heme-containing enzymes present in all living organisms that utilize hydrogen peroxide (H₂O₂) as an electron acceptor to oxidize the donor molecule. Peroxidases in higher plants are categorized into four types: glutathione peroxidase, catalase, ascorbate peroxidase (intracellular class I peroxidase) and classical secretory class III plant peroxidases (PRXs) (Passardi, Penel, and Dunand 2004) (Shigeto and Tsutsumi 2016). Among, these we will focus here on PRXs that are plant-specific heme-oxidoreductases that are also part of a large gene family. All categories of plant peroxidase enzyme have the similar structural arrangement that comprise of ten α-helices and ferriprotoporphyrin IX as a prosthetic group. In addition to this structure, presence of three extra α-helices, highly conserved amino acids and four disulfide bridges distinguishes class III PRXs from other three types of plant peroxidases. They are present in all land plants throughout their life cycle. They share their involvement in plant developmental and physiological processes such as germination, cell growth, stress tolerance, cell wall cross-linking, lignification, senescence to name a few (Passardi, Penel, and Dunand 2004).

It is speculated that mechanistically, H₂O₂ oxidizes the active site of peroxidase enzyme, the substrate binds to this site and gets oxidized to a phenoxy radical (Barceló et al. 2004).

\[
2RH + H_2O_2 \xrightarrow{\text{Peroxidase}} 2R' + 2H_2O
\]

PRXs are highly substrate specific, exclusively coniferyl alcohol (Guaicyl or G units), and some are specific to sinapyl alcohol (Syringyl or S units). They are further classified into
acidic or basic peroxidases due to differences in their isoelectric points and substrate specificity. Acidic peroxidases or anionic peroxidases are poor catalysts for oxidizing sinapyl alcohol as their activity is hindered because of hydrophobic interactions between methoxy atoms of sinapyl alcohol and conserved regions – I-138 and P-139 in the class III peroxidases. This makes them specific for oxidizing coniferyl alcohol (Aoyama et al. 2002). Basic peroxidases or cationic peroxidases can oxidize almost all the three types of monolignols making them less substrate specific compared to acidic peroxidases. The suggested reason for this may be the substitution of the I-138 by L-138 in most basic peroxidase and thereby reducing the hydrophobic interactions exhibited by sinapyl alcohol. (Østergaard et al. 2000) (Passardi et al. 2005). It is still unknown whether this is the only factor that makes them different in their capability of oxidizing monolignols. Lignin polymers rich in coniferyl and sinapyl alcohol are suggested to be oxidized by basic peroxidases. This has been demonstrated in previous studies that explains the existence of a redox shuttle mechanism exhibited by coniferyl alcohol or p-coumaric acid. The guaiacyl radicals – \( G^- \) from coniferyl alcohol or (p-coumaric acid radicals – \( p\text{-CA}^- \) from p-coumaric acid) are capable of withdrawing \( H^- \) atom from sinapyl alcohol that are catalyzed by either acidic or basic peroxidases. This reaction regenerates coniferyl alcohol (G) or p-coumaric acid (p-CA) and sinapyl alcohol-free radical (S'). But, guaiacyl radicals – \( G^- \) or p-coumaric acid radicals – \( p\text{-CA}^- \) radicals are incapable of withdrawing a \( H^- \) atom from sinapyl oligomers. This reaction and thus, forming condensed polymers are catalyzed by only basic peroxidase. This ability of basic peroxidases to oxidize S oligomers makes them

**Modifications in peroxidases**

For the past several decades, researchers have been trying to categorize the exact functions of PRXs using reverse genetics. In one such approach it was observed that constitutive and increased expression of two anionic PRXs led to induced wilting of leaves through loss of turgor pressure in transgenic tobacco (Lagrimini, Bradford, and Rothstein 1990). Another study demonstrated the effects of heterologous expression of chimeric tobacco anionic PRX (TobAnPOD) in tomato plants. The findings of this study suggested involvement of TobAnPOD in wound-induced phenolic metabolism, lignification and thereby increasing lignin levels in wounded-tissues. But it was also observed that elevated levels of this peroxidase did not help to improve the plants resistance to pathogens (Lagrimini et al. 1993). In 1998, Christensen et al. characterized five xylem-specific anionic peroxidases in poplar and suggested PXP3-4 and PXP5 (PRX7 and PRX22 respectively, according to the new nomenclature) to be likely involved in lignin polymerization (Christensen et al. 1998). Antisense suppression of lignin-specific cationic peroxidase in tobacco (NtPrx60) and anionic peroxidase in aspen (PrxA3a) led to vascular tissue modification and reduction in lignin content and composition (Blee et al. 2003; Li et al. 2003).

PRX isozymes function was mostly dependent on the spatiotemporal regulation of PRX gene expression. This was since these isozymes shared high amino acid sequence similarity and other structural traits. Over the years, biochemical strategies were adopted to identify
and develop a network of function and biochemical properties pertaining to PRX isozymes. These strategies considered characteristics such as substrate specificity, selectivity and mode of action. As stated earlier, lignin polymerization step requires an enzyme that can catalyze the oxidation of four lignin precursors, the three monolignols and growing lignin polymer (Sasaki et al. 2004). One of the most commonly studied PRX protein, horseradish peroxidase isoenzyme C (HRP-C) are found in non-lignified tissues. An *in vitro* study performed demonstrated that HRP-C could actively oxidize coniferyl alcohol i.e. involved in the formation of G-lignin. But it failed to oxidize sinapyl alcohol and other lignin oligomers as these polymers could not fit into the heme pocket of the enzyme owing to their large size. This implies that HRP-C and many similar PRX isoenzymes cannot be involved in lignin polymerization due to lower oxidation activity for sinapyl alcohol. Similar studies have determined PRXs with higher oxidation activity for both coniferyl and sinapyl alcohol. These PRXs play a critical role in dehydrogenative polymerization of sinapyl alcohol and its dimer to lignin polymers (Marjamaa et al. 2006). Cationic cell wall peroxidase (CWPO-C) – a peroxidase isoenzyme from poplar callus can oxidize sinapyl alcohol, synthetic lignin polymers and ferrocytochrome c. Studies indicate that the heme pockets in this isoenzyme are similar in size to other plant PRXs. Instead, these isoenzymes display two tyrosine (Tyr) residue – Tyr-177 and Tyr-74 near the heme-pocket on its protein surface. This site is unique and active oxidation site for this isoenzyme as modifications made to these residues suppresses the oxidation activity (Sasaki et al. 2008). PRXs that are like CWPO-C have been identified in other plant species, for example ZePrx from *Zinnia elegans*, BPX1 from *Betula pendula* exhibiting the same oxidation property. CWPO-C and ZePrx play an essential role in lignification of cell walls over the radical
mediator theory like their putative *Arabidopsis* orthologs (AtPrx71 and AtPrx4, 52 and 72 respectively were involved in lignification (Aoyama et al. 2002) (Sasaki et al. 2004) (Pham et al. 2011) (Shigeto et al. 2013) (Shigeto et al. 2014) (Herrero, Carrasco, and Zapata 2014) (Fernández-Pérez, Pomar, et al. 2015) (Fernández-Pérez, Vivar, et al. 2015). A suggestive ability of CWPO-C, AtPrx2, 25 and 71 in oxidation of large lignin polymers was demonstrated by their capability to oxidize ferrocytochrome c (Shigeto et al. 2014). In addition, AtPrx71 also negatively regulates *Arabidopsis* growth and cell wall alterations promotes accumulation of reactive oxygen species (ROS) that affects cell expansion (Raggi et al. 2015). Enhanced activity of apoplastic peroxidase from barley, *HvPrx8* in tobacco plants revealed that an increase in peroxidase activity can affect lignification of cell walls, programmed cell death (PCD) and elevated expression of the antioxidant ascorbate peroxidase (Burbridge et al. 2014).

Numerous similar studies have been performed to understand the role played by peroxidases in cell wall lignification. The most interesting study demonstrated that the alteration of PRXs could lead to increase in saccharification by 200-300% in tobacco plants (Kavousi et al. 2010) (Cook et al. 2012). But it is still unclear whether the peroxidase activity is the rate limiting step for monolignol polymerization. The current study was designed to alter the lignin content in the developing xylem with an aim to comprehend the role of peroxidases in monolignol polymerization and changes in saccharification efficiency in poplar trees. For this purpose, we selected two stem-differentiating xylem (SDX)-specific peroxidase genes from the 93 known peroxidases in poplar trees (Ren et al. 2014) that were overexpressed under a tissue-specific promoter, DX15 (Ko et al. 2012)
that will ensure the changes caused by the manipulation are only seen in the developing xylem.

2.2 Materials and Methods

2.2.1 Gene selection and vector construction

For this study, out of the 93 peroxidase genes present in *Populus trichocarpa*, we selected two peroxidase genes (PRX25 and PRX36) based on their expression in SDX (Ren et al. 2014) (see Appendix B.1). The full-length amplification of the coding region of these genes was performed using gene specific primers (see Appendix A.1). We used QIAquick PCR purification kit (Qiagen) for clean-up of PCR reactions residual components to purify the amplified gene product. The purified gene products were digested using restriction enzymes *Xba*I/*Pac*I for PRX25 gene and *Bam*HI/*Sac*I for PRX36 gene. Digested fragments were cloned using NEB® 5-alpha competent *E. coli* (high-efficiency) cells into modified pBI101 vector with tissue specific promoter DX15 to replace GUS gene. Cloned plasmid was then transformed into *Agrobacterium* strain C58C1 using freeze thaw method (Boerjan et al. 1997) (Wu, Joshi, and Chiang 2000). The transformed plasmids were selected by antibiotic resistance. Verification PCR for the gene of interest on the selected clones were carried out before transforming the plants. The sequences of the selected clones for DX15, PRX25 and PRX36 genes from plasmid and agrobacterium transformation was also confirmed by sanger sequencing (see Appendix A.2, B.2, B.3, B.4, B.5).
2.2.2 Plant transformation and growth conditions

The positively verified Agrobacterium clones were used to transform *Populus tremula x alba* clone 717-1B4 genotype (routinely referred as 717) using leaf-disc transformation protocol as previously described (Boerjan et al. 1997) (Wu, Joshi, and Chiang 2000). First, the *Agrobacterium* clone is allowed to infect the leaf discs at the wound site (where the leaves have been cut) through several initial steps. Later these infected leaves are allowed to grow on callus induction medium (CIM) for three to four weeks to develop callus (unorganized mass of plant cell). Once callus is formed, they were moved to shoot induction medium (SIM) and then to shoot elongation medium (SEM) for three to four weeks each. Shoots generating from separate calli were considered independent lines. Keeping track of each of these independent lines, they were further moved to root induction medium (RIM) until the shoots developed roots. The callus and shoots were grown in a growth chamber (Thermo Scientific Precision model 818 incubator) with a light and dark cycle of 16 hrs and 8 hrs at 25°C. Plants that rooted were selected and genotypically verified for the presence of the gene of interest using genomic PCR. Verified transgenic lines were acclimatized and, moved and maintained in the greenhouse for approximately 3 months before harvesting. The greenhouse growth conditions were kept similar to the growth chamber with light and dark cycle of 16hrs and 8hrs, temperature of 25°C and relative humidity ranging from 40-70%. Upon harvest of the 13 weeks old plants, all leaf material from each plant was weighed before and after drying and stored in paper bags. The stem was cut into approximately four to five pieces (with ten to fifteen internodes each). The bark was cut open with a sharp blade and developing xylem on the wood material was
scraped in liquid N2, collected in aluminum foil and stored in liquid nitrogen until use. The remaining wood material from each plant was air-dried and stored for further analysis.

2.2.3 RNA extraction and qRT-PCR analysis

Total RNA was extracted from SDX (1-10 internodes counted from the base of the plants with 40-50 internodes) using TRIzol (Ambion, Life Technologies) method. First-strand cDNA was synthesized from 1µg of total RNA using high capacity cDNA reverse transcription kit (Applied Biosystems). qRT-PCR was performed using PowerUp SYBR green master mix (Applied Biosystems). Primers were designed for each PRX gene as listed in Appendix A.1. Each reaction mixture was 12 µl containing 6 µl of SYBR, 1 µl of each primer (1 µM), 1 µl of cDNA template and 3 µl of RNase-free water. The reaction for each gene was conducted in triplicates with thermal cycling conditions as follows: 95°C for 10 mins, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The primer specificity was confirmed by melt curve analysis. The relative gene expression was calculated using the 2^ΔΔCt method. Actin gene was used as an internal control and values were normalized to actin and comparing the normalized values to those of WT plants.

2.2.4 Peroxidase enzyme assay

For peroxidase purification and enzyme assay, we followed (Gafur, Schutzendubel, and Polle 2007) with modifications, where half a gram of SDX was homogenized in 5 ml of cold extraction buffer (10mM Tris-HCl, 10mM NaHCO3, 10mM MgCl2, 0.1mM Na-EDTA, 10mM Mercaptoethanol, 1mM PMSF, 2% polyvinylpolypyrrolidone (PVPP), 0.1% [v/v] Triton X-100) at 4°C. After several centrifugation steps, the supernatant was
finally subjected to column chromatography using Sephadex G-25 column for purifying total peroxidase enzyme from each transgenic line for PRX25 and PRX36. The rest of the assay was conducted in triplicates for each sample. The reaction mixture included potassium phosphate buffer pH 5.25, guaiacol as substrate, purified enzyme and H$_2$O$_2$. The addition of H$_2$O$_2$ initiated the reaction and the change in optical density of peroxidase enzyme using guaiacol was monitored at 436 nm using spectrophotometer every minute starting at time zero for a duration of five mins.

2.2.5 Lignin analysis

Dried wood samples from previously harvested poplar plants were used for the analysis. These wood samples were milled to mesh 20 using Willey-mill. Each sample weighing 100 mg was placed in microfuge tubes and sent to National Renewable Energy Laboratory (NREL) for pyrolysis/molecular beam mass spectrometry (Py-MBMS) (Kelley et al. 2004). Each sample was analysed in duplicates for this procedure.

2.2.6 Microscopic image analysis

For microscopy, stems of poplar plants were cut at the tenth internode from the apex of the plant with 40-50 internodes and were fixed in cold FAA (formaldehyde – acetic acid – ethanol) solution. The internode was vacuum infiltrated for 15-20 mins and stored with fresh cold FAA solution overnight at 4°C. After fixation, these internodes were dehydrated and embedded in wax. Seven-micrometer-thick consecutive sections were cut with a microtome, mounted on a slide and de-waxed using xylene and ethanol series. Lignin
autofluorescence from de-waxed sections was photographed using fluorescent light imaging.

2.2.7 Saccharification assay

For performing saccharification assay to estimate glucose release, we followed (Kumar and Turner 2015) with some modifications. Ten milligrams of dried and milled wood samples from previously harvested poplar plants were used for the analysis. Alcohol insoluble residue (AIR) samples were prepared, treated with acetonitrile solution (acetic acid: nitric acid: water 8:2:1) at 100°C for 30 mins. These samples were further subjected to sulfuric acid swelling. Sugar release was measured by anthrone method by monitoring the reactants' solution absorbance at 620 nm. Using 10-250 µg µl⁻¹ of glucose, the standard curve regression line was generated and the glucose release in each sample was calculated.

2.2.8 Growth data analysis

Growth data for height, leaf count and stem girth were measured for all the generated transgenic lines every week (for 13 weeks) until the day of harvest.

2.3 Results

2.3.1 Tissue level expression of PRXs

We tested and compared tissue level expression of all SDX PRXs with in-silico data from (Ren et al. 2014) as well as data obtained from Poplar eFP browser provided in Bio-Analytic Resource for plant biology (BAR) (see Appendix B.1) Out of 93 poplar PRXs, only six were SDX PRXs. Of which, two PRXs – PRX25 and PRX36 were selected for the
experiments as their expression was highest in the developing xylem (DX). The full-length coding region of these PRXs expressed under tissue-specific promoter DX15 (PtrDX15 – Potri.009G012200) were amplified, cloned and transformed into poplar 717 WT plants (Phytozome v12.1 accession numbers: PtrPRX25 – Potri.004G015300, PtrPRX36 – Potri.005G195600). The 13-week old harvested transgenic trees were tested for change in gene expression of PRX25 and PRX36 compared to WT plants.

2.3.2 Gene expression studies in PRX25 and PRX36 transgenic lines

The relative gene expression of PRX25 and PRX36 were quantified using qRT-PCR. It was observed that the attempted over-expression of the PRX25 led to the major down regulation of this gene (co-suppression) in transgenic poplar lines when compared to WT (Figure 2.1 a). We also tested the relative expression of other SDX PRXs – PRX36, PRX12, PRX33, PRX22, PRX23 in these lines. As shown in Figure 2.2 (a-e), an increase in the expression of PRX36, decrease in the expression PRX33 and no significant changes in PRX12, PRX22 and PRX33 was observed in PRX25 transgenic lines.

**Figure 2.1: Relative gene expression of PRX25 and PRX36 in developing xylem of harvested poplar transgenic lines.** (a) relative expression of PRX25 gene in PRX25 transgenic lines (b) relative expression of PRX36 gene in PRX36 transgenic lines, compared to WT plants. Significant compared to WT, ** P-value < 0.009, *** P-value < 0.0001.
The attempted over-expression of PRX36 lines led to a significant increase in the expression of this gene in the six independent lines (Figure 2.1 b). Similarly, we tested the expression of other five SDX PRXs in PRX36 lines (Figure 2.3 a-e). We observed an increase in PRX25 gene expression, decrease in the expression of PRX23 and PRX33 and no significant change to PRX12 and PRX22 gene expression. To further support these findings, we next tested the total enzyme activity in DX of all lines in PRX25 and PRX36.
Figure 2.2: Relative gene expression of other SDX PRXs in developing xylem of harvested PRX25 transgenic lines. (a) relative expression of PRX36 gene in PRX25 transgenic lines. (b) relative expression of PRX12 gene in PRX25 transgenic lines. (c) relative expression of PRX33 gene in PRX25 transgenic lines. (d) relative expression of PRX22 gene in PRX25 transgenic lines. (e) relative expression of PRX23 gene in PRX25 transgenic lines, compared to WT plants. Significant compared to WT, * P-value < 0.04, ** P-value < 0.0076, *** P-value < 0.0007.
Figure 2.3: Relative gene expression of other SDX PRXs in developing xylem of harvested PRX36 transgenic lines. (a) relative expression of PRX25 gene in PRX36 transgenic lines. (b) relative expression of PRX12 gene in PRX36 transgenic lines. (c) relative expression of PRX33 gene in PRX36 transgenic lines. (d) relative expression of PRX22 gene in PRX36 transgenic lines. (e) relative expression of PRX23 gene in PRX36 transgenic lines, compared to WT plants. Significant compared to WT, * P-value < 0.04, ** P-value < 0.0076, *** P-value < 0.0007

2.3.3 Effect on total peroxidase enzyme activity

Purified peroxidase enzyme was extracted from the DX from WT and transgenic PRX25 and PRX36 lines. The enzyme activity was tested for a period of five minutes using guaiacol as substrate for peroxidase. A decrease in total peroxidase enzyme activity was observed in PRX25 lines when compared to WT as shown in Figure 2.4 (a). Down
regulation of PRX25 gene can be suggested as a reason for the decrease in enzyme activity. The increase in PRX36 gene in PRX25 lines did not seem to contribute to increase the activity. As shown in Figure 2.4 (b), an increase in peroxidase activity was observed in PRX36 lines on comparison with WT. The overall increase in total enzyme activity in these lines can be suggested to be due to the increase in expression of PRX36 and PRX25.

Figure 2.4: Quantification of peroxidase activity of purified SDX proteins from WT and transgenic poplar lines using guaiacol as substrate. (a) decrease in peroxidase activity in PRX25 lines (b) increase in peroxidase activity in PRX36 lines, compared to WT plants. Significant compared to WT, *** P-value < 0.0001.

2.3.4 Effect on xylem formation

If peroxidase enzyme plays a significant role in lignin polymerization, the changes in gene expression could also lead to changes in the development of xylem. We, therefore, looked at the stem sections of the tenth internode from the apex in transgenic lines and compared it to WT. Autofluorescence images revealed a significant decrease in the DX region in PRX25 lines as shown in Figure 2.5 (a and b). We also observed decreased autofluorescence in the phloem and sclerenchyma region when compared to wild type (Figure 2.5 a and b). We also calculated the area of xylem region in these lines and an apparent decrease in this region was observed (Figure 2.5 d).
In contrast to the gene expression and enzyme activity findings, the xylem region was significantly reduced in the PRX36 lines (Figure 2.5 a and c). A drastic reduction in the DX rays with reduced to no autofluorescence in the phloem and sclerenchyma of these lines. The area of the xylem region was significantly reduced when compared to WT (Figure 2.5 e).

