Exploring the function of laccases from *Miscanthus sinensis* in lignin biosynthesis

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submitted to the

Combined Faculties for the Natural Sciences and for Mathematics

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Exploring the function of laccases from *Miscanthus sinensis* in lignin biosynthesis

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The present thesis is based on the following papers and conference reports:

I. **He F, Machemer-Noonan K, Golfier P, Unda F, Dechert J, Zhang W, Hoffmann N, Samuels L, Mansfield S, Rausch T and Wolf S. 2019.** Functional characterization of MsLAC1, a *Miscanthus* laccase isoform promoting lignification of interfascicular fibers in transgenic *Arabidopsis* plants. New Phytologist (Under review).

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Summary

Miscanthus, a fast-growing C4-grass with high water use efficiency and low fertilizer demand is an attractive source of lignocellulosic biomass for bioeconomy. While the lignin component causes cell wall recalcitrance towards saccharification, it also offers a novel source for aromatic platform chemicals, thus performing as friend or foe depending on biomass use. Consequently, changing amount and/or quality of *Miscanthus* lignin are important breeding goals. Transcriptional regulation of monolignol biosynthesis genes in *Miscanthus* has been previously studied. However, monolignol polymerization is as of yet poorly understood. Laccases are phenol-oxidizing enzymes which can convert monolignols into free radicals, leading to the formation of lignin polymers. In plants, a number of laccases have been reported to be involved in lignin polymerization in secondary cell walls and middle lamellas, not only affecting lignin content, but also influencing the composition of lignin monomers.

Here, we have explored the link between the lignification process and different laccase isoforms from *Miscanthus*. A comprehensive analysis has been performed including expression profiling, characterization of recombinant MsLAC1 protein, subcellular localization, activation of the *MsLAC1-5* promoters by lignification-promoting R2R3 MYB transcription factors, effective complementation of an *Arabidopsis* laccase double mutant (*lac4-2 lac17*), and impact of ectopic expression. MsLAC1, 2 and 5 are co-expressed with the regulators of secondary cell wall biosynthesis in *Miscanthus*. Those laccases localize to cell walls and are regulated by the *Miscanthus* lignin-related transcription factors. We also established that recombinant MsLAC1 is able to oxidize monolignol *in vitro*, confirming the catalytic ability of laccases for lignin polymerization. The semi-dwarf phenotype and collapsed xylem phenotypes of the *Arabidopsis lac4-2 lac17* knockout mutant were also complemented by *MsLAC1* and *MsLAC2*. Furthermore, transgenic *Arabidopsis* plants over-expressing *MsLAC1* showed higher G-lignin content, while expressing *MsLAC5* in lac4-2 lac17 double mutant significantly increased the H-lignin content in

stem.

The results provide compelling evidence for the involvement of *Miscanthus* laccases in monolignol polymerization. *Miscanthus* laccases are regulated by secondary cell wall MYBs and MsLAC1 is probably involved in lignification of xylem fibers. The role of *Miscanthus* laccases in orchestrating the lignification process is discussed. Its ability to modify lignin content and composition identifies the *Miscanthus* laccase genes as promising breeding targets in *Miscanthus* for biofuel and biomaterial applications.

Zusammenfassung

Miscanthus ist ein schnell wachsendes C4-Gras mit geringem Wasser- und Nährstoffbedarf, welches eine attraktive Quelle von lignozellulosehaltiger Biomasse darstellt. Das darin enthaltene Lignin, welches ursächlich für eine schwierige Verzuckerung von Biomasse ist, ist gleichzeitig ein einzigartiger Ausgangstoff für aromatische Chemikalien und wird daher als Freund und Feind betrachtet, abhängig von der Verwendung der Biomasse. Aus diesem Grund sind Veränderungen der Ligninmenge und/oder -qualität eines der wichtigsten Züchtungsziele bei *Miscanthus*. Die transkriptionelle Regulation von Genen der Monolignolbiosynthese ist bereits zuvor erforscht worden, die Polymerisation der Monolignole dagegen ist bis jetzt nur schlecht ist. Laccasen sind Phenol-oxidierende Enzyme, die durch die Erzeugung von freien Monolignol-Radikalen die Bildung des Ligninpolymers initiieren können. Einer Vielzahl von Laccasen wird eine Rolle in der Ligninpolymerisation von sekundären Zellwänden und Mittellamelle zugeschrieben, was wiederum Einfluss auf Ligninmenge und –zusammensetzung hat.