Figure 2.5: Autofluorescence of lignin in stem sections and estimated xylem area of harvested WT and transgenic poplar lines. Cross section of (a) WT, (b) PRX25 transgenic lines, (c) PRX36 transgenic lines, (d) area of xylem in PRX25 transgenic lines, (e) area of xylem in PRX36 transgenic lines, compared to WT plants. Significant when compared to WT, *** P-value < 0.0001.

2.3.5 Effect of gene manipulation on lignin content

The entire experiment was designed to increase the lignin content in poplar trees and to understand how PRXs plays a role in lignin polymerization. To evaluate the lignin content, we used dried and milled wood between the first and second internode from the base of the tree. Lignin analysis was carried out by Py-MBMS for WT and transgenic lines. The PRX25 lines showed no significant change in lignin content but a significant decrease in
syringyl/guaiacyl (S/G) lignin ratio was observed in these lines as seen in Figure 2.6 (a) and (c), respectively. In PRX36 transgenic lines, there was an overall decrease in lignin content between 3-6% when compared to WT with no significant change in S/G lignin ratio in these lines (Figure 2.6 b and d). These changes could possibly explain the changes in the xylem region respective to the PRXs as mentioned in the previous section. Since, we observed a change in lignin content/composition, we further decided to test the sugar release in these trees.

2.3.6 Saccharification assay in PRX25 and PRX36 lines

We performed the saccharification assay using the anthrone method to test the sugar release in PRX25 and PRX36 lines. With changes in lignin in these lines, we expected to observe
a change in the sugar yield. As expected, there was an overall increase in the sugar release by 40-65% in both PRX25 and PRX36 lines as shown in Figure 2.7 (a) and (b).

![Figure 2.7: Glucose release assay of WT and transgenic poplar lines using anthrone method. Percent change of glucose release in (a) PRX25 transgenic lines (b) PRX36 transgenic lines, compared to WT. Significant when compared to WT, *** P-value < 0.0001.]

### 2.3.7 Effect on biomass and growth of PRX25 and PRX36 lines

Apart from molecular and biochemical analysis, we also studied several phenotypic characteristics in all transgenic lines and compared them to WT. Change in leaf biomass, stem girth, plant height and leaf count were the four traits that we analyzed for this study.

Upon harvest, we weighed the leaf weight that accounted as wet biomass. These leaves were then placed in paper bags, dried and weighed again which accounted as dry biomass. This was done for all controls, transgenics and their biological replicates. We observed slight or no significant change in the wet/dry leaf biomass in PRX25 and PRX36 lines as compared to wild type (Figure 2.8 a and b).
The other traits were monitored weekly (week 0 – week 13, until harvest). Significant changes in height and leaf count of PRX25 lines was observed with no significant change in stem girth. No significant changes were observed in PRX36 transgenic lines when compared to WT (Figure 2.9 a-f). The changes in gene expression and lignin content did not seem to affect the plant growth rate and was like that of WT for the 13 week-period.
Figure 2.9 a: Estimated height (in cm) of WT and PRX25 transgenic lines over a period of 13 weeks until harvest.

Figure 2.9 b: Estimated leaf count of WT and PRX25 transgenic lines over a period of 13 weeks until harvest.
Figure 2.9 c: Estimated stem girth (in mm) of WT and PRX25 transgenic lines over a period of 13 weeks until harvest.

Figure 2.9 d: Estimated height (in cm) of WT and PRX36 transgenic lines over a period of 13 weeks until harvest.
Figure 2.9 e: Estimated leaf count of WT and PRX36 transgenic lines over a period of 13 weeks until harvest.

Figure 2.9 f: Estimated stem girth (in mm) of WT and PRX36 transgenic lines over a period of 13 weeks until harvest.
2.3.8 Correlation analysis

Data from all the experiments were correlated to establish a relationship pattern between different factors using the correlation data analysis feature on Microsoft Excel. In summary, the correlation analysis for PRX25 lines suggested a positive correlation between gene expression and enzyme activity, gene expression and lignin content, lignin content and glucose release and, S/G lignin ratio and lignin content. A negative correlation was observed between lignin content and enzyme activity and, S/G lignin ratio and glucose release when compared to WT. For PRX36 lines, a positive correlation was observed between gene expression and enzyme activity and, S/G lignin ratio and lignin content. A strong negative correlation was observed between gene expression and lignin content, lignin content and enzyme activity, lignin content and glucose release and, S/G lignin ratio and glucose release when compared to WT (Figure 2.10 (a) and (b), Figure 2.11 (a) and (b)).
Figure 2.10: Correlation analysis of relative gene expression, peroxidase enzyme activity and lignin content in WT and transgenic poplar lines. (a) PRX25 transgenic lines (b) PRX36 transgenic lines, compared to WT
Lignins are heteropolymers belonging to the phenylpropanoid family. They have an essential role in maintaining cellular plasticity and rigidity in plant cell wall. About ten different enzymes participate for lignin biosynthesis and produce lignin monomers, termed as monolignols (Nookaraju et al. 2013). Oxidative coupling of the three monolignols (p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol) catalyzed by either peroxidase
and laccase enzymes gives rise to lignin polymers. Lignin polymers form complexes with other polysaccharides within the plant cell wall giving plants its tensile structure. The sustainable nature of the wood obtained from plants makes it desirable for paper and pulp industry, building construction and as biomass for energy production, to name a few. Making the wood readily available for such purposes i.e. increasing cellulose accessibility and reducing or altering lignin/hemicellulose content) involves the use of chemicals and bleaching agents that are considered as environmental pollutants. Through several decades, efforts have been made to engineer plants with less lignin to decrease the innate biomass recalcitrance and making the process more cost effective. Though lignin is credited as a hindrance for wood degradability, there are several other beneficial applications of lignin for generation of value-added products, for instance, energy conversion from biomass into chemicals and fuels, production of carbon fiber and polymers, use as additives and its applications in pharmaceutical sciences (Lange, Decina, and Crestini 2013) (Ragauskas et al. 2014).

Owing to the growing importance of lignin, the current study aimed to interpret the role PRXs during lignin polymerization and the impact of the changes in expression of SDX PRXs on lignin quantity and sugar releases from the plant cell wall. Peroxidases are speculated to function at several developmental stages in plants from germination to senescence. For the past several decades attempts have been made to delineate the exact role Class III heme-PRXs play in lignin polymerization. Also, this understanding will help to generate plants with improved lignin characteristics for the above stated applications. Peroxidases are classified based on their isoelectric point into acidic (anionic) and basic
(cationic) peroxidases with the capability to consume H$_2$O$_2$ and release reactive oxygen species (Passardi, Penel, and Dunand 2004). Previous studies have demonstrated competence of basic PRXs over acidic PRXs due to substrate specificity to catalyze the polymerization of SA and other synthetic lignin oligomers. The substitution of I-138 with L-138 or the presence two Tyr residue (Tyr-74 and Tyr-177) near the heme pockets are the speculated the reasons for this competency.

We attempted the overexpression of PRX25 and PRX36 using xylem-specific promoter in poplar trees. Overexpression of PRX25 led to the co-suppression of this gene in PRX25 transgenic lines which is a known phenomenon called post-transcriptional gene silencing (PTGS) and first defined in plants in the year 1990 as co-suppression (Van der Krol et al. 1990) (Napoli, Lemieux, and Jorgensen 1990). Co-suppression leads to sequence-specific degradation of endogenous mRNA and acts as an epigenetic regulation that has been studied in several plant species (Atanassova et al. 1995) (Burch-Smith et al. 2004) (Jung et al. 2012). The expression analysis of PRX25 corroborated with the total peroxidase enzyme activity where we observed a severe reduction in the activity (Figure 2.4). The co-suppression of PRX25 seems to have affected the xylem development evident by the reduction in the xylem area and reduced autofluorescence in the phloem fibers in these lines that are rich in S-lignin units (Figure 2.5). This reduction in autofluorescence correlates with the decreased S/G lignin ratio though the lignin content was unaffected. The reduction in S/G lignin ratio also makes us to speculate the importance of the presence of this gene to oxidize S-lignin monomers or oligomers. For instance, the expression of PRX36 gene was high in PRX25 transgenic lines but this did not help to maintain the S/G
ratio nor help with xylem formation. We strongly believe that the changes observed in these lines are strictly due to these changes in gene expression of PRX25 because none of the six SDX PRX share gene sequence similarity (see Appendix B.6, B.7, B.8) and therefore would fail to replace any function carried out by the other PRXs. These findings suggest that PRX25 could be a basic peroxidase and it would be interesting to demonstrate either substitution of I-138 with L-138 or the presence two Tyr residue (Tyr-74 and Tyr-177) near the heme pockets in PRX25 to confirm this finding.

The genetic manipulation of PRX36 gene led to significant increase in the expression of PRX36 gene with an increase in the total peroxidase enzyme activity in the PRX36 transgenic lines. With this increase in gene expression of PRX36, we expected to see an increase in the xylem area and lignin content. But contrary to our expectations, we observed a severe reduction in the xylem area and the phloem fibers almost non-existing in these lines (Figure 2.5). The lignin content in these lines showed a downward trend but was not statistically significant. The S/G lignin ratio showed reduction in a few plants with significant increase in glucose release about 40-60%. To sum-up these findings, it can be said that modification of PRX36 did not affect the lignin content. But, the overexpression of PRX36 could be detrimental for lignin production and xylem intactness in SCW.

Studies have demonstrated alteration to the S/G ratio have improved pulpability and improved downstream bioconversion of biomass. We further speculate, the changes in S/G lignin ratio brought about structural changes within the cell wall that led to increased sugar release thereby improving saccharification efficiency in these transgenic PRX lines (Figure 2.7). Previous studies have demonstrated genetically engineered plants with increased
saccharification efficiency but resulting in developmental deformities in plants which limits the use of these plants for bioenergy purposes. We succeeded in generation of plants with improved saccharification efficiency, exhibiting normal phenotype like WT and displays no growth penalty.

We also performed gene expression analysis of SDX LACs in the PRX25 and PRX36 lines which revealed decreased expression of LAC18 and LAC14 in these lines. The expression of LAC27 seemed to be high in both the PRX25 and PRX36 lines (Figure 2.12 a-f). Though previous gene expression studies suggest that LAC27 is one of the highly expressed SDX LAC in poplar developing xylem, our study suggests that LAC27 does not replace the function of these PRXs. This is evident by the reduction in xylem area and autofluorescence of phloem fibers and sclerenchyma, we observed in PRX25 and PRX36 lines. Therefore, suggesting specific role of PRXs and LACs in lignin polymerization with no functional overlap.
Figure 2.12: Relative gene expression of SDX LACs in developing xylem of harvested PRX25 and PRX36 transgenic lines. (a) relative expression of LAC27 gene in PRX25 transgenic lines. (b) relative expression of LAC18 gene in PRX25 transgenic lines. (c) relative expression of LAC14 gene in PRX25 transgenic lines. (d) relative expression of LAC27 gene in PRX36 transgenic lines. (e) relative expression of LAC18 gene in PRX36 transgenic lines. (f) relative gene expression of LAC14 in PRX36 transgenic lines, compared to WT plants.

Conclusion

In conclusion, this study establishes an effort in characterizing the role of PRXs in polymerization of lignin. Based on the gene expression analysis, enzyme assay and wood analysis, it provides proof that explains proportional differences in expression of PRX/LAC. Thus, suggesting that each of these enzymes has a unique role to play in lignin
polymerization and that they do not take over each other’s functions i.e. they could be non-redundant. Nevertheless, this study also stands out as a promising approach to genetically engineer lignin for saccharification purposes without yield penalty as the manipulation of PRXs did not show any morphological defects in plants.

**Summary of manipulation of PRXs**
2.5 Acknowledgements

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3 Chapter 3

Genetic manipulation of secondary wall-specific laccase genes in poplars

Abstract

Lignification takes place in the xylem tissue during plant cell wall differentiation that provides plants with structural stability. In this study, we aimed at understanding the molecular mechanism behind the functioning of laccase gene family which are said to be involved in the lignin polymerization process. We selected and overexpressed two SDX LAC – LAC27 and LAC18 genes using tissue-specific promoter to manipulate the process of lignin formation. To gain comprehensive knowledge about laccase activity, we also adopted the STTM technology to study the effect of loss-of-gene function of miR397a which are suggested to be negative regulators of laccase gene expression. Poplar leaf-disc transformation helped to generate transgenic plants with gene of interest. Gene expression analysis suggested that manipulation of LACs did not significantly change the lignin content but affected the S/G lignin ratio. The manipulation of miR397a led to suppression of miR397a gene in poplars and seemed to increase the S/G lignin ratio. These changes brought about minor structural changes in the plant cell wall leading to improved sugar release but did not affect the plant growth yield making the wood highly desirable for bioconversion of lignocellulosic material with enhanced saccharification efficiency.
3.1 Introduction

Laccases are copper-containing oxidoreductases with highly conserved histidine motifs. They are broadly classified as fungal and plant laccases depending upon their origin. They can oxidize a broad range of aromatic/non-aromatic compounds as they are not substrate specific. Oxygen acts as the electron acceptor and gets reduced to water in the process.

\[
4\text{AH} + \text{O}_2 \xrightarrow{\text{Laccase}} 4\text{A}^\cdot + 2\text{H}_2\text{O}
\]

Plant laccases are highly glycosylated. They are active in the pH range of 5-7.5. Though little is known about plant laccases, polymerization of phenols is assumed to be an act of defense in plants (Ranocha et al. 1999) (Berthet et al. 2012). Earlier studies suggested that laccases do not play a role in lignification in plants (Harkin and Obst 1973). However, later studies suggested their role in lignification where, in the absence of hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), they catalyze a reaction to polymerize monolignols under in-vitro conditions in maple (Driouich et al. 1992) (O'Malley et al. 1993). A similar in-vitro study in sycamore maple suspension cells and loblolly pine xylem suggested a combined role of peroxidase and laccase in dehydrogenation of lignin that contributes to lignin heterogeniety in plant cell walls (Sterjiades, Dean, and Eriksson 1992) (Bao et al. 1993). Therefore, it became increasingly important to understand how laccases could oxidize lignin monomers. A comparison analysis of encoded nucleotide sequences of cDNA clones for laccase genes from sycamore maple and tobacco stem helped to discern this function. The conserved copper-binding domains identified from both the species shared only 48% sequence similarity suggestive of the physical and functional divergence exhibited by laccases from...
different species (LaFayette, Eriksson, and Dean 1995) (Kiefer-Meyer et al. 1996). Overexpression of a laccase (LtLacc2.1-2.4) in yellow poplar (Liriodendron tulipifera) and tobacco (Nicotiana tabacum) cells showed no effect on lignification (LaFayette, Eriksson, and Dean 1999). Downregulation of laccases in poplar with the help of antisense RNA technology led to increased levels of soluble phenols and irregular xylem fibers, but lignin content or composition was unaltered. This study also suggested that a clear connection could not be derived between laccase expression and lignification process, though laccase could be involved to maintain the xylem integrity in the plant cell wall (Ranocha et al. 2002). Mutant analysis of laccase gene in Arabidopsis (AtLAC15) showed decrease in laccase activity as well as in lignin content (Liang, Haroldsen, et al. 2006). Another study generated transgenic poplar with heterologous overexpression of cotton laccase (GaLAC1) that showed a 2.1–13.2-fold increase in laccase activity as well as 2–20% increase in lignin compared to WT (Wang et al. 2008). An extensive study on polymerization of different lignins gave insights on the capability of laccases to oxidize lignin without mediators. It was also determined that the laccase activity is pH dependent, they are capable of polymerizing different lignins and this efficiency was measured by analyzing the degree of polymerization (Mattinen et al. 2008). Arabidopsis have been identified with 17 laccases gene family members and classified into six groups based on sequence similarity (McCaig, Meagher, and Dean 2005). In a few studies using laccase double mutants (lac4-2 lac11-1, lac 4-2 lac17-1 and lac11-1 lac17-1) and triple mutant (lac4, lac11 and lac17) in Arabidopsis, growth defects in these mutants were observed due to monolignol toxicity, extensive loss of lignin causing vascular function impairment and deposition of G units was affected. Some studies also suggested that they function during early xylem
development and peroxidases function in the later stages of xylem development. That study also suggested that laccase and peroxidase serve non-redundant functions in lignification of vascular tissues of stem and roots wherein laccase and peroxidase are involved in the initial and later stages of monolignol polymerization respectively (Zhao et al. 2013a). Poplar has 49 laccases when compared to 17 laccases in Arabidopsis, making it difficult to characterize the role of each laccase. Few recent studies stated below helps to provide an understanding of how laccase could be involved with the lignification process. A unique system comprising of fluorescently tagged monolignol analogs (γ-nitrobenzofuran, NBD-tagged coniferyl alcohol, CA) and VND-7 induced protoxylem system was used to demonstrate that laccase (LAC4 and LAC17) were the essential participant to direct lignification of protoxylem tracheary elements (TE) in Arabidopsis (Schuetz et al. 2014). Mutant analysis of LAC5 and LAC6 in Brachypodium distachyon showed reduced lignin, an increase S-lignin due to change in S/G lignin ratio and improved saccharification efficiency thus revealing the reduced recalcitrance of grass lignocelluloses (Wang et al. 2015). In a recent study, using RNAi to downregulate PdLAC2 from Populus deltoides showed an increase in the S/G lignin ratio with unaltered lignin content when compared to control lines. These RNAi mediated knockdown lines also exhibited reduced recalcitrance to pre-treatment procedures with an increase in the release of five and six carbon sugar (Bryan et al. 2016). These studies suggest that the involvement of laccase may or may not affect the lignin content in the cell walls with an improvement in the saccharification efficiency. Though, it is still a puzzle as to how each enzyme within the gene family functions during this process.
Role of miR397 in lignin biosynthesis

MicroRNAs (miRNAs) are a class of 20-21 nucleotide (nt) long, non-coding upstream gene regulators that are found in almost all eukaryotic organisms. They play an essential role in modulating gene expression at several developmental stages of an organism at pre- and post-transcriptional level, translational modifications and epigenetic factor regulation. Thousands of miRNAs have been identified in plants and animals and functions of many such regulators are still unknown (Gielen et al. 2012) (Zhou and Luo 2013).

One such plant miRNA was identified to play a role in lignin biosynthesis is miR397a as it specifically targets laccase genes involved in lignin polymerization. Computational analysis suggested that miR397 targets laccases such as LAC2, LAC4 and LAC17 and regulates the lignin biosynthesis in *Arabidopsis thaliana* (Berthet et al. 2011). Significant reduction of lignin in poplar was observed due to constitutive overexpression of a regulatory miRNA (miR397a) leading to general silencing of thirteen stem differentiating xylem (SDX) laccases in transgenic poplar (Lu et al. 2013). It was determined that *Ptr*-miR397a and *Ath*-miR397b were negative regulators of laccase gene expression and affected the lignin content in *Populus* and *Arabidopsis*, respectively (Lu et al. 2013) (Wang et al. 2014). These studies helped to provide an insight to understand plant laccases, to a certain extent and the role they play in lignification by exploring the post-transcriptional regulation exerted by miR397 in different plant species.