In dieser Arbeit wurde der Zusammenhang von Lignifizierung und verschiedenen Laccase-Isoformen von *Miscanthus* untersucht. Die umfangreiche Analyse umfasste Expressionsprofile, Charakterisierung rekombinanten MsLAC1 Proteins Untersuchungen der subzellulärer Lokalisation und der Aktivierung von MsLAC1-5 Promotoren durch Lignin-induzierende R2R3 MYB Transkriptionsfaktoren, die effektive Komplementierung einer *Arabidopsis* Laccase Doppelmutante (*lac4-2 lac17*) und Analyse des Einflusses von ektopischer Expression. MsLAC1, 2 und 5 sind mit Regulatoren der sekundären Zellwandsynthese in *Miscanthus* co-exprimiert. Diese Laccasen sind in Zellwänden lokalisiert und werden von Transkriptionsfaktoren aus *Miscanthus*, welche in Verbindung mit Lignifizierung stehen reguliert. Ergänzend wurde gezeigt, dass rekombinates MsLAC1 Protein in der Lage ist, Monolignole in vitro zu oxidieren, was die katalytische Aktivität der Laccase bestätigt. Der teilweise verzwergte Phänotyp der *Arabidopsis lac4-2 lac17* Mutante mit kollabiertem Xylem wurde von *MsLAC1* und *MsLAC2* komplementiert. Außerdem zeigten transgene

Arabidopsis Pflanzen, die *MsLAC1* überexprimierten, einen hohen G-Lignin Gehalt, wogegen Überexpression von MsLAC5 in der *lac4-2 lac17* Doppelmutante zu signifikant mehr H-Lignin im Stängel führte. Diese Ergebnisse liefern starke Hinweise auf die Beteiligung der *Miscanthus* Laccasen an der Polymerisation von Monolignolen. Außerdem werden *Miscanthus* Laccasen von MYB Transkriptionsfaktoren reguliert, die die Bildung sekundäre Zellwände steuern. MsLAC1 ist sehr wahrscheinlich bei der Lignifizierung von Xylem-Fasern beteiligt und die Rolle von weiteren *Miscanthus* Laccasen an der Lignifizierung wird diskutiert. Die Fähigkeit von *Miscanthus* Laccasen, die Ligninmenge und -qualität zu beeinflussen, machen sie zu einem attraktiven Ziel bei gezielten *Miscanthus* Züchtung für Anwendungen im Bereich von Biotreibstoffen und -materialien.

1. Introduction

Two of the main challenges we are facing in the 21st century are energy supply and environment protection. With the development of technology and industry, the increasing demand of energy leads to higher consumption of fossil fuels. Consequently, the growing greenhouse gas emission from mining industry and the sulfur and nitrogen oxides produced by burning fossil fuels are now causing environmental problems including global warming, acid rain and photochemical smog. Taking into account rising problems, seeking a clean, renewable energy source is of great importance worldwide (Venendaal and Jørgensen et al., 1997). Against this background, lignocellulosic biomass, the most extensive and cheapest resource on the planet, stands out as an alternative way to solve the problems.

Miscanthus, a perennial C4 grass, has been promoted as a promising energy crop producing lignocellulosic biomass due to its significant biomass yield and low demand for fertilizer and pesticides (Lewandowski *et al.*, 2000). However, lignin content and quality as well as the cellulose-to-lignin ratio have a substantial impact on the utilization and degradability of *Miscanthus* biomass (Lygin *et al.*, 2011; Adams *et al.*, 2018). Lignin biosynthesis includes three major steps: monolignol biosynthesis in the cytosol, transport of monolignols to the cell wall matrix, and polymerization into the heterogeneous, cross-linked lignin polymer (Barros *et al.*, 2016). Besides many monolignol biosynthetic genes that have been characterized in *Arabidopsis* and shown to impact on lignin content and composition (Vanholme *et al.*, 2013), there is strong experimental support for oxygen-dependent laccases in the lignin biosynthesis process (Berthet *et al.*, 2011; Cesarino *et al.*, 2013; Cho *et al.*, 2014; Bryan *et al.*, 2016). However, there is still very limited knowledge about lignification in *Miscanthus*.