In poplar, seventeen out of the total forty-nine laccases are abundantly expressed in the SDX. Studies performed on these SDX laccases suggested that the expression of thirteen
PtrLACs (PtrLAC2, 7, 11, 12, 14, 15, 18, 23, 24, 26, 30, 40, and 49) was regulated by miR397a. The other four – PtrLAC17, 19, 25, and 27 were not targets of this miRNA. For the current study, we selected PtrLACs based on their expression level in the SDX (Lu et al. 2013), their phylogenetic similarity with previously studied AtLACs and whether these LACs were targets of miR397a that indirectly affected the lignification. We selected and overexpressed PtrLAC27, which is the most abundant laccase present in the SDX, it belonged to clade one and closely related with AtLAC4 and AtLAC11 and it was not a target of miR397a. Next, we also selected PtrLAC18 which was a target of miR397a but was expressed at lower levels compared to PtrLAC27. We also used the short tandem target mimic (STTM) technology to target miR397a which has been successfully used in previous studies to analyze the regulatory functions exhibited by several miRNAs in plants and animals (Tang et al. 2012) (Yan et al. 2012). The STTM designed to target miR397a will block and knockdown the expression of these miRNAs in poplar trees and in turn will not control expression of laccases. Due to loss of function of this specific miRNA, we expect to see alterations in laccase gene regulation and consequently in lignin polymerization. This study was also developed to demarcate the functional role of laccase and peroxidase enzymes involved in lignin polymerization as an extended hypothesis from chapter one. We expect this study to help provide additional information on the significant role played by these two gene families in wood development in poplar.
3.2 Materials and Methods

3.2.1 Gene selection and vector construction

For this study, out of the forty-nine laccase genes present in *Populus trichocarpa*, we selected two laccase genes (LAC27 and LAC18) based on their expression in SDX (Lu et al. 2013) (see Appendix C.1). The full-length amplification of the coding region of these genes was performed using gene specific primers (see Appendix A.1). We used QIAquick PCR purification kit (Qiagen) for clean-up of PCR reactions residual components to purify the amplified gene product. The purified gene products were digested using restriction enzymes *Bam*HI/*Sac*I for LAC27 and LAC18. Digested fragments were cloned using NEB® 5-alpha competent *E. coli* (high-efficiency) cells into modified pBI101 with tissue specific promoter DX15 to replace GUS gene.

Cloned plasmid was then transformed into *Agrobacterium* strain C58C1 using freeze thaw method (Boerjan et al. 1997; Wu, Joshi, and Chiang 2000). The transformed plasmids were selected for antibiotic resistance. Verification PCR for the gene of interest on the selected clones were carried out before transforming the plants. The sequences of the selected clones for LAC27, LAC18 and miR397a genes from plasmid and agrobacterium transformation was also confirmed by sanger sequencing (see Appendix C.2, C.3, C.4, C.5).

3.2.2 Plant transformation and growth conditions

The positively verified *Agrobacterium* clones were transformed into *Populus tremula* x *alba* clone 717-1B4 genotype (routinely referred as 717) using leaf-disc transformation
protocol previously described in (Boerjan et al. 1997; Wu, Joshi, and Chiang 2000). First, the Agrobacterium clone is allowed to infect the leaf discs at the wound site (where the leaves have been cut) through several initial steps. Later these infected leaves are allowed to grow on callus induction medium (CIM) for three to four weeks to develop callus (unorganized mass of plant cell). Once callus is formed, they were moved to shoot induction medium (SIM) and then to shoot elongation medium (SEM) for three to four weeks each. Shoots generating from each callus was considered as an independent line. Keeping track of each of these independent lines, they were further moved to root induction medium (RIM) until the shoots developed roots. The callus and shoots were grown in a growth chamber (Thermo Scientific Precision model 818 incubator) with a light and dark cycle of 16 hrs and 8 hrs at 25°C. Plants that rooted were selected and genotypically verified for the presence of the gene of interest using genomic PCR. Verified transgenic lines were acclimatized and, moved and maintained in greenhouse for approximately 3 months before harvesting. The greenhouse growth conditions were kept similar to the growth chamber with light and dark cycle of 16hrs and 8hrs, temperature of 25°C and relative humidity ranging from 40-70%. Upon harvest of the 13-week old (LAC27 and LAC18) and 16-week old (STTM:miR397a) plants, all leaf material from each plant was weighed before and after drying and stored in paper bags. The stem was cut into approximately four to five pieces (with ten to fifteen internodes each). The bark was cut open with a sharp blade and developing xylem on the wood material was scraped in liquid N2, collected in aluminum foil and stored in liquid nitrogen until use. The remaining wood material from each plant was air-dried and stored for further analysis.
3.2.3 RNA extraction and RT-PCR analysis

Total RNA was extracted from SDX (1-10 internodes counted from the base of the plants with 40-50 internodes) using TRIzol (Ambion, Life Technologies) method. First-strand cDNA was synthesized from 1µg of total RNA using high capacity cDNA reverse transcription kit (Applied Biosystems). qRT-PCR was performed using PowerUp SYBR green master mix (Applied Biosystems). Primers were designed for each LAC gene as listed in Appendix A.1. Each reaction mixture was 12 µl containing 6 µl of SYBR, 1 µl of each primer (1 µM), 1 µl of cDNA template and 3 µl of RNase-free water. The reaction for each gene were conducted in triplicate with thermal cycling conditions as follows: 95°C for 10 mins, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The primer specificity was confirmed by melt curve analysis. The relative gene expression was calculated using the 2−ΔΔCt method. Actin gene was used as an internal control and values were normalized to actin and comparing the normalized values to those of WT plants.

3.2.4 Laccase enzyme assay

For laccase purification and enzyme assay, we followed (Berthet et al. 2011) (Lu et al. 2013) with modifications, where one gram of SDX was homogenized in 10 ml of extraction buffer [25 mM Tris, pH 7.0, 200 mM CaCl₂, 10% (vol/vol) glycerol, 4 µM sodium cacodylate, 1 mM PMSF and 1/200 (vol/vol) protease inhibitor cocktail (P-9599); Sigma-Aldrich]. After several extraction and centrifugation steps, the supernatant was finally subjected to affinity column chromatography using Concanavalin-A Sepharose beads. Methyl-α-glucopyranoside was used to elute proteins from each transgenic line for LAC27,
LAC18 and STTM:miR397a as stated in (Berthet et al. 2011). The rest of the experiment was conducted in triplicates for each sample. The generation of stable cationic radical by laccase enzyme during the oxidation of ABTS was measured at 420 nm for 5 mins using a spectrophotometer.

3.2.5 Lignin analysis

Dried wood samples from previously harvested poplar plants were used for the analysis. These wood samples were milled to mesh 20 using Willey-mill. Each sample weighing 100 mg were placed in microfuge tubes and sent to NREL for pyrolysis-molecular beam mass spectrometry (Py-MBMS) (Kelley et al. 2004). Each sample was analyzed in duplicates for this procedure.

3.2.6 Microscopic image analysis

For microscopy, stems of poplar plants were cut at tenth internode from the apex of the plant with 40-50 internodes and were fixed in cold FAA (formaldehyde – acetic acid – ethanol) solution. The internode was vacuum infiltrated for 15-20 mins and stored with fresh cold FAA solution overnight at 4°C. after fixation, these internodes were dehydrated and embedded in wax. Seven-micrometer-thick consecutive sections were cut with a microtome, mounted on a slide and de-waxed using xylene and ethanol series. Lignin autofluorescence from de-waxed sections was photographed using fluorescent light imaging.
3.2.7 Saccharification assay

For performing saccharification assay to estimate glucose release, we followed (Kumar and Turner 2015) with some modifications. Ten milligrams of dried and milled wood samples from previously harvested poplar plants were used for the analysis. Alcohol insoluble residue (AIR) samples were prepared, treated with acetonitrile solution (acetic acid: nitric acid: water 8:2:1) at 100°C for 30 mins. These samples were further subjected to sulfuric acid swelling. Sugar release was measured by anthrone method by monitoring the reactants’ solution absorbance at 620 nm. Using 10-250 µg µl\(^{-1}\) of glucose, the standard curve regression line was generated and the glucose yield in each sample were calculated.

3.2.8 Growth data analysis

Growth data for height, leaf count and stem girth were measured for all the generated transgenic lines every week (for 13-weeks – LAC27 and LAC18 lines, for 16-weeks – STTM:miR397a lines) until the day of harvest.

3.3 Results

3.3.1 Tissue level expression of LACs

We compared the tissue level expression of all SDX LACs with in-silico data from (Lu et al. 2013) as well as data obtained from Poplar eFP browser provided in Bio-Analytic Resource for plant biology (BAR) (see Appendix C.1). Out of the 17 SDX LACs, we verified the presence of six SDX LACs in six different regions of WT poplar – apex, young leaf (YL), mature leaf (ML), young stem (YS), mature stem (MS) and developing xylem (DX). We selected LAC27 and LAC18 after the expression analysis for the following
reasons. First, they are among the highly expressed LACs (see Appendix C.1), they belong to different clades in the phylogenetic tree – LAC27 in clade 1 and LAC18 in clade 5 (Lu et al. 2013). Lastly, among the two LACs, LAC27 is not a target of miR397a whereas LAC18 is a target of miR397a. The miR397a is said to act as a negative regulator of SDX LACs and indirectly play a role in suppressing lignin polymerization. The full-length coding region of these LACs expressed in modified pBI101 under DX15 promoter were then amplified, cloned and transformed into poplar 717 plants (Phytozome v12.1 accession numbers: PtrLAC27 – Potri.010G193100.1, PtrLAC18 – Potri.008G073700.1). The 13-weeks and 16-weeks old harvested transgenic trees were tested for change in gene expression of LAC27, LAC18 and compared to WT plants whereas STTM:miR397a was compared to vector control.

3.3.2 Gene expression studies in LAC27, LAC18 and STTM:miR397a transgenic lines

We attempted to overexpress previously selected LAC27 and LAC18 genes in poplar 717 lines and quantified the relative expression by qRT-PCR. The overexpression of LAC27 led to significant increase in LAC27 gene expression in transgenic lines compared to WT (Figure 3.1 a). Similarly, we also tested the relative expression of other SDX LACs in these lines. Downregulation of LAC18 and LAC14 genes was observed whereas the expression of other LACs (LAC11, LAC15, and LAC23) remained undetermined (i.e. even after 40 cycles of amplification the presence of these genes could not be detected in the sample) in these lines (Figure 3.2 (a) and (b)).
Figure 3.1: Relative gene expression of LAC27, LAC18 and miR397a in developing xylem of harvested poplar transgenic lines. Relative expression of (a) LAC27 gene in LAC27 transgenic lines (b) LAC18 gene in LAC18 transgenic lines, compared to WT plants. Significant compared to WT, *** P-value < 0.0001, ** P-value < 0.006. Relative expression of (c) miR397a gene in STTM:miR397a transgenic lines, compared to vector control. Significant compared to vector control, * P-value < 0.01.

A significant increase in LAC18 gene expression was observed in LAC18 transgenic lines as shown in Figure 3.1 (b). Among the other SDX LACs, relative expression of LAC27 was significantly increased but a decrease in LAC14 expression was observed in LAC18 lines (Figure 3.2 (c) and (d)). Like LAC27 lines, the other three SDX LACs tested remained undetermined.
Figure 3.2: Relative gene expression of other SDX LACs in developing xylem of harvested LAC27, LAC18 and STTM:miR397a transgenic lines. Relative expression of (a) LAC18 gene in LAC27 transgenic lines. (b) LAC14 gene in LAC27 transgenic lines. (c) LAC27 gene in LAC18 transgenic lines. (d) LAC14 gene in LAC18 transgenic lines, compared to WT plants. Significant compared to WT, *** P-value < 0.0006, ** P-value < 0.004, * P-value < 0.02 Relative expression of (e) LAC27 gene in STTM:miR397a transgenic lines. (f) LAC18 gene in STTM:miR397a transgenic lines. (g) LAC14 gene in STTM:miR397a transgenic lines, compared to vector control plants. *** P-value < 0.0001.
The STTM strategy has been proven to be prudent for functional characterization of miRNAs in several plant species (Tang et al. 2012) (Yan et al. 2012) (Wong et al. 2014) (Zhang et al. 2017). With this aim, we designed a construct with the STTM cassette with miR397a sequence in pBI121 vector under CaMV 35S promoter and studied the changes in gene expression for miR397a. The relative gene expression of miR397a and its target genes were quantified using qRT-PCR. For STTM lines, all transgenic lines were compared to vector control which had the empty STTM cassette with no specific miRNA region. The STTM:miR397a lines showed significant downregulation of miR397a in transgenic lines when compared to control (Figure 3.1 c). The miR397a has been previously demonstrated to be a negative regulator of laccase genes present in developing xylem. So, we also tested the target SDX laccase genes in the STTM:miR397a lines. An increase in LAC27 and LAC14 gene expression was observed in these lines while LAC18 expression was not significant when compared to the vector control (Figure 3.2 (e) – (g)).

The other SDX LACs that were tested – LAC11, LAC15 and LAC23 could not be determined. We next analyzed total laccase enzyme activity due to changes in gene expression pattern for laccase in these lines.

3.3.3 Effect on total laccase enzyme activity

Total laccase enzyme was extracted from DX of WT and LAC27 and LAC18 transgenic lines which were purified using affinity chromatography. To test the total laccase enzyme activity, ABTS was used as a substrate and monitored for a period of 5 mins. There was an overall increase in total laccase activity in LAC27 and LAC18 lines when compared to WT
(Figure 3.3 (a) and (b)). This extends a correlation with the observation made from gene expression analysis.

Since laccase genes are targets of miR397a, we also tested the total enzyme activity in the STTM lines. There was an overall increase in the total enzyme activity in the STTM lines when compared to control (Figure 3.3 (c)) which also correlates with the gene expression data.

![Figure 3.3: Quantification of total laccase enzyme activity of purified SDX proteins from WT and transgenic poplar lines using ABTS as substrate.](image)

(a) increase in total laccase activity in LAC27 transgenic lines (b) increase in total laccase activity in LAC18 transgenic lines, compared to WT plants. Significant compared to WT, *** P-value < 0.0002 (c) increase in total laccase activity in STTM:miR397a transgenic lines, compared to vector control plants. Significant compared to vector control, *** P-value < 0.0002.

### 3.3.4 Effect on xylem formation

As laccases are suggested to play a role in monolignol polymerization, the changes in gene expression in transgenic lines could possibly bring about a change in xylem development.
Stem sections of the tenth internode from the apex was used to study any such changes in development. On comparing autofluorescence images of LAC27 lines with WT, an increase in the DX region in LAC27 lines was observed as shown in Figure 3.4.1 (a) and (b). We also calculated the area of xylem region in these lines and an evident increase in this region was observed (Figure 3.4 (d)).

![Figure 3.4-1](image_url)

**Figure 3.4-1:** Autofluorescence of lignin in stem sections and estimated xylem area of harvested WT and transgenic poplar lines. Cross section of (a) WT, (b) LAC27 transgenic lines, (c) LAC18 transgenic lines, (d) area of xylem in LAC27 transgenic lines, (e) area of xylem in LAC18 transgenic lines, compared to WT plants.

With an increase in gene expression and enzyme activity in LAC18 lines, we expected an increase in xylem region like LAC27 lines. Contradicting the previous findings, the xylem region was reduced in the LAC18 lines when compared to WT (Figure 3.4 (a) and (c)). Irregular xylem vessels and reduced DX rays were observed in these sections. The area of the xylem region was also significantly reduced when compared to WT (Figure 3.4 (e)).
drastic reduction in autofluorescence in the phloem and sclerenchyma was also observed in these lines.

Stem sections of STTM:miR397a lines were also observed to study the effect of gene expression changes on xylem development. An increase in xylem region with irregular xylem vessels were observed in STTM lines (Figure 3.4.2 (f) and (g)). The area of xylem region in these lines were significantly increased when compared to vector control (Figure 3.4 (h)). Next, we analyzed the lignin content/composition to assess the changes caused by the manipulation of laccase genes and miR397a.

![Cross section of (f) vector control, (g) STTM:miR397a transgenic lines, (h) area of xylem in STTM:miR397a transgenic lines, compared to vector control plants.](image)

Figure 3.4-2: Autofluorescence of lignin in stem sections and estimated xylem area of harvested vector control and transgenic poplar lines. Cross section of (f) vector control, (g) STTM:miR397a transgenic lines, (h) area of xylem in STTM:miR397a transgenic lines, compared to vector control plants.
3.3.5 Effect of gene manipulation on lignin content and composition

Using Py-MBMS, we evaluated the lignin content/composition in dried and milled wood from transgenic LAC and WT lines. An overall 5-8% decrease in lignin content was observed in LAC27 lines but there was no change in the S/G lignin ratio (Figure 3.5 (a) and 2.6 (a)). In LAC18 lines, no change in lignin content was observed whereas a decrease in S/G lignin ratio was observed as shown in Figure 3.5 (b) and 3.6 (b). These changes in lignin content and composition could possibly explain difference in the observation made in DX region of LAC27 and LAC18 lines. A six percent decrease in lignin content and an increase in S/G lignin ratio was estimated in the STTM lines (Figure 3.5 (c) and 2.6 (c)). Owing to these changes, we also further analyzed the glucose release in LAC and STTM lines.

**Figure 3.5: Estimation of lignin content in WT, vector control and transgenic poplar lines.**

Lignin content in (a) LAC27 transgenic lines, significant when compared to WT (b) LAC18 transgenic lines, compared to WT. Lignin content in (c) STTM:miR397a transgenic lines, compared to vector control plants. Significant when compared to vector control, *** P-value < 0.0001.
Figure 3.6: Estimation of syringyl/guaiacyl (S/G) lignin ratio in WT, vector control and transgenic poplar lines. S/G lignin ratio in (a) LAC27 transgenic lines (b) LAC18 transgenic lines, compared to WT. Significant when compared to WT, ** P-value < 0.008, * P-value < 0.04. S/G lignin ratio in (c) STTM:miR397a transgenic lines, compared to vector control plants. Significant when compared to vector control, *** P-value < 0.0001.

3.3.6 Saccharification assay in LAC27, LAC18 and STTM:miR397a lines

Saccharification assay showed an increase in glucose release in both LAC27 and LAC18 lines compared to WT (Figure 3.7 (a) and (b)). In LAC27 lines, there was an overall 28-45% increase in glucose release whereas the release was between 32-76% in LAC18 lines. This increase could be attributed to the lignin content and S/G lignin ratio changes observed in these lines. An increase in glucose release in the range of 56-76% was observed in the STTM:miR397a lines (Figure 3.7 (c)).
Figure 3.7: Glucose release assay of WT, vector control and transgenic poplar lines using anthrone method. Percent change of glucose release in (a) LAC27 transgenic lines (b) LAC18 transgenic lines, compared to WT. Significant when compared to WT, *** P-value < 0.0002, ** P-value < 0.002. Percent change of glucose release in (c) STTM:miR397a lines, compared to vector control. Significant when compared to vector, *** P-value < 0.0002.

3.3.7 Effect on biomass and growth of LAC27 and LAC18 lines

We measured four phenotypic traits – wet/dry biomass, stem girth, height and leaf count in the transgenic LAC lines. There was no significant change in the wet/dry biomass in LAC27 and LAC18 lines when compared to WT (Figure 3.8 (a) and (b)). Similarly, no significant change in other traits - stem girth, height or leaf count was observed in these lines (Figure 3.9 (a) – (f)). In the 13-week period, there was no detectable change in growth rate of these lines and WT.
Figure 3.8: Estimated wet/dry leaf biomass of WT, vector control and transgenic poplar lines. Leaf biomass change in (a) LAC27 transgenic lines (b) LAC18 transgenic lines, compared to WT. Leaf biomass change in (c) STTM:miR397a transgenic lines, compared to vector control.
Figure 3.9 a: Estimated height (in cm) of WT and LAC27 transgenic lines over a period of 13 weeks until harvest.

Figure 3.9 b: Estimated leaf count of WT and LAC27 transgenic lines over a period of 13 weeks until harvest.
Figure 3.9 c: Estimated stem girth (in mm) of WT and LAC27 transgenic lines over a period of 13 weeks until harvest.

Figure 3.9 d: Estimated height (in cm) of WT and LAC18 transgenic lines over a period of 13 weeks until harvest.
Figure 3.9 e: Estimated leaf count of WT and LAC18 transgenic lines over a period of 13 weeks until harvest.

Figure 3.9 f: Estimated stem girth (in mm) of WT and LAC18 transgenic lines over a period of 13 weeks until harvest.
We analyzed the four same phenotypic traits in STTM lines. There was a slight to significant increase in wet/dry biomass in these lines (Figure 3.8 c). There was no significant change in the stem girth, height and leaf count traits in these lines (Figure 3.9 (g) – (i)).

Figure 3.9 g: Estimated height (in cm) of vector control and STTM:miR397a transgenic lines over a period of 16 weeks until harvest.
Figure 3.9 h: Estimated leaf count of vector control and STTM:miR397a transgenic lines over a period of 16 weeks until harvest.

Figure 3.9 i: Estimated stem girth (in mm) of vector control and STTM:miR397a transgenic lines over a period of 16 weeks until harvest.
3.3.8 Correlation analysis

Correlation data analysis using Microsoft Excel was used to summarize the findings for this chapter. In LAC27 lines, the only positive correlation was established between gene expression and enzyme activity. The other factors – gene expression and lignin content, lignin content and enzyme activity, lignin content and glucose release, S/G lignin ratio and lignin content and, glucose release and S/G lignin ratio – correlates negatively with each other (Figure 3.10.1 (a) and 3.10.2 (a)). For LAC18 lines, a positive correlation was observed between gene expression and enzyme activity, gene expression and lignin content and, S/G lignin ratio and lignin content. lignin content and enzyme activity, lignin content and glucose release and, glucose release and S/G lignin ratio correlates negatively with each other in LAC18 lines on comparison with WT (Figure 3.10.1 (b) and 3.10.2 (b)). In the STTM:miR397a lines, we observed a positive correlation between gene expression and lignin content and, S/G lignin ratio and glucose release. A negative correlation existed between gene expression and enzyme activity, lignin content and enzyme activity, lignin content and glucose release and, S/G lignin ratio and lignin content (Figure 3.10.1 (c) and 3.10.2 (c)).
Figure 3.10-1: Correlation analysis of relative gene expression, laccase enzyme activity and lignin content in WT and transgenic poplar lines. (a) LAC27 transgenic lines (b) LAC18 transgenic lines, compared to WT. (c) STTM:miR397a transgenic lines, compared to vector control.
Figure 3.10-2: Correlation analysis of lignin content, S/G lignin ratio and glucose release content in WT and transgenic poplar lines. (d) LAC27 transgenic lines (e) LAC18 transgenic lines, compared to WT. (f) STTM:miR397a transgenic lines, compared to vector control.
3.4 Discussion

The development and regulation of primary and secondary plant cell wall plays a critical role in vascular plants. The inelastic nature of the secondary cell wall provides structural rigidity and protects the plant from mechanical stress and pathogens. Cellulose, hemicellulose, lignin, pectins and other proteins builds a strong intricate network of polymers within the secondary cell wall. Due to its abundance, cellulose is considered the most essential component for bioconversion of plant biomass for production of several wood-based products. Recalcitrant to enzymatic and microbial degradation, lignin and hemicellulose are major hindrances for accessibility of cellulose and other fermentable sugars to be used for bioconversion. Extensive research has been carried out to manipulate the biosynthesis pathway these three components individually to understand the structural and functional complexity of cell wall. This understanding has helped to create new methods for generating plants with less or easily digestible lignin/hemicellulose for downstream applications (Boudet et al. 2003) (Chen and Dixon 2007) (Nookaraju et al. 2013).

Recent findings suggest plant laccases play significant role at different developmental stages like peroxidases, especially during lignin polymerization. This family of copper-containing oxidoreductases can oxidize a broad range of substrates in presence of oxygen. Their expression during lignin biosynthesis and wood formation has been demonstrated to be regulated by miR397a (Lu et al. 2013). It has also been suggested that LAC and PRXs share non-redundant functions and have a combinatorial participation in lignin polymerization (Zhao et al. 2013a). Our study is aimed at expanding this knowledge by
manipulating certain SDX laccases and miR397a gene expression with an attempt to determine the impact caused by these changes and for functional characterization of laccase and peroxidase.

The two SDX LACs selected for this study were LAC27 and LAC18 based on their tissue gene expression profile, their sequence similarity with each other and if they were targets of miR397a. An attempted overexpression of these LACs in poplar using a xylem-specific promoter led to the overexpression of LAC27 and LAC18 in their respective transgenic lines. The overexpression of LAC27 brought about an increase in total laccase enzyme activity, decreased the lignin content, increased the xylem area but did not affect the S/G ratio in LAC27 transgenic lines. We speculate the changes observed in LAC27 lines were dependent mostly on the expression profile of LAC27. We believe this to be true because the expression of the other tested SDX LACs were significantly low in these lines. This could be suggested to be either due to the regulation exerted by miR397a or redundancy among the laccase gene family due to overexpression of LAC27 (Figure 3.2 (a) and (b)).

On the other hand, though the overexpression of LAC18 saw an increase in the total laccase enzyme activity, it did not affect the lignin content, severely reduced the xylem area and significantly decreased the S/G ratio in LAC18 transgenic lines. This reduction in S/G lignin ratio also correlates with the reduced autofluorescence observed in the phloem fibers which are otherwise rich in S-lignin units. The gene expression analysis suggests that the increase in expression of LAC27 did not seem to contribute to maintain the S/G ratio neither the xylem development in LAC18 lines. The possible reason could be that they do
not share high sequence similarity and therefore do not share a functional overlap (see Appendix C.6, C.7, C.8, C.9, C.10).

Figure 3.11: Relative gene expression of SDX PRXs in developing xylem of harvested LAC27 transgenic lines. Relative expression of (a) PRX25 gene in LAC27 transgenic lines. (b) PRX12 gene in LAC27 transgenic lines. (c) PRX33 gene in LAC27 transgenic lines. (d) PRX22 gene in LAC27 transgenic lines. (e) PRX23 gene in LAC27 transgenic lines, compared to WT plants, *** P-value < 0.0009.
Similarly, the gene expression analysis of the six SDX PRXs in LAC27 (Figure 3.11 (a) – (e)) and LAC18 (Figure 3.12 (a) – (e)) lines were tested. Though we observed an increase in PRX25 expression, we do not believe it participated in the changes observed in LAC27 lines because these two enzymes are among the highly expressed LACs and PRXs in SDX and could exhibit a level of competition to polymerize lignin in this region. On the other hand, the expression of PRX25 was significantly higher in LAC18 lines and we believe PRXs did not compensate for the function exhibited by LAC18.

Therefore, it would be appropriate to say that each enzyme in the laccase gene family is different from the other and PRX gene family in their effect they display during the cell wall biosynthesis. This is evident by the difference in effect seen by overexpression of LAC27 and LAC18 which demonstrated contrary impact on the cell wall.  could possibly exist a level of cross talk between the functioning of LACs and PRX25 or competition for substrates (monolignols) due to the difference in substrate specificity.
Figure 3.12: Relative gene expression of SDX PRXs in developing xylem of harvested LAC18 transgenic lines. Relative expression of (a) PRX25 gene in LAC18 transgenic lines. (b) PRX12 gene in LAC18 transgenic lines. (c) PRX33 gene in LAC18 transgenic lines. (d) PRX22 gene in LAC18 transgenic lines. (e) PRX23 gene in LAC18 transgenic lines, compared to WT plants, *** P-value < 0.0002, ** P-value < 0.009, * P-value < 0.02.

We used STTM technology to manipulate the expression of miR397a that negatively regulates expression laccases in SDX. So, suppression of miR397a expression should ideally emancipate the negative regulation exerted on laccase activity and which in turn should increase lignin polymerization if these LACs are involved in this process. Using this approach, we clearly observed a reduction in the miR397a gene expression with an
increase in laccase gene activity in these lines. Gene expression analysis of the SDX LACs that are targets of miR397a, displayed an increase only in LAC14 expression whereas the other tested LACs – LAC18, LAC11, LAC23 and LAC30 were either reduced or undetermined in the STTM lines. We believe that the change in miR397a expression did bring about a change in the expression pattern of LACs but it is undetectable due to the differential expression of these genes in the xylem. This is evident by the increase in xylem area and increase in autofluorescence in the phloem fibers. The manipulation of miR397a led to an increase in the S/G lignin ratio with a 6% decrease in lignin with no significant changes to the xylem formation in STTM:miR397a lines. Thus, the increase in S-lignin would reduce the branching within the lignin backbone that will help to make the lignocellulosic biomass easy to digest making the wood obtained from these lines more desirable for bioenergy purposes.

The manipulations of LACs and miRNA also witnessed a significant increase in sugar release. We believe the reason for this to be the changes within the internal structure of SCW brought about by changes in lignin content and/or composition. This could have resulted in improved saccharification efficiency in LAC and STTM lines which again was not as significant as demonstrated by (Kavousi et al. 2010; Cook et al. 2012). But making this approach beneficial for cell wall deconstruction by producing wood with easily digestible lignin when subjected to acid pre-treatment as evident with increased sugar release. Successful attempts to engineer lignin have been performed in the past but it has been evident that these plants suffer severe growth abnormalities and therefore, limiting their use for bioenergy purposes (Nookaraju et al. 2013). In our current study with the
manipulation of LAC or miR397, we did observe changes in lignin content and composition, but this did not affect the growth characteristics of the LAC or STTM lines.

**Conclusion**

Overall, the manipulation of laccases and miR397a help to ascertain the role they play in lignin polymerization. We did not detect any co-suppression phenomena exhibited by LAC27 or LAC18 lines as observed during the overexpression of PRXs (Chapter 2). We were able to decipher a relation between LACs and PRXs with respect to polymerization. We could draw conclusions that LACs and PRXs share proportional differences in their expression and they act independently of the other with non-redundant functions. This study was novel in its own way due to the application of STTM technology to study the regulation of miRNA in trees. This study provides a better platform to manipulate lignocellulosic biomass efficiently with no yield penalty which is important due to the growing demand for plant-based bioproducts.
Summary of LAC and miR397a manipulation

Manipulation of LAC27
- overexpression of the gene
  - increase in total enzyme activity
  - increase in xylem area
  - reduction in lignin content
  - no change in S/G lignin ratio
  - increase in saccharification efficiency

Lignin Polymerization
- unique role of LACs
- healthy plants with no growth defects

Manipulation of LAC18
- overexpression of the gene
  - decrease in total enzyme activity
  - decrease in xylem area and phloem fibers
  - no change in lignin content
  - decrease in S/G lignin ratio
  - increase in saccharification efficiency

Lignin Polymerization
- unique role of LACs
- healthy plants with no growth defects
3.5 Acknowledgements

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4 Chapter 4

Secondary cell wall regulation by miRNA166

Abstract

Plant miRNAs regulate target gene expressions at transcriptional or post-translational level for most of the biological process that takes place in plants. Over the years, understanding the role of miRNAs in secondary cell wall biosynthesis has been gaining importance. The functional characterization of the mechanism behind miRNAs regulating wood development will prove to be beneficial to design and generate plants with wood characteristics essential for bioconversion of lignocellulosic biomass. We used STTM strategy to manipulate the regulation of miR166 which is suggested to be involved in cambial and wood development. We performed leaf disc-transformation of poplar plants and generated transgenic lines with reduced expression of miR166. This is the first study to use STTM technology for studying regulation of miR166 in trees. The study revealed that miR166 acts as positive and negative regulator of the target HD-ZIPIII homeobox (HB) genes. The changes in these regulations exerted by miR166 led to changes in the growth characteristics of the transgenic plants. One of the significant finding was the pleotropic effect seen on the leaf polarity exhibiting the curled-leaf phenotype. Improved saccharification efficiency with no growth penalty were other significant findings of this study. Overall, the study was helpful to understand wood development regulated by miR166 and generated trees with superior wood quality that can be used for bioenergy applications.
4.1 Introduction

Plant miRNAs belong to a large class of endogenous small non-coding RNAs with potential regulatory functions. It has been shown that they play a modulatory role in gene expression either by transcriptional modification causing methylation of target DNA or post-transcriptional cleavage or translational inhibition of target mRNAs (Baulcombe 2004) (Kume et al. 2014). They are involved in several biological processes such as developmental stages, environmental stress responses, and secondary cell wall biosynthesis. They can act as positive or negative regulators and the effects of this regulation on their targets can either be studied by silencing or constitutive overexpression of miRNAs, or creating artificial target mimics against miRNAs (Gielen et al. 2012) (Zhou and Luo 2013) (Tang and Tang 2013).

In the 20-21nt (nucleotide) long mature miRNA sequence, the second to eighth position of the miRNA is called as the seed region. This region is suggested to be involved in recognizing the binding site on the target genes. The miRNA cutting region is believed to be between the tenth and the eleventh nucleotide. This characteristic being almost universal for all plant miRNAs identified so far, the next step was to characterize their function in plants (Baulcombe 2004) (Kume et al. 2014). A method called short tandem target mimic (STTM) was pioneered and designed by Dr. Guiliang Tang’s group. These STTM structures were capable to block and knockdown specific miRNAs and helped to reveal effects caused due to their loss of function in plants. A weak stem-loop structure linked with two miRNA-binding sites on either end defines the functional STTM, artificially designed non-coding RNA sequence. There exists a partial complementarity between the
miRNA-binding site in the STTM structure and its target gene. There exists a 3-nt bulge that corresponds to the tenth and eleventh nucleotide of the target miRNA. This bulge also prevents the cleavage of STTM by the targeted miRNA. The efficacy of STTMs to knockdown miRNA activity has been successfully demonstrated in several plant and animal species (Yan et al. 2012) (Tang et al. 2012; Tang and Tang 2013).

With recent developments in understanding the role of miRNA in regulation of lignin biosynthesis and secondary cell wall formation, we decided to adopt the STTM strategy for our current study. Previous studies suggested that overexpression of several miRNAs such as miR156, miR166 and miR397 in poplar led to the decrease in lignin, enhanced plant biomass for sustainable biofuel and reduce recalcitrance (Lu et al. 2013) (Trumbo, Zhang, and Stewart 2015) (Chen et al. 2016). We have attempted to suppress miRNA166 formation using STTM strategy.

**Role of miR166 in cambial and vascular tissue development**

In one of our previous studies, we used the STTM strategy to characterize the role of miR397a in lignin polymerization (Chapter 2). Similarly, for this study we selected miR166 due to its suggested involvement in active cambial cells and vascular tissue development. miR166 family comprises of 7 family members – miR166a to miR166g. This family of miRNAs regulates the class III *HOMEODOMAIN-LEUCINE ZIPPER* (HD-ZIP III) transcription factor family and binds to GTAAT(G/C)ATTAC sequence within the promoter region (Sessa et al. 1998) (Zhong and Ye 2007). They exert their regulation by epigenetic silencing of a few enzymes involved in secondary cell wall formation and by
increased expression in tension wood when compared to opposite wood in *Populus*, *Arabidopsis* and *Acacia* (Yan et al. 2012) (Ong and Wickneswari 2012) (Ding, Zeng, and He 2014). The HD-ZIPIII homeobox (HB) genes present in *Populus trichocarpa* shares a high sequence similarity with the five known HB genes in *Arabidopsis*. But due to the duplication event of poplar genome, there are eight HB proteins in poplar. They share high sequence similarity with genes within a specific group but dissimilar between groups (see Appendix D.2, D.3). The role played by each HB protein, therefore seems to be independent of the other HB protein. An inverse correlation between developmental and seasonal regulation of *PtaHB1* and *Pta.miR166* was demonstrated in a study (Ko, Prassinos, and Han 2006). The PHB, PHV and REV genes regulate polarity patterning of lateral organs and vascular bundles. PopREV, a transcription factor is suggested to be negatively regulated by miR165/166 that functions in the initiation of cambium development and patterning of secondary vascular tissue (Robischon et al. 2011). Studies also suggest that miR166 might have prospective application in biofuel production, recalcitrance and bio-confinements due to its participation in shoot and floral development (Trumbo, Zhang, and Stewart 2015).

A detailed miRNA profiling is necessary to understand plant vascular development and wood formation. For this purpose, in our study, we used the STTM strategy with an aim to suppress the activity of miR166 to study the changes occurring in the vascular development. We expect the target HD-ZIPIII HB genes to be free from the regulation exerted by this miRNA and would help provide insights to understand their functions during wood development in poplar trees.
4.2 Materials and methods

4.2.1 Vector construction

STTM constructs were generated as previously stated in (Tang et al. 2012). Modifications were made to facilitate successful transformation in poplar. The final STTM-pFGC5941 plasmid was digested with PacI to generate the STTM fragment. This fragment was incorporated into pBI121 vector with Cauliflower mosaic virus (CaMV) 35S promoter for STTM:miR166. Cloned plasmid was then transformed into Agrobacterium strain C58C1 using freeze-thaw method (Boerjan et al. 1997) (Wu, Joshi, and Chiang 2000). The transformed plasmids were selected for antibiotic resistance. Verification PCR for the gene of interest on the selected clones were carried out before transforming the plants. The sequences of the selected clones for STTM:miR166 genes from plasmid and agrobacterium transformation was also confirmed by sanger sequencing (see Appendix D.1).

4.2.2 Plant transformation and growth conditions

The verified clones were transformed into Populus tremula x alba clone 717-1B4 genotype (routinely referred as 717) using leaf-disc transformation protocol previously described in (Boerjan et al. 1997) (Wu, Joshi, and Chiang 2000). First, the Agrobacterium clone is allowed to infect the leaf discs at the wound site (where the leaves have been cut) through several initial steps. Later these infected leaves are allowed to grow on callus induction medium (CIM) for three to four weeks to develop callus (unorganized mass of plant cell). Once callus is formed, they were moved to shoot induction medium (SIM) and then to shoot elongation medium (SEM) for three to four weeks each. Shoots generating from each callus was considered as an independent line. Keeping track of each of these independent
lines, they were further moved to root induction medium (RIM) until the shoots developed roots. The callus and shoots were grown in a growth chamber (Thermo Scientific Precision model 818 incubator) with a light and dark cycle of 16 hrs and 8 hrs at 25°C. Plants that rooted were selected and genotypically verified for the presence of the gene of interest using genomic PCR. Verified transgenic lines were acclimatized and, moved and maintained in greenhouse for approximately 4 months before harvesting. The greenhouse growth conditions were kept similar to the growth chamber with light and dark cycle of 16hrs and 8hrs, temperature of 25°C and relative humidity ranging from 40-70%. Upon harvest of the 16-week old STTM:miR166 transgenic plants, all leaf material from each plant was weighed before and after drying and stored in paper bags. The stem was cut into approximately four to five pieces (with ten to fifteen internodes each). The bark was cut open with a sharp blade and developing xylem on the wood material was scraped in liquid N2, collected in aluminum foil and stored in liquid nitrogen until use. The remaining wood material from each plant was air-dried and stored for further analysis.

4.2.3 RNA extraction and qRT-PCR analysis

Total RNA was extracted from SDX (1-10 internodes counted from the base of the plants with 40-50 internodes) using TRIzol (Ambion, Life Technologies) method. Primers were designed for each miRNA and target gene are as listed in (see Appendix A.1). Stem-loop qRT-PCR was performed to measure the targeted miRNA expression level and detection of miRNA target gene expression was performed by traditional qRT-PCR. For both, first-strand cDNA synthesis from 1µg of total RNA was performed using high capacity cDNA reverse transcription kit (Applied Biosystems). The analysis was performed with an
Applied Biosystems Step-one instrument using PowerUp SYBR green master mix (Applied Biosystems). The reaction for each gene were conducted in triplicate with thermal cycling conditions as follows: 95°C for 10 mins, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The primer specificity was confirmed by melt curve analysis. The relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method. Actin gene was used as an internal control and values were normalized to actin and comparing the normalized values to those of control plants.

4.2.4 Lignin analysis

Dried wood samples from previously harvested poplar plants were used for the analysis. These wood samples were milled to mesh 20 using Wiley-mill. Each sample weighing 100 mg were placed in microfuge tubes and sent to NREL for pyrolysis/molecular beam mass spectrometry (Py-MBMS) (Kelley et al. 2004). Each sample was analyzed in duplicates for this procedure.

4.2.5 Microscopic image analysis

For light microscopy, stems of poplar plants were cut at tenth internode from the apex of the plant with 40-50 internodes and were fixed in cold FAA (formaldehyde – acetic acid – ethanol) solution. The internode was vacuum infiltrated for 15-20 mins and stored with fresh cold FAA solution overnight at 4°C. after fixation, these internodes were dehydrated and embedded in wax. Seven-micrometer-thick consecutive sections were cut with a microtome, mounted on a slide and de-waxed using xylene and ethanol series. Lignin
autofluorescence from de-waxed sections was photographed using fluorescent light imaging.