In this section, the advantages of using *Miscanthus* as an energy crop will be reviewed, and previous studies on lignin biosynthesis and its regulation will be summarized. The research on plant laccases in model organisms and economically important species links their functions with lignin biosynthesis, thus those studies will also be

introduced.

1.1 *Miscanthus*.

Miscanthus is a fast growing perennial C4 grass originated from East and Southeast Asia and native to subtropical and tropical regions, and currently grown mainly in Europe (Lygin *et al.*, 2011). In total, there are around 17 species of *Miscanthus* (Brosse *et al.*, 2012), among which *M. sacchariflorus*, *M. sinensis* and their interspecific hybrids *M.* × *giganteus*, are most important for bioenergy use (Fig. 1.1-1). As a typical C4 plant with *Miscanthus* has an efficient photosynthesis system, leading to significant biomass yield and low demand for fertilizer and pesticides (Lewandowski *et al.*, 2000). Compared with other C4 grasses, *Miscanthus* has higher cold tolerance, even maintaining activity of photosynthesis at 10 ^{o}C (Wang *et al.*, 2008; Dohleman & Long, 2009), making it more adaptable to different environments.

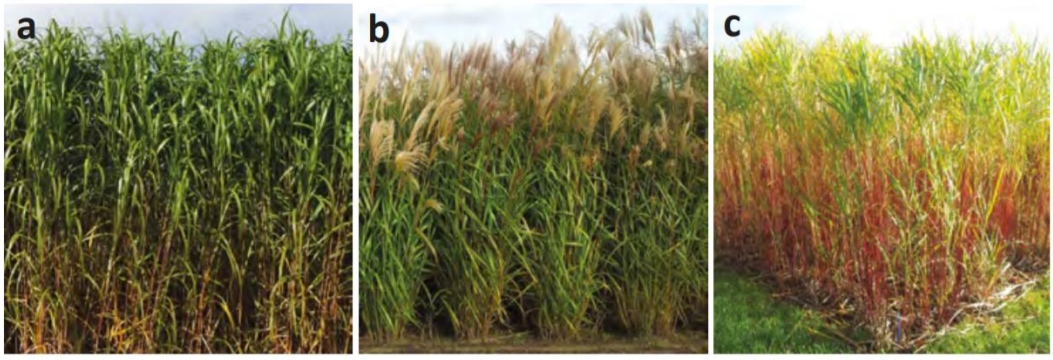

Fig. 1.1-1 Plant phenotype of three species of *Miscanthus*. a, *M.* × *giganteus*, b, *M. sinensis* and c, *M. sacchariflorus* (van der Weijde, 2016).

1.1.1 *Miscanthus* is a promising energy crop.

Miscanthus is a highly productive crop growing 2-4 meters tall in one season and produces lignocellulosic biomass (Anderson *et al.*, 2011). As a perennial grass, once established, *Miscanthus* can maintain production of high yield for more than 20 years (Clifton Brown *et al.*, 2007). Highest yield of *M.* × *giganteus* was found in the 10th year after breeding (Christian *et al.*, 2008). Compared with switchgrass, which is considered as one of the best candidate for bioenergy production, *Miscanthus* showed

much higher production of biomass. In a period of 3 years, *M.* × *giganteus* grown in the Midwestern US achieved harvestable biomass between 30 and 61 t ha^{-1} which is much higher than the yield of switchgrass (10 t ha^{-1}) (Heaton *et al.*, 2008). To achieve the potential yield, switchgrass requires more N fertilization. Therefore, *Miscanthus* has significant advantages in ecological level (Iqbal *et al.*, 2015). *M.* × *giganteus* also revealed to be 59% more productive than *Zea mays*, with twice the size of leaf area and up to 60% higher leaf-level photosynthetic CO_2 uptake (Dohleman & Long, 2009).