4.2.6 Saccharification assay for STTM:miR166 lines

For performing saccharification assay to estimate glucose release, we followed (Kumar and Turner 2015) with some modifications. Ten milligrams of dried and milled wood samples from previously harvested poplar plants were used for the analysis. Alcohol insoluble residue (AIR) samples were prepared, treated with acetonitrile solution (acetic acid: nitric acid: water 8:2:1) at 100°C for 30 mins. These samples were further subjected to sulfuric acid swelling. Sugar release was measured by anthrone method by monitoring the reactants' solution absorbance at 620 nm. Using 10-250 µg µl⁻¹ of glucose, the standard curve regression line was generated and the glucose release in each sample were calculated.

4.2.7 Growth data analysis

Growth data for height, leaf count and stem girth were measured for all the generated transgenic lines (for STTM:miR166) every week (for 16 weeks) until the day of harvest.

4.3 Results

4.3.1 Gene expression study of miR166 by STTM strategy

In this study, all transgenic lines were compared to vector control which had the empty STTM cassette with no specific miRNA region. The relative gene expression of miR166 and its target genes were quantified using qRT-PCR. A significant downregulation of miR166 was observed in the STTM lines when compared to vector control (Figure 4.1). It
has been previously demonstrated that miR166 exhibits a regulation over the HD-ZIPIII family genes. Since the expression profile of the miR166 changed, we decided to test the expression of its target genes. It was observed that there was a significant decrease in the expression of HB1 and HB5 whereas a significant increase in the expression of HB4 and HB7 in STTM:miR166 lines (Figure 4.2 (a) – (d)).

**Figure 4.1: Relative gene expression of miR166 in developing xylem of harvested poplar transgenic lines.** Relative expression of miR166 gene in STTM:miR166 transgenic lines, compared to vector control. Significant compared to vector control, ** P-value < 0.002, * P-value < 0.07
Figure 4.2: Relative gene expression of target HB gene in developing xylem of harvested SSTM:miR166 transgenic lines. Relative expression of (a) HB1 gene in SSTM:miR166 transgenic lines. (b) HB4 gene in SSTM:miR166 transgenic lines. (c) HB5 gene in SSTM:miR166 transgenic lines. (d) HB7 gene in SSTM:miR166 transgenic lines, compared to vector control plants. *** P-value < 0.0007, ** P-value < 0.003.
4.3.2 Effect on xylem formation

The HD-ZIPIII genes are suggested to play a role in cambial development. Stem sections of the tenth internode from the apex of the transgenic STTM lines were compared to vector control. The lignin autofluorescence images of the STTM lines demonstrated a decrease of cells in the cambial region whereas a slight increase around xylem region. (Figure 4.3 (a) and (b)). We calculated the xylem area in these lines but did not show any significant change in this region (Figure 4.3 (c)).

![Image of lignin autofluorescence and xylem area](image)

Figure 4.3: Auto fluor escence of lignin in stem sections and estimated xylem area of harvested vector control and transgenic poplar lines. Cross section of (a) vector control, (b) STTM:miR166 transgenic lines, (c) area of xylem in STTM:miR166 transgenic lines, compared to vector control plants.
4.3.3 Effect of gene manipulation on lignin content and saccharification assay in STTM:miR166 lines

Evaluation of lignin content/composition in dried and milled wood from transgenic STTM and vector lines was carried out using Py-MBMS. An overall decrease of only 2-4% in lignin content was observed in the STTM lines. The S/G lignin ratio was significantly higher in these lines as shown in Figure 4.4 (a) and (b). Glucose release had also increased in transgenic STTM lines and calculated to be in the range of 55-57% when compared to vector controls (Figure 4.4 (c)).

![Lignin content, S/G lignin ratio, and glucose release assay](image)

Figure 4.4: Estimation of lignin content, S/G lignin ratio and glucose release assay in vector control and transgenic poplar lines. (a) Lignin content, (b) S/G lignin ratio, (c) percent change of glucose release, in STTM:miR166 transgenic lines, compared to vector control plants. Significant when compared to vector control, *** P-value < 0.0005.

4.3.4 Effect on growth and biomass of STTM:miR166 lines

As demonstrated in previous studies, the leaves of transgenic STTM:miR166 lines exhibited curled leaf phenotype and loss of leaf polarity in the early stages of development.
It was observed that once the plants were moved to the greenhouse, the leaf polarity gradually shifted to normal and did not exhibit this curled leaf phenotype.

The growth characteristics such as height and leaf count showed a significant increase in these lines when compared to control (Figure 4.5 (b) and (c)). There was no significant difference in the stem girth of these lines (Figure 4.5 (d)). The wet and dry leaf biomass also exhibited a significant increase in the STTM lines. (Figure 4.5 (e)).

**Figure 4.5 a: Comparison of phenotypes between vector control and STTM:miR166 transgenic lines.** (i – iii) Altered leaf and root development was observed after knock down of miR166, (iv & v) curled leaf and loss of leaf veins and polarity observed in STTM:miR166 lines, compared to vector control.
Figure 4.5 b: Estimated height (in cm) of vector control and STTM:miR166 transgenic lines over a period of 16 weeks until harvest.

Figure 4.5 c: Estimated leaf count of vector control and STTM:miR166 transgenic lines over a period of 16 weeks until harvest.
Figure 4.5 d: Estimated stem girth (in mm) of vector control and STTM:miR166 transgenic lines over a period of 16 weeks until harvest.

Figure 4.5 e: Estimated wet/dry leaf biomass change in STTM:miR166 transgenic lines, compared to vector control.
4.3.5 Correlation analysis

Correlation analysis helped to summarize the findings of this study. Here, a positive correlation was observed between gene expression of miR166 and lignin content and, S/G lignin ratio and glucose release in STTM lines. Negative correlation was exhibited by STTM lines for lignin content and glucose release and, S/G lignin ratio and lignin content (Figure 4.6 (a) and (b)).

![Graph (a)](image1)

![Graph (b)](image2)

Figure 4.6: (a) Correlation analysis of relative gene expression and lignin content in vector control and STTM:miR166 transgenic lines. (b) Correlation analysis of lignin content, S/G lignin ratio and glucose release content in vector control and STTM:miR166 transgenic lines.
4.4 Discussion

A class of small non-coding RNAs, called miRNAs that are ~21 nt long are said to play a significant role in gene, epigenetic and chromatin regulation. They are believed to be a class of post-transcriptional negative regulators active during plant development and stress responses. Functional genomics is making it easier to ascertain the exact relation between miRNA and its target genes and how it affects downstream processes in an organism. The discovery of miRNA has made it possible to believe that the hidden puzzles of plant cell wall biosynthesis can be determined. These miRNAs are highly conserved between plant species and are involved in the regulation of same or similar processes in these species. This makes it easier to translate the findings and exhibit similar results in another plant species. There have been several studies stating HD-ZIP III proteins have a regulatory role in wood formation and vascular differentiation (Du et al. 2011) (Robischon et al. 2011) (Zhu et al. 2013) (Zhu et al. 2017). We used STTM strategy for the first time in poplars to understand the mechanism behind the regulation exerted by miR166 on their target genes in poplar trees.

The application of STTM technology to study the regulation of miR166 on wood development was successful as suggested by gene expression analysis that demonstrated downregulation of miR166 gene in the STTM:miR166 lines. Gene expression analysis of the miR166 target genes (homeobox HB) in our study suggested that miR166 can act as positive and negative regulator of HB genes in poplar. It positively regulates the expression of \( Ptr\text{HB1} \) and \( Ptr\text{HB5} \) genes whereas it negatively regulates the expression of \( Ptr\text{HB4} \) and \( Ptr\text{HB7} \) genes (Figure 4.2). The suppression of miR166 caused pleiotropic changes to
the morphology of these lines and exhibited the curly leaf phenotype during tissue culture phase. This finding corroborates with the increased expression of \textit{PtrHB7} gene in STTM lines which has previously been demonstrated to be the reason for exhibiting this severe phenotype (Zhu et al. 2013). Interestingly, this curled leaf phenotype was reversed once these plants were grown in the green house for a period of 16-weeks. The reason for the reversal of phenotype is still unclear. In 2017, Zhu et al demonstrated the importance of HB4 in enhancing cambial activity (Zhu et al. 2017). Our study demonstrated an increase in the cambial region but no ectopic effects of cambial or phloem activity. In contrast, the expression of HB1 was reduced with the downregulation of miR166, the expression of which was previously stated to be inversely correlated (Ko, Prassinos, and Han 2006). It is not clear how HB5 is involved in cambial and wood development in poplar. We believe a concerted regulation of many factors control the process of cambial and wood development. We tested only four out of eight HB genes controlled by miR166 as they shared high sequence similarity.

The downregulation of miR166 using STTM strategy saw a reduction in lignin content which was not a significant change when compared to previous studies. The increase in S/G lignin ratio leading to structural changes within the plant cell wall was another significant observation of this study. The STTM lines also exhibited increase in growth characteristics such as height and leaf count thereby increasing the biomass content. The increase in release of sugars helped to improve saccharification efficiency in these transgenic lines. These phenotypic and genotypic changes did not affect the plant growth
and help generate plants without any growth compensation increasing their value for wood production.

**Conclusion**

Overall, this study helped to understand the regulation miR166 exerts on its targets in poplar trees. We predict that the HB gene family in poplar plays a concerted role in cambial development as evident with reversal of leaf polarity, no ectopic effects in the cambial and wood development, increase in plant growth without growth penalty and improved saccharification efficiency. Thus, making it a desirable approach for generating trees to be used in the downstream application such as paper and pulp or biofuel production.

**Summary of manipulation of miR166**
4.5 Acknowledgements

I would like to thank NSF-SEP grant (# 1230803) for providing funding for successful completion of this project. I would like to thank Surattana Boonsai (SB), Dane Wouri (DW), Robert Sykes (RS), Anne E. Harman-Ware (AH), Crissa Doeppke (CD), and Shawn Mansfield (SM), for their contributions and assisting in performing parts of the research. SB and DW were involved in vector construction, plant transformation and harvesting. RS, AH, CD from NREL performed Py-MBMS to provide data concerning wood characteristics. SM performed Klason lignin analysis as a part of wood analysis. This work would not have been possible without your sincere efforts.

4.6 References


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5 Chapter 5
Conclusion and future perspectives

Understanding plant cell wall development with respect to lignin biosynthesis and polymerization has been a challenging task. Several attempts have been made over the decades to manipulate and engineer lignin to understand the functions of every gene and enzyme involved in its synthesis. We, now have a better understanding of how lignin biosynthesis occurs and how its alterations could be beneficial for its downstream applications such as lignin valorization. Despite the progress made, the mechanism behind lignin polymerization still needs to be deciphered. It is now known that peroxidase and laccase gene families catalyze this process but their exact role and how they function during the process is still unclear. Current research on lignin mostly aims on improving lignin degradability for sugar accessibility (saccharification) from lignocellulosic biomass. Understanding lignin polymerization in plants, especially in trees like poplars (*Populus* species) might enable genetic improvement of lignin for better wood utilization in the future due to the growing importance of lignin-based bioproducts.

The first step towards this goal was to identify specific SDX-PRXs and -LACs from the entire gene family in *Populus*. In the current study, we identified SDX-PRXs and -LACs each based on previous *in-silico* studies. We verified the expression of these SDX genes in different parts of the poplar plant. We selected two SDX genes for PRX and LAC and attempted to overexpress these genes in transgenic poplar plants. We further decided to manipulate these genes using a tissue-specific, in this case developing xylem-specific
promoter (DX15) rather than using a constitutive promoter (CaMV35S) that typically produces many growth abnormalities in transgenic trees. This was mainly to observe the downstream changes only within the expected developing xylem region where wood development occurs and to avoid ectopic expression of these genes in different parts of the plant.

The overexpression of PRX25 and its expression analysis indicated that this gene was co-suppressed in PRX25 transgenic lines that altered the S/G lignin ratio. The overexpression of PRX36 was detrimental for lignin production and xylem intactness. Manipulation of PRXs increased the sugar release and improved the saccharification. Expression analysis of the six SDX-PRXs in these lines also suggests that they do not share redundant functions and each enzyme seems to play a significant role in lignin polymerization. The study also implies that SDX LACs do not compensate for the alterations in PRX expression. Changes within the cell wall due to PRXs manipulation did not affect the plant physiology and generated transgenics with no growth abnormalities.

The overexpression of LAC27 and LAC18 and its expression analysis indicated that these genes were overexpressed in their respective transgenic lines with no or less significant changes observed in lignin content and S/G ratio. This study also implies that there exists a level of cross talk in functioning between LACs and PRXs but they do not override other genes function. This is evident in LAC18 transgenic lines where PRX25 is expressed at higher levels than LAC18 and LAC27 but we see a reduction in the S/G lignin ratio and xylem formation. The manipulation of miR397a using STTM strategy also helped to understand the role it plays in regulating lignin polymerization. The suppression of
miR397a increased the S/G lignin ratio and improved saccharification efficiency of these plants.

We also attempted to understand the changes in cambial and wood development via manipulation of miR166 using STTM strategy. This is the first study to demonstrate the effect of changes in cambial development owing to the regulation of all the Homeobox (HB) genes. miR166 acts as a positive and negative regulator of HB genes which in turn affects the xylem and cambial formation. These changes improved growth characteristics and biomass and, increased S/G lignin ratio and improved saccharification efficiency. This study also suggests that this approach could be adapted for downstream application to procure improved biomass for bioenergy production.

In conclusion, we could decipher that there exists a communication between the PRXs and LACs which is strongly sensed by the plant system and regulates its function. Each enzyme in the pathway plays a specific role which is unknown and how they balance their function is still unclear. Each enzyme is different in their effect that they have on the plant cell wall as seen with the manipulation of PRXs/LACs. Their manipulation affects the expression of the other gene but the net effect is not lignin increase. The most significant finding was that none of our transgenic lines showed any growth penalty and/or ectopic expression of lignin and exhibited a normal phenotype. To lay emphasis over again, any minor alterations in expression of these genes led to structural changes within the cell wall and this is evident by the increased improved saccharification efficiency. Thus, making it highly desirable to use of wood efficiently for future applications.
**Future goals**

Both PRXs and LACs were shown to exhibit non-redundant functions during lignin polymerization. An in-depth study of SDX-PRXs/-LACs to validate their role during this process can be performed using gene editing techniques (CRISPR/Cas9). This study was performed by homologous expression of poplar genes in polar plants. To take a step further to understand the function of PRXs and LACs, our group is also working on heterologous expression of these genes i.e. expressing *Arabidopsis* PRX and LAC in poplar plants. It will also be interesting to group HB genes that were manipulated positively and negatively by miR166 in poplar and to study the changes in cambial and wood development. Looking at the larger picture, being able to answer these proposed questions and changes could strengthen the knowledge about lignin polymerization, improve the commercially available lignin in economically important tree species making it beneficial to produce value-added bioproducts from lignocellulosic biomass.
Appendix A

A.1 The entire list of primers used for all experiments includes primers for full-length amplification of genes, genomic PCR verification primers, stem-loop PCR primers for miRNAs and qRT-PCR primers for all genes

Table A 1: Highly expressed LAC and PRX in *Ptr* for full-length DNA amplification

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<th>Primer sequence 5’ - 3’ with adaptors</th>
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<td><em>PtrLAC18_SacI_R</em></td>
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<th>quantitative-PCR primers (Seq 5' to 3')</th>
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A.2 Sequencing data comparison of plasmid clones with full length CDS region of DX15 promoter

CLUSTAL 2.1 Multiple Sequence Alignments

Sequence type explicitly set to DNA
Sequence format is Pearson
Sequence 1: DX15_Promoter 1025 bp
Sequence 2: DX15_HSbf_3_joined 1027 bp
Sequence 3: DX15_HSbf_5_joined 1027 bp
Sequence 4: DX15_Sbf_6_joined 1027 bp
Sequence 5: DX15_HSbf_8_joined 1027 bp
Start of Pairwise alignments
Aligning...

Sequences (1:2) Aligned. Score: 98.1463
Sequences (1:3) Aligned. Score: 98.1463
Sequences (1:4) Aligned. Score: 98.1463
Sequences (1:5) Aligned. Score: 98.1463
Sequences (2:3) Aligned. Score: 100
Sequences (2:4) Aligned. Score: 100
Sequences (2:5) Aligned. Score: 100
Sequences (3:4) Aligned. Score: 100
Sequences (3:5) Aligned. Score: 100
Sequences (4:5) Aligned. Score: 100
Guide tree file created: [clustalw.dnd]

There are 4 groups
Start of Multiple Alignment
Aligning...
Group 1: Sequences: 2 Score:19513
Group 2: Sequences: 3 Score:19513
Group 3: Sequences: 4 Score:19513
Group 4: Sequences: 5 Score:19268
Alignment Score 67916

CLUSTAL-Alignment file created [clustalw.aln]

clustalw.aln

CLUSTAL 2.1 multiple sequence alignment

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****** ******************************************************************************
### Appendix B

#### B.1 Complete list of the ninety-three peroxidases in *Populus trichocarpa* with tissue gene expression profile comparison

*Table B 1:* Complete list of 93 PRX genes in *Populus trichocarpa* and their expression profile in difference parts of the tree

<table>
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<tr>
<th>Gene Model (Ren et al &amp; Phytozone)</th>
<th>Alias</th>
<th>Old nomenclature (Christensen et al)</th>
<th>Expression (Gene Atlas)</th>
<th>Protein Homolog</th>
<th>Ortholog in <em>Arabidopsis thaliana</em></th>
<th>Ortholog gene expression</th>
<th>Expression (Poplar eFP Browser)</th>
<th>Absolute Value (Poplar eFP browser)</th>
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<tbody>
<tr>
<td><strong>Ptr PRX1</strong></td>
<td>Potri.001G145800.1</td>
<td>POPTR_0001s02870</td>
<td>Roots 32.201</td>
<td>AT2G35380.1</td>
<td>Light grown seedling</td>
<td>Light grown seedling</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ptr PRX2</strong></td>
<td>Potri.001G011000.1</td>
<td>POPTR_0001s04820</td>
<td>immature leaf</td>
<td>Potri.001G013000.1 (95.0%)</td>
<td>Leaf and root tip</td>
<td>Light grown seedling</td>
<td>Light grown seedling</td>
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</tr>
<tr>
<td><strong>Ptr PRX3</strong></td>
<td>Potri.001G011200.1</td>
<td>POPTR_0001s04840</td>
<td>Leaf and root tip</td>
<td>Potri.001G011000.1 (90.1%), Potri.001G011300.1 (89.2%)</td>
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</tbody>
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<table>
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<tr>
<th>PRX</th>
<th>Potri.001G011300.1</th>
<th>POTPR_0001s04850</th>
<th>Root 12.119</th>
<th>Potri.001G011000.1 (97.7%), Potri.001G013000.1 (94.2%)</th>
<th>Not in db</th>
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<td>PRX4</td>
<td>Potri.001G011400.1</td>
<td>Gene not found</td>
<td>PXP22</td>
<td>Not in db</td>
<td>Not in db</td>
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<tr>
<td>PRX5</td>
<td>Potri.001G011500.1</td>
<td>POTPR_0001s04870</td>
<td>Roots 1.148</td>
<td>Male catkins</td>
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<td>PRX6</td>
<td>Potri.001G013000.1</td>
<td>POTPR_0001s05050</td>
<td>PXP3-4</td>
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<td>PRX7</td>
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<td>POTPR_0001s05060</td>
<td>PXP11</td>
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<td>Ptr PRX9</td>
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<td>POPT R_0001s18270</td>
<td>leaf</td>
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<td>roots</td>
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<td>Male catkins</td>
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<td>Ptr PRX11</td>
<td>Potri.001G329200.1</td>
<td>POPT R_0001s33680</td>
<td>not expressed in any sample</td>
<td>Potri.001G064100.1 (89.5%)</td>
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<td>Not in db</td>
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<td>Ptr PRX12</td>
<td>Potri.001G351000.1</td>
<td>POPT R_0001s34660</td>
<td>Stem internode 30.355</td>
<td>At3g28200, At5g40150; similar to Atperox P63/ATP26a</td>
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<tr>
<td>At3g28200 - top of Stem 365.92, Whole stem top 227.62 bottom of Stem 117.64, whole stem bottom 171.09, At5g40150 SAM pWUS (Rib Meristem) 780.28, pFIL (PZ) 700.66, pLAS (organ Xylem)</td>
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<td>POPT R_000 2s032 60</td>
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<td>POPT R_000 2s065 90</td>
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<td>At1g71695.1</td>
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<td>Stem Internode 962.818, Stem Node 892.617, Root 715.983, Leaf 558.952</td>
<td>Potri.004G0 15300.2 (100%)</td>
<td>At4g21960 - Top of stem 6430.3, Bottom of stem 7021.52, whole stem 6309.13, Stem internode 4985.81</td>
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<td>Potri.004</td>
<td>Potri.004</td>
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<td>similar to Peroxidase 17 precursor (EC 1.11.1.7) (Atperox P17) (ATP25a); [ co-ortholog (1of2) of At2g22420, ]</td>
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<td>Stem internode 156.854, Stem node 92.599</td>
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<td>Ptr PRX34</td>
<td>Potri.005G118700.1</td>
<td>POPT R_0005s12070</td>
<td>Roots 19.545</td>
<td>Potri.007G019300.1</td>
<td>Roots</td>
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<td>Ptr PRX35</td>
<td>Potri.005G135300.1</td>
<td>POPT R_0005s14190</td>
<td>Roots 26.983</td>
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<td><strong>Ptr PRX36</strong></td>
<td>Potri.005 G195600.1</td>
<td>POPT R_000 5s217 40</td>
<td>Roots 134.633, Stem internode 70.742, Stem node 47.371</td>
<td>similar to peroxidase precursor. [ORG:Euphorbia characias]; [co-ortholog (2of3) of At1g71695, AAS97959,]</td>
<td>Cotyledon and top epidermal peel 542.85, pFIL (PZ) 803.42</td>
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<td><strong>Ptr PRX37</strong></td>
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<td>POPT R_000 5s217 50</td>
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<td>similar to peroxidase precursor. [ORG:Euphorbia characias]; [co-ortholog (3of3) of At1g71695, AAS97959,]</td>
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<td>POPT R_000 6s068 90</td>
<td>Roots 6.448 Potri.018G1 31600.1</td>
<td>similar to Peroxidase 25 precursor (EC 1.11.1.7) (Atperox P25); [ortholog of At2g41480,]</td>
<td>Female Catkins</td>
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<td><strong>Ptr PRX40</strong></td>
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<td>POPT R_000</td>
<td>Roots 7.609</td>
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<td>Roots 6.734</td>
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<td>POPT R_000 7s025 80</td>
<td>Roots 202.075</td>
<td>Potri.017G0 37900.1 (90.5%)</td>
<td>similar to peroxidase precursor. [ORG:Glycine max]; [ortholog of At1g05260, AAD11482, AA D11481,]</td>
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<td>[co-ortholog (2of2) of At2g22420, Trichomes Not in db]</td>
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<td><strong>Ptr PRX48</strong></td>
<td>Potri.007 G096200 .1</td>
<td>POPT R_000 7s051 00</td>
<td>Stem node 11.925</td>
<td>Potri.005G0 72800.1 (94.2%)</td>
<td>similar to Peroxidase 17 precursor (EC 1.11.1.7) (Atperox P17) (ATP25a).; [co-ortholog (2of2) of At2g22420, Trichomes Not in db]</td>
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<td>Leaf 0.187</td>
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<td>similar to Peroxidase 50 precursor (EC 1.11.1.7) (Atperox P50) (PRXR2) (ATP9a); [ortholog of At4g37530,At4g37520]</td>
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<td>POPT R.0007s134 20</td>
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<td>POPT R.0008s023 30</td>
<td>Root 5.485</td>
<td>Potri.008G022700.1(99.4%), Potri.T160000.1(99.4%), Potri.008G022600.1(97.7%)</td>
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<td>Root 2.969</td>
<td>similar to Peroxidase 11 precursor (EC 1.11.1.7) (Atperox P11) (ATP23a/ATP23b); [co-ortholog (2of2) of At1g68850,]</td>
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<td>POPT R_001 0s04550</td>
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<td>POPT R_001 0s144 60</td>
<td>Root 13.805</td>
<td>similar to Peroxidase 11 precursor (EC 1.11.1.7) (Atperox P11) (ATP23a/ATP23b);</td>
<td>co-ortholog (1of2) of At1g68850,</td>
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<td>Potri.T1631 00.1 (99.4%), Potri.T1630 00.1 (97.8%), Potri.T1632 00.1 (96.2%)</td>
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<td>Potri.T0993 00.1 (98.2%), Potri.019G0</td>
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<td>POPT R_001 1s060 80</td>
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<td>POPT R_001 3s063 00</td>
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<td>POPT R_001 3s152 40</td>
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<td>POPT R_001 3s152 50</td>
<td>Stem node 19.253, Stem node 10.412</td>
<td>Dark etiolated seedling, 3 hr light seedling, male catkins, roots</td>
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<td><strong>Ptr PRX75</strong></td>
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<td>POPT R_001 3s152 70</td>
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<td>POPT R_001 4s140 00</td>
<td>Roots 1.184</td>
<td>Light grown seedling</td>
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Potri.013 G156400.1 and Potri.013 G156500.1 were not found in the database.
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<td><strong>Potri.015G0 03500.2 (99.7%)</strong></td>
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<td><strong>Roots 37.547</strong></td>
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<td><strong>Roots 12.019</strong></td>
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similar to Peroxidase 16 precursor (EC 1.11.1.7) (Atperox P16) (ATP22a); [ortholog of At2g18980,At4g30170,]
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<td><strong>POPT R_001 9s091 50</strong></td>
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<td><strong>Root 19.433</strong></td>
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<td>Potri.006G0 69600.1 (95.5%)</td>
<td>Potri.T0455 00.1 (99.2%), Potri.004G1 34800.1 (94.5%)</td>
<td>Potri.T0993 00.1 (99.7%), Potri.011G0 27300.1 (97.9%)</td>
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B.2 Sequencing data comparison of plasmid clones with full length CDS region of PRX25_F

CLUSTAL 2.1 Multiple Sequence Alignments

Sequence type explicitly set to DNA
Sequence format is Pearson
Sequence 1: DXMF 766 bp
Sequence 2: DXMFP25 766 bp
Start of Pairwise alignments
Aligning...

Sequences (1:2) Aligned. Score: 98.3029
Guide tree file created: [clustalw.dnd]

There are 1 groups
Start of Multiple Alignment
Aligning...
Group 1: Sequences: 2 Score:14373
Alignment Score 5323

CLUSTAL-Alignment file created [clustalw.aln]

clustalw.aln

CLUSTAL 2.1 multiple sequence alignment

DXMF

ACTACACCCCAACACTTCTGCAAGCACAACACTCCATCTCAAGAGATATAGGCGGC
ACTACACCCCAACACTTCTGCAAGCACAACACTCCATCTCAAGAGATATAGGCGGC
*******************************************************************************

DXMF

CGCTGCAACAAAAACGACTCTAGATCGTAAGATGAGGCCACACACTTTCACTCCTTCC
CACTGCAACAAAAACGACTCTAGATCGTAAGATGAGGCCACACACTTTCACTCCTTCC
* *******************************************************************************

DXMF

TGCAGGTCGACTCTAGATGCCAAAGCTCTCTTCTTTGCTTGGTCTTCTCTTCTC
TGCAGGTCGACTCTAGATGCCAAAGCTCTCTTCTTTGCTTGGTCTTCTCTTCTC
*******************************************************************************

DXMF

CAGCTGTGGCTGTCAGGCGGGCATTAGAAGAAATGGAAGAAACCTGGGCTTTTGGGAGAAATGGAAGAAACCTGGGCTTTTGGGAGAAATGGAAGAAACCT
*******************************************************************************

DXMF

ACTTTTACAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGCTTTATGTTACATGCAAAAAGGATACATGCGC
DXMF
DXMFP25
CTGTTCAAGTCATGTGATGCTTCACTGCTGCTGACTCAAACAAGGAGGACCTTGTCCGAGA
CTGTTCAAGTCATGTGATGCTTCACTGCTGCTGACTCAAACAAGGAGGACCTTGTCCGAGA
****************************************************************************** ****

AGGAGACAGACAGGGACTTTTGCCCTAGGAAACTTTTAGATACCTTTGAGCATAATCAAGAGAG
AGGAGACAGACAGGGACTTTTGCCCTAGGAAACTTTTAGATACCTTTGAGCATAATCAAGAGAG
****************************************************************************** ****

DXMF
DXMFP25
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CTGTTGAAGAAGAGTGTCTGGAAGTGTTCTCTCTGCTGATATTGTCTTGTCTGCTGCTA
****************************************************************************** ****

GAGATGGCATTGTTCTGCTAAGGAGGACTATACCTTCCAGACCACAAATGAAAGCATAT
GAGATGGCATTGTTCTGCTAAGGAGGACTATACCTTCCAGACCACAAATGAAAGCATAT
****************************************************************************** ****

GCAGGGAAGACAGGACAGGACATGCTGACTGGGACTATCTTTGCTGCATCAGCCCTGAGACTGATTTTGACTGCTGCTGCTGCT
GCAGGGAAGACAGGACAGGACATGCTGACTGGGACTATCTTTGCTGCATCAGCCCTGAGACTGATTTTGACTGCTGCTGCTGCT
****************************************************************************** ****

DTMF
DXMFP25
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CTGTTGTCTTCTTACAGGGTTTGGCTTCCATGAGGACTGATTTTGACTGCTGCTGCTGCTGCT
****************************************************************************** ****

TAGGAGCTCACAGTGTTGGAAGACTCAGTGTGGAAGCTGCTGCTGCTGCT
TAGGAGCTCACAGTGTTGGAAGACTCAGTGTGGAAGCTGCTGCTGCTGCT
****************************************************************************** ****

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(DMF:0.0084856, DXMFP25:0.0084856);
B.3 Sequencing data comparison of plasmid clones with full length CDS region of PRX25_MF

CLUSTAL 2.1 Multiple Sequence Alignments

Sequence type explicitly set to DNA
Sequence format is Pearson
Sequence 1: P25MF 834 bp
Sequence 2: DXP25MF 834 bp
Start of Pairwise alignments
Aligning...

Sequences (1:2) Aligned. Score: 97.8417
Guide tree file created: [clustalw.dnd]

There are 1 groups
Start of Multiple Alignment

Aligning...
Group 1: Sequences: 2 Score:15639
Alignment Score 5679

CLUSTAL-Alignment file created [clustalw.aln]

clustalw.aln

CLUSTAL 2.1 multiple sequence alignment

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<th>DXP25MF</th>
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<td>AAGCATATCTGTGTTCATTGACAGTTTGTCTCCATGGGAATTGACACCATCCTGGACTGATG</td>
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<tr>
<td>********************************************* **</td>
<td></td>
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<tr>
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<tr>
<td>TGCCCTTGTACAGGAGTCACAAGTGTGGGAAAGACTGACTGATGGAAGCTGGGACCCAGGGTCTT</td>
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</tr>
<tr>
<td>********************************************* **</td>
<td></td>
</tr>
<tr>
<td>GTACCGGGAAGTTGACCAACCCGCTTGAACCATGTTGAGCAATGCAAGCCGACCCGTATACCTGAGAAATGCAAGGACGACACC</td>
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</tr>
<tr>
<td>GTACCGGGAAGTTGACCAACCCGCTTGAACCATGTTGAGCAATGCAAGCCGACCCGTATACCTGAGAAATGCAAGGACGACACC</td>
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</tr>
<tr>
<td>********************************************* **</td>
<td></td>
</tr>
<tr>
<td>CCCTGATTTCAATCCGAGCCCTAAAGCGTAATGTCAGGAGCTGAGAATGACACAGGACACACC</td>
<td></td>
</tr>
<tr>
<td>CCCTGATTTCAATCCGAGCCCTAAAGCGTAATGTCAGGAGCTGAGAATGACACAGGACACACC</td>
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</tr>
<tr>
<td>********************************************* **</td>
<td></td>
</tr>
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</tr>
<tr>
<td>CATGGTTCTAGAAAACACACTACAGAAACACATATTGGGAAACGAGGGCTGTGTGATAGT</td>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>GGATCCAAAATGGGACCTAGAAAGGAAAGAAGCTGACTTGGTAAGAAAATGCGGCAAGAG</td>
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<tr>
<td>********************************************* **</td>
<td></td>
</tr>
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<td>DXP25MF</td>
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<tr>
<td>---------------</td>
<td>--------------------------------------------------------------------------</td>
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<td>GTTAATTAATCGAGAGTCTCGAATTTCCTCCCGATCCTTCAAAACATTGTGCAATAAAGTTTCT</td>
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<td>TAAGATTGGAATCTCGGTGCGGTCGGTGATATTACCCATTACCTGAATTCTGTGGAATTACG</td>
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<tr>
<td>TTAAGGATGTGATAATCTGGATAGCTGACGTTATTATGAGATGGGTGTATTATGAATTTATGA</td>
<td>TTAAGGATGTGATAATCTGGATAGCTGACGTTATTATGAGATGGGTGTATTATGAATTTATGA</td>
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<td>AGGATAAAATTATCGGGGCGGCTGTTGATTACATTGTGTTACGATCGGGAAA-TTCACGTGGCC-G AGGATAAAATTATCGGGGCGGCTGTTGATTACATTGTGTTACGATCGGGAAA-TTCACGTGGCC-G</td>
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<td>TCGTTTTGCAACGTCGACTGGGAAAACCCTGGGCTTACCCCAAACCTAACGGCCTT TCGTTTTGCAACGTCGACTGGGAAAACCCTGGGCTTACCCCAAACCTAACGGCCTT</td>
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`clustalw.dnd`

(P25MF: 0.010791, DXP25MF: 0.010791);
B.4 Sequencing data comparison of plasmid clones with full length CDS region of PRX36_F

CLUSTAL 2.1 Multiple Sequence Alignments

Sequence type explicitly set to DNA
Sequence format is Pearson
Sequence 1: DXMF 611 bp
Sequence 2: DXMFP36 611 bp
Start of Pairwise alignments
Aligning...

Sequences (1:2) Aligned. Score: 97.3813
Guide tree file created: [clustalw.dnd]

There are 1 groups
Start of Multiple Alignment
Aligning...
Group 1: Sequences: 2 Score:11400
Alignment Score 4350

CLUSTAL-Alignment file created [clustalw.aln]

clustalw.aln

CLUSTAL 2.1 multiple sequence alignment

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<td>DXMFP36</td>
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<tr>
<td>DXMF</td>
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</tr>
<tr>
<td>DXMFP36</td>
<td>CGCTGCAACAAACACAGCACTCCTTAGCTTCAAGATGAGAGCCACAAACTTTCTCCTTCC</td>
</tr>
<tr>
<td>DXMF</td>
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</tr>
<tr>
<td>DXMFP36</td>
<td>TGCAGTGCAGCCTAGAGATCCATGAGCCAGAGCTGCTATGCTCTTTACCTCTTCTTCTTC</td>
</tr>
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<tr>
<td>DXMFP36</td>
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<td>DXMFP36</td>
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<tr>
<td>ATTATAATGTT</td>
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</table>

** clustalw.dnd **

(DXM:0.013093,DXMFP36:0.013093);
B.5 Sequencing data comparison of plasmid clones with full length CDS region of PRX36_MF

CLUSTAL 2.1 Multiple Sequence Alignments

Sequence type explicitly set to DNA
Sequence format is Pearson
Sequence 1: P36MF 575 bp
Sequence 2: DXP36MF 575 bp
Start of Pairwise alignments
Aligning...

Sequences (1:2) Aligned. Score: 97.7391
Guide tree file created: [clustalw.dnd]

There are 1 groups
Start of Multiple Alignment
Aligning...
Group 1: Sequences: 2 Score:10783
Alignment Score 3765

CLUSTAL-Alignment file created [clustalw.aln]

clustalw.aln

CLUSTAL 2.1 multiple sequence alignment

P36MF  AATCTCAAGGAAGTTGCCCCACAAGAGACTTCAATAACAACAAACGTTGATATTCTGA  
       AATCTCAAG-AAGTTGCCCCACAAGAGACTTCAATAACAACAAACGTTGATATTCTGA  
               **************************************************

DXP36MF  TCTCCTATAAAAATTGACAAAGAAGTGATCTGACATGAATGCGCAAGGCTGTTTT  
         TCTCCTATAAAAATTGACAAAGAAGTGATCTGACATGAATGCGCAAGGCTGTTTT  
               **************************************************  ** **************************************************

P36MF  ACTTCAGACCAAGACTTGTACACGAACAAGAAGGCCATTTGTCACAGCTTCTTCTCTC  
       ACTTCAGACCAAGACTTGTACACGAACAAGAAGGCCATTTGTCACAGCTTCTTCTCTC  
               **************************************************

DXP36MF  GTTAATCAAAAGTTTGTCTTCTGATAAGTTTGTGCTGATCAAAATGTCACAGCTC  
         GTTAATCAAAAGTTTGTCTTCTGATAAGTTTGTGCTGATCAAAATGTCACAGCTC  
               ** **************************************************

P36MF  AAGGTCTTGGACAGGAATCAAGGTGAATACCGCGCCAGTTGCGGAGAGAGAAATTCAGGC  
       AAGGTCTTGGACAGGAATCAAGGTGAATACCGCGCCAGTTGCGGAGAGAGAAATTCAGGC  
               **************************************************  ***

DXP36MF  TACAGTTACTTGAGTCTGTTGTGAAGAGGGTTCTCGACGACGACTGTCCGAGATTAAATGA  
         TACAGTTACTTGAGTCTGTTGTGAAGAGGGTTCTCGACGACGACTGTCCGAGATTAAATGA  
               **************************************************
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<th>P36MF</th>
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<td>TGTTGCGGCGTCTTGCGATGATTATCATATAAATTTCTGTTGAAATTACGTTAAGCATGTAAT TGTTGCGGCGTCTTGCGATGATTATCATATAAATTTCTGTTGAAATTACGTTAAGCATGTAAT</td>
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(P36MF:0.011304, DXP36MF:0.011304);
B.6

Sequence similarity of the CDS region for PRX25 and PRX36

160


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(Ptr_PRX25_CDS:0.31928,Ptr_PRX36_CDS:0.31928);
### B.7 Sequence similarity of the CDS region for all six SDX PRXs

CLUSTAL 2.1 Multiple Sequence Alignments

Sequence type explicitly set to DNA
Sequence format is Pearson
Sequence: 1: Ptr_PRX12_Potri.001G351000 963 bp
Sequence: 2: Ptr_PRX22_Potri.003G214800 1050 bp
Sequence: 3: Ptr_PRX23_Potri.003G214700 1065 bp
Sequence: 4: Ptr_PRX25_Potri.004G015300 996 bp
Sequence: 5: Ptr_PRX33_Potri.005G108900 1137 bp
Sequence: 6: Ptr_PRX36_Potri.005G195600 1065 bp
Start of Pairwise alignments
Aligning...