Compared to other species, *Miscanthus* biomass contains significantly less moisture and produces less ash, making it suitable for biofuel generation and production of valuable chemicals via bio-conversion and bio-refinery processes (Brosse *et al.*, 2012). The concentration of ash in the biomass will significantly affect heating value, thus influence the quality of combustion. The ash content of different biomass species including wheat straw, barley straw, corn stover and switchgrass is between 5.5 and 10.7 %wt (Mani *et al.*, 2006). Based on dry material, the ash content of different species of *Miscanthus* was only 2.2-3.0 %wt in February and 2.3-3.5 %wt in November (Hodgson *et al.*, 2011), indicating that harvesting in February generally leads to lower ash content for most *Miscanthus* species. High content of moisture will cause problems for storage and transportation and raises the emission of unburned components (Lewandowski & Kicherer, 1997a). In wet basis, the moisture of different Miscanthus species is less than 5 %wt (Brosse *et al.*, 2012), much less comparing to other biomass materials (Mani *et al.*, 2006). For bioconversion, the low moisture content in *Miscanthus* increased the heating value and also decreased the energy input for pretreatment (Iqbal & Lewandowski, 2016).

The composition of *Miscanthus* lignocellulose biomass is also an advantage for using *Miscanthus* as an energy crop. Lignocellulose has three main components, including cellulose (C), hemicellulose (H) and lignin (L). The H:L ratio is considered as a good indicator of ethanol production efficiency from *Miscanthus* biomass (Adams *et al.*, 2018); decrease of lignin content correspondingly increased the degradability of lignocellulose materials. Compared with other typical plant resources of biomass (Fig.

1.1-2), *Miscanthus* has the highest amount of cellulose (45% - 52%) as well as lowest content of lignin (9% - 13%), indicating a promising potential to use *Miscanthus* for bioethanol production.