Sequences (1:2) Aligned. Score: 31.7757
Sequences (1:3) Aligned. Score: 31.568
Sequences (1:4) Aligned. Score: 32.2949
Sequences (1:5) Aligned. Score: 33.541
Sequences (1:6) Aligned. Score: 35.0987
Sequences (2:3) Aligned. Score: 35.8095
Sequences (2:4) Aligned. Score: 32.0281
Sequences (2:5) Aligned. Score: 33.1429
Sequences (2:6) Aligned. Score: 38.4762
Sequences (3:4) Aligned. Score: 31.1245
Sequences (3:5) Aligned. Score: 33.0516
Sequences (3:6) Aligned. Score: 35.3991
Sequences (4:5) Aligned. Score: 31.3253
Sequences (4:6) Aligned. Score: 36.1446
Sequences (5:6) Aligned. Score: 32.3005
Guide tree file created: [clustalw.dnd]

There are 5 groups
Start of Multiple Alignment
Aligning...
Group 1: Sequences: 2 Score:19013
Group 2: Sequences: 3 Score:11575
Group 3: Sequences: 4 Score:18384
Group 4: Sequences: 2 Score:9783
Group 5: Sequences: 6 Score:9787
Alignment Score 39873

CLUSTAL-Alignment file created [clustalw.aln]
Phylogenetic tree of all six SDX PRXs

- Ptr PRX22 Potri.003G214800
- Ptr PRX23 Potri.003G214700
- Ptr PRX36 Potri.005G195600
- Ptr PRX25 Potri.004G015300
- Ptr PRX12 Potri.001G351000
- Ptr PRX33 Potri.005G108900
### Appendix C

#### C.1 Complete list of the forty-nine laccases in *Populus trichocarpa* with tissue gene expression profile comparison

*Table C1*: Complete list of all 49 LAC genes in *Populus trichocarpa* and their expression profile in different parts of the tree

<table>
<thead>
<tr>
<th>Laccase name</th>
<th>Gene model (Lu et. al)</th>
<th>Phytozome (Alias)</th>
<th>Expression (Gene Atlas)</th>
<th>Protein Homolog</th>
<th>Ortholog in At</th>
<th>Expression (Lu et. al)</th>
<th>Gene model number (Ren et. al)</th>
<th>Expression</th>
<th>Absolute value</th>
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<td>POPTR_0001s14010</td>
<td>Potri.001G054600.1</td>
<td>Stem</td>
<td>Potri.001G401300.1 (92.8%), Potri.001G401100.1 (95.0%)</td>
<td>At5g06020 (Lac 17)</td>
<td>Young stem (S)</td>
<td>Potri.001G054600.1</td>
<td>not in db</td>
<td></td>
</tr>
<tr>
<td><strong>Ptr LAC2</strong></td>
<td>POPTR_0001s18500</td>
<td>Potri.001G184300.1</td>
<td>Stem</td>
<td>Potri.009G156800.1 (99.0%), Potri.009G156600.1 (98.0%)</td>
<td>Stem differentiating xylem (X)</td>
<td>Xylem 1848.83</td>
<td>Potri.001G184300.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ptr LAC3</strong></td>
<td>POPTR_0001s21380</td>
<td>Potri.001G206200.1</td>
<td>not expressed</td>
<td>No Homologs</td>
<td>At5g09360 (lac 14)</td>
<td>Young Leaf (YL)</td>
<td>Potri.001G206200.1</td>
<td>Male catkins</td>
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</tr>
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<td></td>
<td>LAC</td>
<td>POPTR_0001s25580</td>
<td>Potri.001G 248700.1</td>
<td>Roots and Stem internode</td>
<td>Potri.009G 042500.1 (95%)</td>
<td>X</td>
<td>All tissues</td>
<td>Potri.005G 200600.1</td>
<td>(96.1%), Potri.005G 200700.1 (92.4%), Potri.005G 102700.1</td>
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<td>LAC4</td>
<td>POPTR_0001s35740</td>
<td>Potri.001G 341600.1</td>
<td>Stem internode</td>
<td>Potri.009G 054600.1 (95%)</td>
<td>X</td>
<td>Roots [R]</td>
<td>Potri.001G 341600.1</td>
<td>(95%), Potri.009G 401300.1 (92.1%)</td>
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**Legend:**
- **R, P, X**
- **X, P**
- **Xylem**
- **not in db**
- **dark etiolated seedling**
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Potri.019G 088900.1 (97.9%), Potri.019G 088600.1 (97.0%), Potri.019G 088500.1 (96.8%), Potri.019G 088700.1 (96.6%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Potri.019G 088900.1 (98.2%), Potri.019G 088800.1 (97.9%), Potri.019G 088700.1 (97.7%), Potri.019G 088500.1 (92.1%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Potri.019G 124300.1 (99.8%)</td>
</tr>
</tbody>
</table>

**LAC45**

**LAC46**

**LAC47**

Continuous light grown seedling

Roots
<table>
<thead>
<tr>
<th>LAC48</th>
<th>POPTR_0091s002</th>
<th>Potri.016G 107900.1</th>
<th>root</th>
<th>Potri.016G 106100.1 (96.0%), Potri.016G 107500.1 (93.7%), Potri.016G 106000.1 (93.5%), Potri.016G 106300.1 (91.7%)</th>
<th>R</th>
<th>Potri.016G107900.1</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAC49</td>
<td>POPTR_0958s002</td>
<td>Potri.006G 097100.1</td>
<td>stem internode 5.026</td>
<td>Potri.006G 097000.1 (97.7%), Potri.006G 096900.1 (98.9%), Potri.016G 112000.1 (97.0%), Potri.016G 112100.1 (94.8%)</td>
<td>X</td>
<td>Potri.006G097100.1</td>
<td>Xylem 687.56</td>
</tr>
</tbody>
</table>
C.2 Sequencing data comparison of plasmid clones with full length CDS region of LAC27_F

CLUSTAL 2.1 Multiple Sequence Alignments

Sequence type explicitly set to DNA
Sequence format is Pearson
Sequence 1: DX15MF  725 bp
Sequence 2: DX15MFL27  725 bp
Start of Pairwise alignments
Aligning...

Sequences (1:2) Aligned. Score: 97.7931
Guide tree file created: [clustalw.dnd]

There are 1 groups
Start of Multiple Alignment

Aligning...
Group 1: Sequences:  2   Score:13594
Alignment Score 5089

CLUSTAL-Alignment file created [clustalw.aln]

clustalw.aln

CLUSTAL 2.1 multiple sequence alignment

<table>
<thead>
<tr>
<th>DX15MF</th>
<th>TATCACTACACCCAAACTTTCTGCAAGCCAACAACTCCATTCAAAGACATCAAGAGTAGAGG</th>
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</thead>
<tbody>
<tr>
<td>DX15MFL27</td>
<td>TCTCACTACACCCAAACTTTCTGCAAGCCAACAACTCCATTCAAAGACATCAAGAGTAGAGG</td>
</tr>
<tr>
<td></td>
<td>*****************************************************************</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DX15MF</th>
<th>CGGCAGCTGCAAACAAACAGCAGCTTCTAGCTACTTCAAGATGAGGCACACATCTTTTACATC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DX15MFL27</td>
<td>CGGCAGCTGCAAACAAACAGCAGCTTCTAGCTACTTCAAGATGAGGCACACATCTTTTACATC</td>
</tr>
<tr>
<td></td>
<td>*****************************************************************</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DX15MF</th>
<th>TTCTTGCAAGTCGACTTACAGAGATCCATGAGACTATACATGAGCAGACCAGTTGCTCCTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DX15MFL27</td>
<td>TTCTTGCAAGTCGACTTACAGAGATCCATGAGACTATACATGAGCAGACCAGTTGCTCCTA</td>
</tr>
<tr>
<td></td>
<td>*****************************************************************</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DX15MF</th>
<th>GTATTTTTCATTTTTCCGAGCTTTGCTGAGTGCAAGGCTGCTGCTGCTGCTGCTGCTGCTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>DX15MFL27</td>
<td>GTATTTTTCATTTTTCCGAGCTTTGCTGAGTGCAAGGCTGCTGCTGCTGCTGCTGCTGCTG</td>
</tr>
<tr>
<td></td>
<td>*****************************************************************</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DX15MF</th>
<th>GTCTCGAAGCAACACAAAGCTTTGTTCGTTCTAACTAGTCCATCCCCACATCAAGTGGAAAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>DX15MFL27</td>
<td>GTCTCGAAGCAACACAAAGCTTTGTTCGTTCTAACTAGTCCATCCCCACATCAAGTGGAAAG</td>
</tr>
<tr>
<td></td>
<td>*****************************************************************</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DX15MF</th>
<th>TTCTCTGGACACTTTTATTCAGCAAGGAGTGTGAATGTCATTGTAATAGGTGTACACTAAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DX15MFL27</td>
<td>TTCTCTGGACACTTTTATTCAGCAAGGAGTGTGAATGTCATTGTAATAGGTGTACACTAAC</td>
</tr>
<tr>
<td></td>
<td>*****************************************************************</td>
</tr>
</tbody>
</table>
DX15MF  CAGGTCCTCAATATACATTTGCTCTTGCAATGGAAGAGTGGAGCTGTCTACCTG
DX15FL27  CAGGATCTCCTCAATATACATTTGCTCTTGCAATGGAAGAGTGGAGCTGTCTACCTG

DX15MF  GCCCATGGCGCCACATACACAGTGGCAATACGCTGAGGCTGGCAGCTTCTCTCAC
DX15FL27  GCCCATGGCGCCACATACACAGTGGCAATACGCTGAGGCTGGCAGCTTCTCTCAC

DX15MF  AATTTTACTATCTTTACAGCAGAGAGCACTTCTTGCAATCAGATTTTCTAGGCAGTTA
DX15FL27  AATTTTACTATCTTTACAGCAGAGAGCACTTCTTGCAATCAGATTTTCTAGGCAGTTA

DX15MF  AGGGCAACGATACATTGGCGCCATTGGCATCTTCTTCTAACCCATTT
DX15FL27  AGGGCAACGATACATTGGCGCCATTGGCATCTTCTTCTAACCCATTT

DX15MF  CCCAAAACCTGACAGGAAAGGTCAATCATATTGGATGAGTGGAGAAGGCTGATGTGTGGAA
DX15FL27  CCCAAAACCTGACAGGAAAGGTCAATCATATTGGATGAGTGGAGAAGGCTGATGTGTGGAA

DX15MF  CAGTGAGCTACCAAGACTGCGCCATTGACTGCTCTTGGCTCAATATATACAGATGCACACACC
DX15FL27  CAGTGAGCTACCAAGACTGCGCCATTGACTGCTCTTGGCTCAATATATACAGATGCACACACC

DX15MF  GTTAA
DX15FL27  GTTAA

---

`clustalw.dnd`

(DX15MF:0.011034,DX15FL27:0.011034);
C.3 Sequencing data comparison of plasmid clones with full length CDS region of LAC27_MF

CLUSTAL 2.1 Multiple Sequence Alignments

Sequence type explicitly set to DNA
Sequence format is Pearson
Sequence 1: LAC27MF    744 bp
Sequence 2: DXL27MF    744 bp
Start of Pairwise alignments
Aligning...

Sequences (1:2) Aligned. Score: 93.2796
Guide tree file created: [clustalw.dnd]

There are 1 groups
Start of Multiple Alignment
Aligning...
Group 1: Sequences: 2 Score: 13616
Alignment Score 4907

CLUSTAL-Alignment file created [clustalw.aln]

---

clustalw.aln

CLUSTAL 2.1 multiple sequence alignment

LAC27MF    TXCCCTTAAGGGAACCCCTTGCTTCTCCCACCTGCTCTAACCACACCTTCTGCTATAA
DXL27MF    TGCCCTATA---GGGAACCTTTGCTTCCACCTGTCTAACCACACCTTCTGCTATAA

LAC27MF    ACGGAACTAACCTGGAACCCCTTGCTTCTCCCACCTGCTCTAACCACACCTTCTGCTATAA
DXL27MF    ACGGAACTAACCTGGAACCCCTTGCTTCTCCCACCTGCTCTAACCACACCTTCTGCTATAA

LAC27MF    ACCCAGAAATGTCCCATTAAACAGTAGACCATGATCTACTCTACTCAGATGAGGCTGTCGTTGCGGA
DXL27MF    ACCCAGAAATGTCCCATTAAACAGTAGACCATGATCTACTCTACTCAGATGAGGCTGTCGTTGCGGA

LAC27MF    TTTGACCCCTTGCAACAGATGTACTAATGTAAGCAAGGCTGTCGAGGATATAAAATAAAATGTTTT
DXL27MF    TTTGACCCCTTGCAACAGATGTACTAATGTAAGCAAGGCTGTCGAGGATATAAAATAAAATGTTTT

LAC27MF    CTCTTTTAAATGCAACTAGCTACTATTCTCTTAGAAGACACACTACTATAACATAAGGCTGTCGAGGATATAAAATAAAATGTTTT
DXL27MF    CTCTTTTAAATGCAACTAGCTACTATTCTCTTAGAAGACACACTACTATAACATAAGGCTGTCGAGGATATAAAATAAAATGTTTT

LAC27MF    TCACAGATGTTTCCCTAACAAACCCACGATTCTTTTAAATCTGAGGAGAATATAACCG
DXL27MF    TCACAGATGTTTCCCTAACAAACCCACGATTCTTTTAAATCTGAGGAGAATATAACCG

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LAC27MF  CAATGAATTCTAAAGACCAACAATGGGACTAGCTAGTATAGGGCTGTTTATAATTCAGCAG  
DXL27MF  CAATGAATTCTAAAGACCAACAATGGGACTAGCTAGTATAGGGCTGTTTATAATTCAGCAG  

LAC27MF  TTCAAGTGGTGCTGCAAAGAACCACATGACGTGTTGCACTATTCATTTCAATCTTCTTC  
DXL27MF  TTCAAGTGGTGCTGCAAAGAACCACATGACGTGTTGCACTATTCATTTCAATCTTCTTC  

LAC27MF  ATGGGATTAAATTTCAATCTGTGATCTCTGTTGAAAGGAAACACAGTGAAGTGATGACTC  
DXL27MF  ATGGGATTAAATTTCAATCTGTGATCTCTGTTGAAAGGAAACACAGTGAAGTGATGACTC  

LAC27MF  CAAAGAATTTAATCTGGCTGATCTCTGTTGAAAGGAAACACAGTGAAGTGATGACTC  
DXL27MF  CAAAGAATTTAATCTGGCTGATCTCTGTTGAAAGGAAACACAGTGAAGTGATGACTC  

LAC27MF  GATGGGATTGGATGAAATCCAGAATCTGATCTCTGTTGAAAGGAAACACAGTGAAGTGATGACTC  
DXL27MF  GATGGGATTGGATGAAATCCAGAATCTGATCTCTGTTGAAAGGAAACACAGTGAAGTGATGACTC  

LAC27MF  TTTGGAAGTACACACACGACTGGGACTAATGATGACTTTTGTGGGGGCTGAAAGTGAAGGGC  
DXL27MF  TTTGGAAGTACACACACGACTGGGACTAATGATGACTTTTGTGGGGGCTGAAAGTGAAGGGC  

LAC27MF  CCAATGAGTCATACACTACCCGCTCCCAATCTGGA  
DXL27MF  CCAATGAGTCATACACTACCCGCTCCCAATCTGGA  

```
clustalw.dnd

(LAC27MF:0.033602,DXL27MF:0.033602);
```
C.4 Sequencing data comparison of plasmid clones with full length CDS region of LAC18_F

CLUSTAL 2.1 Multiple Sequence Alignments

Sequence type explicitly set to DNA
Sequence format is Pearson
Sequence 1: DXL18 774 bp
Sequence 2: DX118S 774 bp
Start of Pairwise alignments
Aligning...

Sequences (1:2) Aligned. Score: 97.5452
Guide tree file created: [clustalw.dnd]

There are 1 groups
Start of Multiple Alignment

Aligning...
Group 1: Sequences: 2 Score:14479
Alignment Score 5371

CLUSTAL-Alignment file created [clustalw.aln]

clustalw.aln

CLUSTAL 2.1 multiple sequence alignment

<table>
<thead>
<tr>
<th>Cloning</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DXL18</td>
<td>AGCACAACCTCCATTCAAGAAACATCAAGAGTATAGGCCGCACTGCAACAAAAACAGCACTC</td>
</tr>
<tr>
<td>DX118S</td>
<td>-GCACAACCTCCATTCAAGAAACATCAAGAGTATAGGCCGCACTGCAACAAAAACAGCACTC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cloning</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DXL18</td>
<td>CTAGCTACTTCAAGATGAGGCCAAACATTCTTTTCATCTGCAAGGTCGACTCTAGAGGAT</td>
</tr>
<tr>
<td>DX118S</td>
<td>CTAAGCTACTTCAAGATGAGGCCAAACATTCTTTTCATCTGCAAGGTCGACTCTAGAGGAT</td>
</tr>
</tbody>
</table>

*******************************************************************************
185

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DNL18  CCATGGAGGTTATCAACCCGTATCTTGTGCCATGCCATTTCTTCTTCTCTTCTGTCCTG
DNL18  CCATGGAGGTTATCAACCCGTATCTTGTGCCATGCCATTTCTTCTTCTCTGTCCTG
DNL18  TCTCGGCTTCAGCAATGTCATTAGCTATGCAAAAAACCCACCACTGATTTAAATTG
DNL18  TCTCGGCTTCAGCAATGTCATTAGCTATGCAAAAAACCCACCACTGATTTAAATTG
DNL18  AAGCAGCAGAAAAGGTAAAAGGCTTTGCAAAAAACCCACCACTGATTTAAATTG
DNL18  AAGCAGCAGAAAAGGTAAAAGGCTTTGCAAAAAACCCACCACTGATTTAAATTG
DNL18  TCCGGGGGCAAGCTTTGAAAGTGAAGAGCAGACATCTAAGTCTAAGGTGAACA
DNL18  TCCGGGGGCAAGCTTTGAAAGTGAAGAGCAGACATCTAAGTCTAAGGTGAACA
DNL18  GAGCAGCAGTTAATGGTACCATCAAGGAGCAGACATCTAAGTCTAAGGTGAACA
DNL18  GAGCAGCAGTTAATGGTACCATCAAGGAGCAGACATCTAAGTCTAAGGTGAACA
DNL18  CAGATGGGCAAGAATTGGGCAAAAAACCCACCACTGATTTAAATTG
DNL18  CAGATGGGCAAGAATTGGGCAAAAAACCCACCACTGATTTAAATTG
DNL18  GGTCTTACTATTGAAGGACGAAGAGAACACTCTTGGAAGCGAGCGTACAGCTGACCT
DNL18  GGTCTTACTATTGAAGGACGAAGAGAACACTCTTGGAAGCGAGCGTACAGCTGACCT
DNL18  GAGCCACTGTTTATGGGTCTATACCATCCATCCAAGAGGAGATCTGATCCATTCT
DNL18  GAGCCACTGTTTATGGGTCTATACCATCCATCCAAGAGGAGATCTGATCCATTCT
DNL18  TAAAGCCCAAAGCGTAAAAACCCCACTTCTTGGTGAAATTGGTGGAACAGCCACCCTATTG
DNL18  TAAAGCCCAAAGCGTAAAAACCCCACTTCTTGGTGAAATTGGTGGAACAGCCACCCTATTG
DNL18  ATGTGTGGAAGGAGCAACTGAAGAGCGAGCGTACAGCTGACCTAATATAGTTAAGC
DNL18  ATGTGTGGAAGGAGCAACTGAAGAGCGAGCGTACAGCTGACCTAATATAGTTAAGC
DNL18  TTAACCGTCAACCTGTTGATCTTTTATTGCTGACCGGAAAGATTACATCAGCCTAG
DNL18  TTAACCGTCAACCTGTTGATCTTTTATTGCTGACCGGAAAGATTACATCAGCCTAG
```