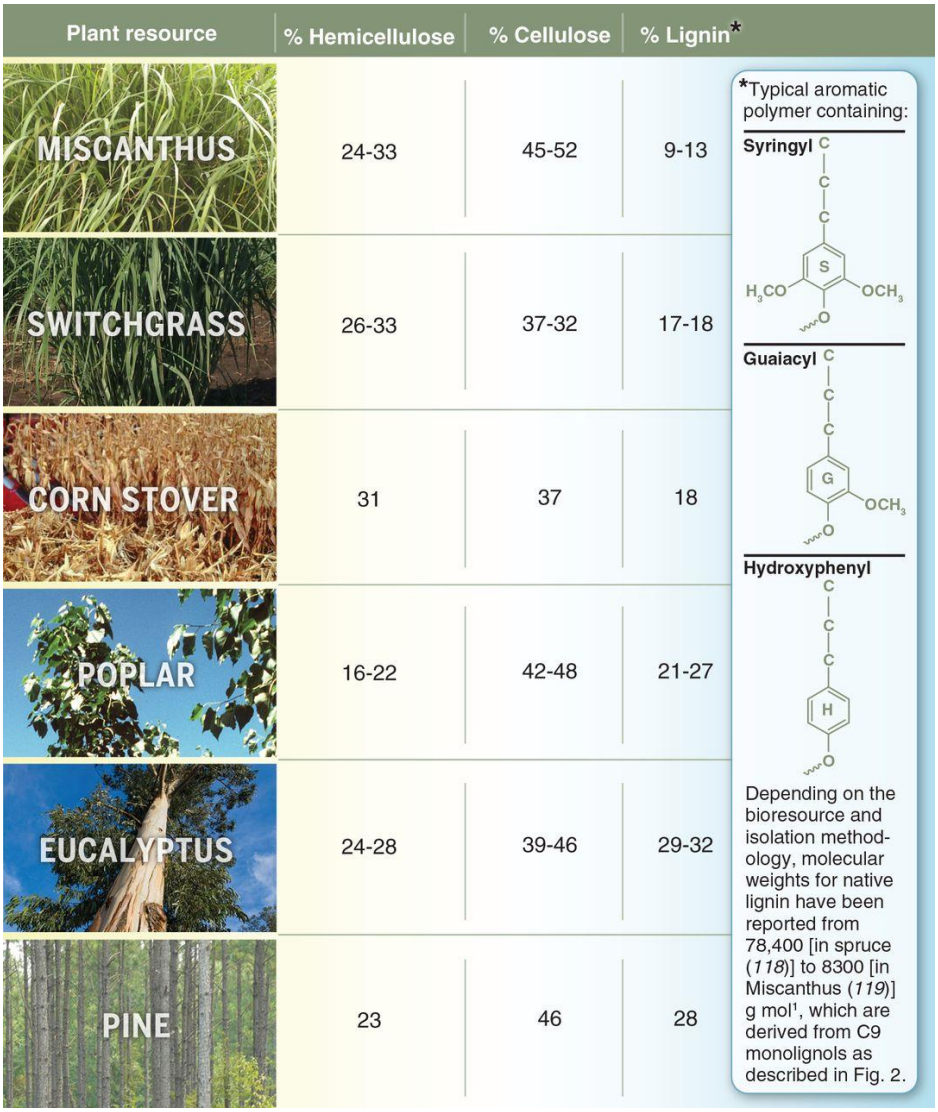

Fig. 1.1-2 Composition of lignocellulose biomass for different plant species (Ragauskas *et al.*, 2014).

1.1.2 Application of *Miscanthus* in bioenergy production.

Originated in Asia, *Miscanthus* was first introduced to Europe in the 1930s, and the research on using *Miscanthus* as energy crop has been started and developed since the 1980s. In 1983, Danmark established the first experimental field of *Miscanthus* for pulping or energy (Venendaal *et al.*, 1997). With the aim to develop new *Miscanthus* hybrids and improve breeding methods, a project under the EU FAIR program was

funded in 1997 to screen different *Miscanthus* genotypes. In addition to burning *Miscanthus* directly or co-fired with coal as a fuel, more applications including bioethanol and biogas production were developed (Brosse et al., 2012; Hodgson et al., 2011).

Combustion:

As mentioned above, the biomass of *Miscanthus* has low moisture and ash content, as well as low mineral content including K, Cl, N and S, thus positively affects the combustion quality (Brosse *et al.*, 2012). The heating value of 20 t *Miscanthus* biomass equals to the value of 12 t hard coals. Furthermore, compared with pure hard coal combustion, biomass combustion using *Miscanthus* reduces CO_2 emission by up to 90% (Lewandowski *et al.*, 1995). In Europe, *Miscanthus* has been widely used for power generation, potentially contribute to about 9% of the total gross electricity production in the European Union in 2000 (Clifton Brown *et al.*, 2004).

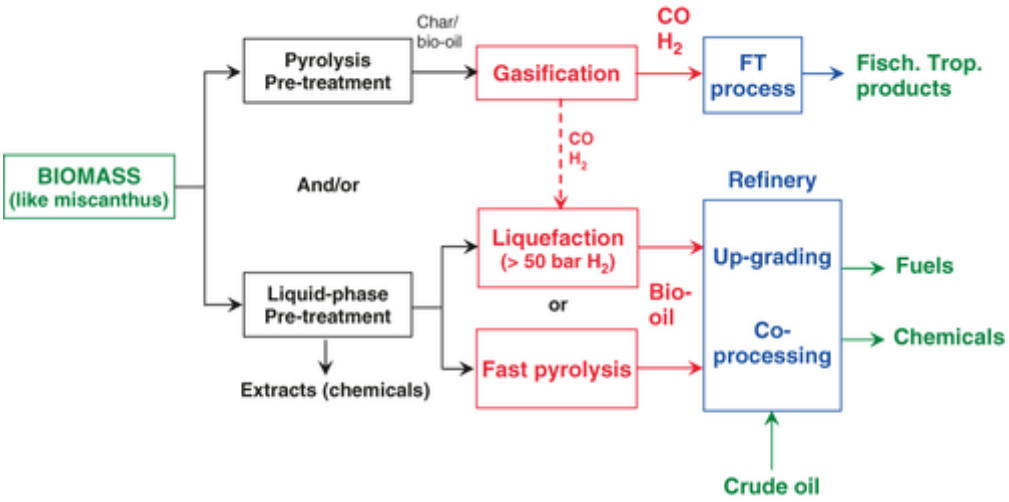

Fig. 1.1-3 Thermochemical conversion of *Miscanthus* biomass for energy and chemical production (Brosse *et al.*, 2012).