```
clustalw.dnd

(DNL18:0.012274, DNL18S:0.012274);
```
C.5 Sequencing data comparison of plasmid clones with full length CDS region of LAC18_MF

CLUSTAL 2.1 Multiple Sequence Alignments

Sequence type explicitly set to DNA
Sequence format is Pearson
Sequence 1: DX15L18 434 bp
Sequence 2: DX15L18Mf 434 bp
Start of Pairwise alignments
Aligning...

Sequences (1:2) Aligned. Score: 93.0876
Guide tree file created: [clustalw.dnd]

There are 1 groups
Start of Multiple Alignment
Aligning...
Group 1: Sequences: 2 Score:7916
Alignment Score 2908

CLUSTAL-Alignment file created [clustalw.aln]

clustalw.aln

CLUSTAL 2.1 multiple sequence alignment

<p>| DX15L18 | CGACGATATTGAGAACATCTCTTTCATCAACGCTAGGACTCAAACAGATTGTCCTA |</p>
<table>
<thead>
<tr>
<th>DX15L18Mf</th>
<th>CGACGATATTGAGAACATCTCTTTCATCAACGCTAGGACTCAAACAGATTGTCCTA</th>
</tr>
</thead>
</table>

| DX15L18 | AAAACTTTAGAGCTAGGGGTGTCAAGGACCAACGGCACTCGTTTTACTGCTAGTATGA |
| DX15L18Mf | AAAACTTTAGAGCTAGGGGTGTCAAGGACCAACGGCACTCGTTTTACTGCTAGTATGA |

*******************************************************************
C.6 Sequence similarity of the CDS region for LAC27 and LAC18

CLUSTAL 2.1 Multiple Sequence Alignments

Sequence type explicitly set to DNA
Sequence format is Pearson
Sequence 1: Ptr_LAC27_CDS 1668 bp
Sequence 2: Ptr_LAC18_CDS 1725 bp
Start of Pairwise alignments
Aligning...

Sequences (1:2) Aligned. Score: 45.2638
Guide tree file created: [clustalw.dnd]

There are 1 groups
Start of Multiple Alignment

Aligning...
Group 1: Sequences: 2 Score:20432
Alignment Score 5776

CLUSTAL-Alignment file created [clustalw.aln]

---

clustalw.aln

CLUSTAL 2.1 multiple sequence alignment

Ptr_LAC27_CDS  ATGGAGTACTATCAACGGCTGACTTCTTTATCTTTTTGCAATCTGGGACACAGTTGTAGG
Ptr_LAC18_CDS  ATGGAGTTATTCAACCGTATCTTTTCTCTTTCTCTTCTTCCTGGCTGTT

Ptr_LAC27_CDS  ATTTTCATTTTTTCCGACATTTCGAGTGACAGTTGTCCGCTTTTATATTTACAGGTAGTC
Ptr_LAC18_CDS  CTGCGCTCAGCAGATATCACTTTATCTTTCTCTTTGTTAGTCA

Ptr_LAC27_CDS  CTGACGCGGACCACAACACAAACAGGCTGTTGACACTACGGTCAAGGCTGAAAGTT
Ptr_LAC18_CDS  GCCCAACAAAATGTTGAAGGCTGGAAGGACACACATGCATTGAAACGGGAACTT

Ptr_LAC27_CDS  CCGGGCCACACCTGTGAAAGTGAAGGACAAGGACACACATGCAATGAAACGGGAACTT
Ptr_LAC18_CDS  CCGGGGCTCGGACATTTCGAGTGACAGTTGTCCGCTTTTATATTTACAGGTAGTC

Ptr_LAC27_CDS  ATGCGCTGCGGACCACAACACAAACAGGCTGTTGACACTACGGTCAAGGCTGAAAGTT
Ptr_LAC18_CDS  ATGCGCTGCGGACCACAACACAAACAGGCTGTTGACACTACGGTCAAGGCTGAAAGTT

Ptr_LAC27_CDS  ATGCGCTGCGGACCACAACACAAACAGGCTGTTGACACTACGGTCAAGGCTGAAAGTT
Ptr_LAC18_CDS  ATGCGCTGCGGACCACAACACAAACAGGCTGTTGACACTACGGTCAAGGCTGAAAGTT

Ptr_LAC27_CDS  ATGCGCTGCGGACCACAACACAAACAGGCTGTTGACACTACGGTCAAGGCTGAAAGTT
Ptr_LAC18_CDS  ATGCGCTGCGGACCACAACACAAACAGGCTGTTGACACTACGGTCAAGGCTGAAAGTT

Ptr_LAC27_CDS  ATGCGCTGCGGACCACAACACAAACAGGCTGTTGACACTACGGTCAAGGCTGAAAGTT
Ptr_LAC18_CDS  ATGCGCTGCGGACCACAACACAAACAGGCTGTTGACACTACGGTCAAGGCTGAAAGTT

Ptr_LAC27_CDS  ATGCGCTGCGGACCACAACACAAACAGGCTGTTGACACTACGGTCAAGGCTGAAAGTT
Ptr_LAC18_CDS  ATGCGCTGCGGACCACAACACAAACAGGCTGTTGACACTACGGTCAAGGCTGAAAGTT

Ptr_LAC27_CDS  ATGCGCTGCGGACCACAACACAAACAGGCTGTTGACACTACGGTCAAGGCTGAAAGTT
Ptr_LAC18_CDS  ATGCGCTGCGGACCACAACACAAACAGGCTGTTGACACTACGGTCAAGGCTGAAAGTT

Ptr_LAC27_CDS  ATGCGCTGCGGACCACAACACAAACAGGCTGTTGACACTACGGTCAAGGCTGAAAGTT
Ptr_LAC18_CDS  ATGCGCTGCGGACCACAACACAAACAGGCTGTTGACACTACGGTCAAGGCTGAAAGTT

Ptr_LAC27_CDS  ATGCGCTGCGGACCACAACACAAACAGGCTGTTGACACTACGGTCAAGGCTGAAAGTT
Ptr_LAC18_CDS  ATGCGCTGCGGACCACAACACAAACAGGCTGTTGACACTACGGTCAAGGCTGAAAGTT

---
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clustalw.dnd

(Ptr_LAC27_CDS:0.27368,Ptr_LAC18_CDS:0.27368);
C.7 Sequence similarity of the CDS region for thirteen SDX LACs which are targets of miR397a

CLUSTAL 2.1 Multiple Sequence Alignments

Sequence type explicitly set to DNA
Sequence format is Pearson
Sequence 1: Ptr_LAC2_Potri.001G184300.1 1731 bp
Sequence 2: Ptr_LAC15_Potri.006G097000.1 1680 bp
Sequence 3: Ptr_LAC24_Potri.009G156800.1 1731 bp
Sequence 4: Ptr_LAC40_Potri.016G112000.1 1674 bp
Sequence 5: Ptr_LAC49_Potri.006G097100.1 1680 bp
Sequence 6: Ptr_LAC18_Potri.008G073700.1 1725 bp
Sequence 7: Ptr_LAC26_Potri.010G183600.1 1728 bp
Sequence 8: Ptr_LAC7_Potri.001G401300.1 1746 bp
Sequence 9: Ptr_LAC11_Potri.006G087100.1 1743 bp
Sequence 10: Ptr_LAC12_Potri.006G087500.1 1740 bp
Sequence 11: Ptr_LAC14_Potri.006G096900.1 1677 bp
Sequence 12: Ptr_LAC23_Potri.009G156600.1 1731 bp
Sequence 13: Ptr_LAC30_Potri.011G120300.1 1746 bp

Start of Pairwise alignments
Aligning...

Sequences (1:2) Aligned. Score: 58.6548
Sequences (1:3) Aligned. Score: 98.3247
Sequences (1:4) Aligned. Score: 51.9116
Sequences (1:5) Aligned. Score: 50.8929
Sequences (1:6) Aligned. Score: 45.6232
Sequences (1:7) Aligned. Score: 44.8495
Sequences (1:8) Aligned. Score: 66.7244
Sequences (1:9) Aligned. Score: 63.8359
Sequences (1:10) Aligned. Score: 64.8758
Sequences (1:11) Aligned. Score: 50.328
Sequences (1:12) Aligned. Score: 97.9203
Sequences (1:13) Aligned. Score: 64.8758
Sequences (2:3) Aligned. Score: 58.9524
Sequences (2:4) Aligned. Score: 98.5615
Sequences (2:5) Aligned. Score: 99.2857
Sequences (2:6) Aligned. Score: 48.5119
Sequences (2:8) Aligned. Score: 53.9286
Sequences (2:9) Aligned. Score: 51.4881
Sequences (2:10) Aligned. Score: 53.5119
Sequences (2:11) Aligned. Score: 98.0322
Sequences (2:12) Aligned. Score: 50.6548
Sequences (2:13) Aligned. Score: 52.2619
Sequences (3:4) Aligned. Score: 51.6726
Sequences (3:5) Aligned. Score: 50.9524
Sequences (3:6) Aligned. Score: 45.5652
Sequences (3:7) Aligned. Score: 44.9074
Sequences (3:8) Aligned. Score: 67.1288
Sequences (3:9) Aligned. Score: 64.067
Sequences (3:10) Aligned. Score: 65.2802
Sequences (3:11) Aligned. Score: 50.3876
Sequences (3:12) Aligned. Score: 99.3645
Sequences (3:13) Aligned. Score: 64.7603
Sequences (4:5) Aligned. Score: 91.8394
Sequences (4:7) Aligned. Score: 49.1637
Sequences (4:8) Aligned. Score: 54.6595
Sequences (4:9) Aligned. Score: 50.5376
Sequences (4:10) Aligned. Score: 54.3911
Sequences (4:11) Aligned. Score: 90.9797
Sequences (4:12) Aligned. Score: 51.4337
Sequences (4:13) Aligned. Score: 53.5842
Sequences (5:6) Aligned. Score: 48.75
Sequences (5:7) Aligned. Score: 48.5119
Sequences (5:8) Aligned. Score: 53.8095
Sequences (5:9) Aligned. Score: 52.2619
Sequences (5:10) Aligned. Score: 53.75
Sequences (5:11) Aligned. Score: 58.8918
Sequences (5:12) Aligned. Score: 50.8929
Sequences (5:13) Aligned. Score: 53.0357
Sequences (6:7) Aligned. Score: 89.5072
Sequences (6:8) Aligned. Score: 45.2754
Sequences (6:9) Aligned. Score: 43.1884
Sequences (6:10) Aligned. Score: 45.1594
Sequences (6:11) Aligned. Score: 48.2409
Sequences (6:12) Aligned. Score: 45.5072
Sequences (6:13) Aligned. Score: 44.8116
Sequences (7:8) Aligned. Score: 46.5278
Sequences (7:9) Aligned. Score: 42.5347
Sequences (7:10) Aligned. Score: 45.6597
Sequences (7:11) Aligned. Score: 47.6446
Sequences (7:12) Aligned. Score: 44.9653
Sequences (7:13) Aligned. Score: 45.1968
Sequences (8:9) Aligned. Score: 65.8635
Sequences (8:10) Aligned. Score: 66.3218
Sequences (8:11) Aligned. Score: 54.3232
Sequences (8:12) Aligned. Score: 66.84
Sequences (8:13) Aligned. Score: 90.5498
Sequences (9:10) Aligned. Score: 64.0805
Sequences (9:11) Aligned. Score: 51.8187
Sequences (9:12) Aligned. Score: 63.7284
Sequences (9:13) Aligned. Score: 65.8661
Sequences (10:11) Aligned. Score: 53.8462
Sequences (10:12) Aligned. Score: 64.7025
Sequences (10:13) Aligned. Score: 66.6092
Sequences (11:12) Aligned. Score: 50.328
Sequences (11:13) Aligned. Score: 52.7132
Guide tree file created: [clustalw.dnd]

There are 12 groups
Start of Multiple Alignment

Aligning...

Group 1: Sequences: 2  Score:31768
Group 2: Sequences: 3  Score:31461
Group 3: Sequences: 4  Score:29906
Group 4: Sequences: 2  Score:32765
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Group 7: Sequences: 3  Score:25683
Group 8: Sequences: 6  Score:24631
Group 9: Sequences: 7  Score:25168
Group 10: Sequences: 11  Score:21423
Group 11: Sequences: 2  Score:30711
Group 12: Sequences: 13  Score:28400
Alignment Score 578831

CLUSTAL-Alignment file created [clustalw.aln]

clustalw.aln

CLUSTAL 2.1 multiple sequence alignment
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**clustalw2.dnd**

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(  
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Ptr_LAC26_Potri.010G183600.1:0.05268)
:0.24436)
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:0.00616)
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C.8 Sequence similarity of the CDS region for all seventeen SDX LACs

CLUSTAL 2.1 Multiple Sequence Alignments

Sequence type explicitly set to DNA
Sequence format is Pearson

Sequence 1: PIR_LAC2_Potri.001G184300.1 1731 bp
Sequence 2: PIR_LAC15_Potri.006G097000.1 1680 bp
Sequence 3: PIR_LAC24_Potri.009G156800.1 1731 bp
Sequence 4: PIR_LAC40_Potri.016G112000.1 1674 bp
Sequence 5: PIR_LAC49_Potri.006G097100.1 1680 bp
Sequence 6: PIR_LAC18_Potri.008G073700.1 1725 bp
Sequence 7: PIR_LAC26_Potri.010G183600.1 1728 bp
Sequence 8: PIR_LAC7_Potri.001G461300.1 1746 bp
Sequence 9: PIR_LAC11_Potri.006G087100.1 1743 bp
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Sequence 12: PIR_LAC23_Potri.009G156600.1 1731 bp
Sequence 13: PIR_LAC30_Potri.011G120300.1 1746 bp
Sequence 14: PIR_LAC17_Potri.008G064000.1 1671 bp
Sequence 15: PIR_LAC19_Potri.008G073800.1 1749 bp
Sequence 16: PIR_LAC25_Potri.010G183500.1 1749 bp
Sequence 17: PIR_LAC27_Potri.010G193100.1 1668 bp

Start of Pairwise alignments
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Sequences (1:5) Aligned. Score: 50.8929
Sequences (1:6) Aligned. Score: 45.6232
Sequences (1:7) Aligned. Score: 44.8495
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Sequences (10:16) Aligned. Score: 45.8846
Sequences (11:12) Aligned. Score: 50.328
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Sequences (12:14) Aligned. Score: 49.2138
Sequences (12:15) Aligned. Score: 44.3896
Sequences (12:16) Aligned. Score: 43.9053
Sequences (12:17) Aligned. Score: 49.5803
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Guide tree file created: [clustalw.dnd]

There are 16 groups
Start of Multiple Alignment

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Group 2: Sequences: 3  Score:31459
Group 3: Sequences: 4  Score:29901
Group 4: Sequences: 2  Score:29656
Group 5: Sequences: 6  Score:24201
Group 6: Sequences: 2  Score:32765
Group 7: Sequences: 3  Score:32513
Group 8: Sequences: 2  Score:31179
Group 9: Sequences: 3  Score:25684
Group 10: Sequences: 6  Score:24631
Group 11: Sequences: 7  Score:25168
Group 12: Sequences: 13  Score:20603
Group 13: Sequences: 2  Score:30711
Group 14: Sequences: 2  Score:31454
Group 15: Sequences: 4  Score:27637
Group 16: Sequences: 17  Score:19997
Alignment Score 956795

CLUSTAL-Alignment file created  [clustalw.aln]
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Ptr_LAC40_Potri.016G112000.1:0.04397)
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Ptr_LAC27_Potri.010G193100.1:0.04952)
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:0.08931)
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C.9 Phylogenetic tree of thirteen SDX LACs which are targets of miR397a

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C.10 Phylogenetic tree of all seventeen SDX LACs
Appendix D

D.1 Sequence similarity for miRNAs

121STTM166-C-2-R

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GGAGGTTAGCATAGCAGTCTGGCAGTCAGTGTTTTTATTTTAGGAAATAGATGATAGATAGATAGA
ATACTGGAAAGGATGGAAGAACCCTGGCCGAGTTATACCA

Reverse complement(pBI121 STTM165/166)

5’GGGGGATGAAGCTACCTGGTCCGA3’GTTGTTGTTATGTTGCTATTATTTAATATGGAATTAGA
AGAAGAAGAAT5’GGGGAATGAAGCTACCTGGTCCGA3’GAATTCGGTACGCTGAAATCACCA

121STTM397-C-2-R

GTCAACGTAACCAACCCTGGCCCT TAGATCTTTTCTAGCTGCCGAGTCTCGGGGTGTCAGCTGCTCAGCTGTGTGGTTATATTACCA
GGAGGTTAGCATAGCAGTCTGGCAGTCAGTGTTTTTATTTTAGGAAATAGATGATAGATAGA
ATACTGGAAAGGATGGAAGAACCCTGGCCGAGTTATACCA

Reverse complement(pBI121 STTM165/166)

5’GGGGGATGAAGCTACCTGGTCCGA3’GTTGTTGTTATGTTGCTATTATTTAATATGGAATTAGA
AGAAGAAGAAT5’GGGGAATGAAGCTACCTGGTCCGA3’GAATTCGGTACGCTGAAATCACCA
D.2 Sequence similarity of all eight HB genes that are targets of miR166

CLUSTAL 2.1 Multiple Sequence Alignments

Sequence type explicitly set to DNA
Sequence format is Pearson
Sequence 1: Ptr_HB1_Potri.0006014500.1_CDS 2568 bp
Sequence 2: Ptr_HB2_Potri.0004211100.1_POPREV_CDS 2541 bp
Sequence 3: Ptr_HB3_Potri.0116098300.1_PHABULOSA_CDS 2532 bp
Sequence 4: Ptr_HB4_Potri.0001637200.1_CDS 2535 bp
Sequence 5: Ptr_HB5_Potri.0001618800.1_PCN_CDS 2559 bp
Sequence 6: Ptr_HB6_Potri.0003005000.1_CDS 2514 bp
Sequence 7: Ptr_HB7_Potri.0180054500.1_CDS 2532 bp
Sequence 8: Ptr_HB8_Potri.00060237500.1_CDS 2535 bp
Start of Pairwise alignments
Aligning...

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Sequences (2:7) Aligned. Score: 52.4882
Sequences (2:8) Aligned. Score: 49.1913
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Sequences (7:8) Aligned. Score: 93.0095

Guide tree file created: [clustalw.dnd]

There are 7 groups
Start of Multiple Alignment
Aligning...
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Group 2: Sequences: 2 Score:45284
Group 3: Sequences: 4 Score:40248
Group 4: Sequences: 2 Score:46082
Group 5: Sequences: 6 Score:34697
Group 6: Sequences: 2 Score:45259
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Ptr_H5_Potri_001G098300_1_PHA
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Ptr_H1_Potri_009G145000_1_CDS
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Ptr_H5_Potri_001G098300_1_PHA
Ptr_H4_Potri_001G572300_1_CDS
Ptr_H1_Potri_009G145000_1_CDS
Ptr_H2_Potri_004G6211300_1_POP

Ptr_H7_Potri_018G045100_1_CDS
Ptr_H8_Potri_006G237500_1_CDS
Ptr_H5_Potri_001G188800_1_CPN
Ptr_H6_Potri_003G950100_1_CDS
Ptr_H5_Potri_001G098300_1_PHA
Ptr_H4_Potri_001G572300_1_CDS
Ptr_H1_Potri_009G145000_1_CDS
Ptr_H2_Potri_004G6211300_1_POP

Ptr_H7_Potri_018G045100_1_CDS
Ptr_H8_Potri_006G237500_1_CDS
Ptr_H5_Potri_001G188800_1_CPN
Ptr_H6_Potri_003G950100_1_CDS
Ptr_H5_Potri_001G098300_1_PHA
Ptr_H4_Potri_001G572300_1_CDS
Ptr_H1_Potri_009G145000_1_CDS
Ptr_H2_Potri_004G6211300_1_POP

Ptr_H7_Potri_018G045100_1_CDS
Ptr_H8_Potri_006G237500_1_CDS
Ptr_H5_Potri_001G188800_1_CPN
Ptr_H6_Potri_003G950100_1_CDS
Ptr_H5_Potri_001G098300_1_PHA
Ptr_H4_Potri_001G572300_1_CDS
Ptr_H1_Potri_009G145000_1_CDS
Ptr_H2_Potri_004G6211300_1_POP

Ptr_H7_Potri_018G045100_1_CDS
Ptr_H8_Potri_006G237500_1_CDS
Ptr_H5_Potri_001G188800_1_CPN
Ptr_H6_Potri_003G950100_1_CDS
Ptr_H5_Potri_001G098300_1_PHA
Ptr_H4_Potri_001G572300_1_CDS
Ptr_H1_Potri_009G145000_1_CDS
Ptr_H2_Potri_004G6211300_1_POP

Ptr_H7_Potri_018G045100_1_CDS
Ptr_H8_Potri_006G237500_1_CDS
Ptr_H5_Potri_001G188800_1_CPN
Ptr_H6_Potri_003G950100_1_CDS
Ptr_H5_Potri_001G098300_1_PHA
Ptr_H4_Potri_001G572300_1_CDS
Ptr_H1_Potri_009G145000_1_CDS
Ptr_H2_Potri_004G6211300_1_POP

Ptr_H7_Potri_018G045100_1_CDS
Ptr_H8_Potri_006G237500_1_CDS
Ptr_H5_Potri_001G188800_1_CPN
Ptr_H6_Potri_003G950100_1_CDS
Ptr_H5_Potri_001G098300_1_PHA
Ptr_H4_Potri_001G572300_1_CDS
Ptr_H1_Potri_009G145000_1_CDS
Ptr_H2_Potri_004G6211300_1_POP

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(Ptr_HB3_Potri.011G098300.1_PHABULOSA_CDS:0.04035, 
Ptr_HB4_Potri.001G372300.1_CDS:0.02600) :0.16099, 
:0.10074, 
(Ptr_HB5_Potri.001G188800.1_PCN_CDS:0.05242, 
Ptr_HB6_Potri.003G050100.1_CDS:0.01918) :0.09000, 
(Ptr_HB7_Potri.018G045100.1_CDS:0.04187, 
Ptr_HB8_Potri.006G237500.1_CDS:0.02803) :0.09384);
D.3 Phyllogetic tree of all eight HB genes that are targets of miR166

![Phylogenetic tree diagram showing relationships between different HB genes and their identifiers.](image-url)
Appendix E

Copyright documentation

All images in this document are original graphics or photos created by the first author of this dissertation.