Pyrolysis, Liquefaction and Gasification:

Compared with traditional strategies mentioned above, thermochemical methods are considered as promising alternatives for biomass utilization (Fig. 1.1-3). The aims of pyrolysis, liquefaction and gasification are to obtain high quality of solid fuel (char), bio-oil and syngas, respectively. The cell wall composition correlates with the heating value and the ratios of C, H and O which are crucial for the production of high quality fuels (Brosse *et al.*, 2012). *Miscanthus* biomass was shown to be suitable for different

thermochemical conversions (Hodgson *et al.*, 2011; Tanger *et al.*, 2013; Hafez, 2015; Jayaraman & Gökalp, 2015).

Pyrolysis has been developed for charcoal production from biomass material under absence of oxygen. With a high temperature (400-600 ^{o}C), lignocellulose biomass can be decomposed into charcoal, tar and gas (Haarlemmer *et al.*, 2016). Compared with sewage sludge, *Miscanthus* was more suitable for pyrolysis (Jayaraman & Gökalp, 2015). Phenolic and aromatic compounds can be produced after pyrolysis of *Miscanthus* biomass (Park *et al.*, 2012). On the one hand, the quality of biochar can be affected by the pyrolysis conditions, including temperature (Mimmo *et al.*, 2014) and pretreatment (Hodgson *et al.*, 2010); on the other hand, genotypes with different cell wall composition greatly influence the range and yield of products (Hodgson *et al.*, 2011).

To replace fossil liquid fuels, different conversion techniques have been developed to convert biomass into bio-oils. Hydrothermal liquefaction (HTL) takes place at lower temperatures and high pressure and thus is suited for wet biomass, while fast pyrolysis requires very high heating rate (500-1000 ^{o}C s^{-1}) for a short time (Haarlemmer *et al.*, 2016). Using carboxymethylcellulose as a stabilizer, in combination with K_2CO_3 Miscanthus biomass produces the highest bio-oil yield (43%) and a low amount of solid residue (7%) (Lappa *et al.*, 2016). The liquid oil can be refined to biofuels or chemicals.

Gasification refers to the energetic conversion of biomass under controlled air supply into syngas (CO, H_2, CH_4 and CO_2) which is subsequently chemically or biologically cleaned and considered as reliable energy source (Jayaraman & Gökalp, 2015). Around 1.1 m^3 of dry gas was produced after gasification of 1 kg *Miscanthus* stem, containing 46% of H_2 and 24% of CO (Michel *et al.*, 2011). With syngas obtained, electricity can be produced and chemicals including methanol can be synthesized.

Biological conversion:

Biological conversion is a fermentative process of saccharides into gaseous/liquid biofuels or chemicals by microorganisms or enzymes. Lignocellulosic feedstock is by nature recalcitrant to biological degradation. Therefore, a pretreatment is the key to

separate the main constituents that are cellulose, hemicellulose and lignin. Because pretreatment strongly affects subsequent hydrolysis and fermentation and depends on the type of biomass, the numerous pretreatments have to be carefully evaluated. Important criteria are i) enhanced sugar release during enzymatic hydrolysis, ii) minimized formation of fermentation inhibitors like furans that are derived from hemicellulose, and iii) cost effectiveness (Vanholme *et al.*, 2013). Even though fermentation of sugars into fuels, chemicals or materials has a long history, biological conversion of the entire lignocellulose feedstock remains a huge challenge mainly because of the lignin content. Lignin covalently cross-links cell wall polysaccharides and thereby strengthens and rigidifies the cell wall which makes it recalcitrant to biological conversion (Sattler & Funnell-Harris, 2013). For *Miscanthus*, various pretreatment technologies have been developed at laboratory scale in order to optimize subsequent fermentation (Brosse *et al.*, 2012). Biological conversion of *Miscanthus* was shown to hold potentials especially for bioethanol (Boakye-Boaten *et al.*, 2016) but also biogas production (Whittaker *et al.*, 2016). However, economic competitiveness of biological conversion of *Miscanthus* still needs to be evaluated.

1.2 Lignin in *Miscanthus*.

The lignocellulosic biomass from *Miscanthus* has great potential to be used in biorefinery industry because of its high yield and low cost (Lewandowski & Kicherer, 1997b; Yoshida *et al.*, 2008). As we know, cellulose can be hydrolysed by cellulase into monosaccharides directly. Lignin is crucial for cell wall stability and integrity (Boerjan *et al.*, 2003) and forms a barrier against microbial infections (Zhao, 2016). The study on composition of lignocellulose from *Miscanthus* revealed that the cellulose content varied between 32% and 55% among different genotypes while there was little variation in lignin content (22%-28%) (Lygin *et al.*, 2011). Delignification of *Miscanthus sinensis* significantly improved the initial rate of enzyme hydrolysation (Yoshida *et al.*, 2008), suggesting that lignin is a significant resistance factor against enzymatic treatment. Genetic research on different Alfalfa lines also indicated the

relationship between lignin and saccharification, identifying lignin as the major factor in recalcitrance of cell walls to saccharification (Chen & Dixon, 2007). Reducing the content of lignin or changing the composition of monolignols can help to improve the application of lignocellulosic biomass (Giordano *et al.*, 2014; Sykes *et al.*, 2015).

The chemical complexity of polymeric lignin makes it refractory to enzymatic degradation, which becomes a major challenge to improving industrial biomass processing such as biofuel industry (Chen & Dixon, 2007), but also suggesting that it could play a central role as a novel chemical feedstock (Holladay *et al.*, 2007). Due to the polyelectrolyte properties of lignin polymer, traditionally lignin products can be used as adhensives and fillers (Graupner, 2008). Lignin can also be selectively converted into single chemicals (Okuda *et al.*, 2004), leading to release of high-value phenolic compounds like ferulic acid, coumaric acid, vanillic acid and vanillin (Tapin *et al.*, 2006). Recently, the application of lignin-based nanocomposites has attracted increasing interest since they have huge potential to be used in food, packaging, engineering, energy, and others (Wang, X *et al.*, 2015). However, identifying products from lignin was more challenging compared with other carbon resources because of its complex nature. Currently, the main utilization of lignin is burn as fuels for heating and power production (Holladay *et al.*, 2007; Wang, X *et al.*, 2015). In order to seek a balance between the high yield of lignin production and low biological usability, lignin biosynthesis could be modified or 'designed' to reduce the recalcitrance of lignin (Mottiar *et al.*, 2016).

Studies in recent decades showed that the content and composition of lignins differed enormously among different plant taxa and even different cell types in same plant (Weng & Chapple, 2010; Mottiar *et al.*, 2016). A study from the European Miscanthus Improvement (EMI) project indicates that both genetic and environmental factors strongly influence cell wall composition of *Miscanthus*. Interestingly, the *M.* × *giganteus* and *M. sacchariflorus* genotypes were found to produce the highest concentration of cellulose but also contain the highest contents of lignin, which means they would yield the highest heating values theoretically but are less suitable for fermentation or anaerobic digestion (Hodgson *et al.*, 2010).

The evolution from protists to vascular plants is accompanied with the appearance of lignin (Weng & Chapple, 2010). As one of the major division of angiosperms, monocots have the most complex composition of lignin and most complex lignin biosynthesis pathway (Vanholme *et al.*, 2010; Barros *et al.*, 2016). Biosynthesis of lignin in monocots like *Miscanthus* starts with synthesis of hydroxycinnamyl alcohols (monolignols), followed with transportation of those precursors across plasma membrane to form the lignin polymer within the cell wall (Liu, 2012).

1.2.1 Biosynthesis of Lignin.

With studies on monolignol biosynthesis for over a century, the main synthesis route of three dominant monolignol units namely p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units were elucidated. The well-established pathway originates from phenylalamine (Phe) that is obtained from the shikimate pathway (Fig 1.2-1). Phe is then converted via the phenylpropanoid and monolignol-specific pathways for production of various compounds, amongst others the H, G and S units (Vanholme *et al.*, 2010). The modification processes include the deamination and consequential hydroxylation and methylation on the aromatic-ring (Weng & Chapple, 2010). Enzymes involved in the process are summarized in Table. 1.2-1.

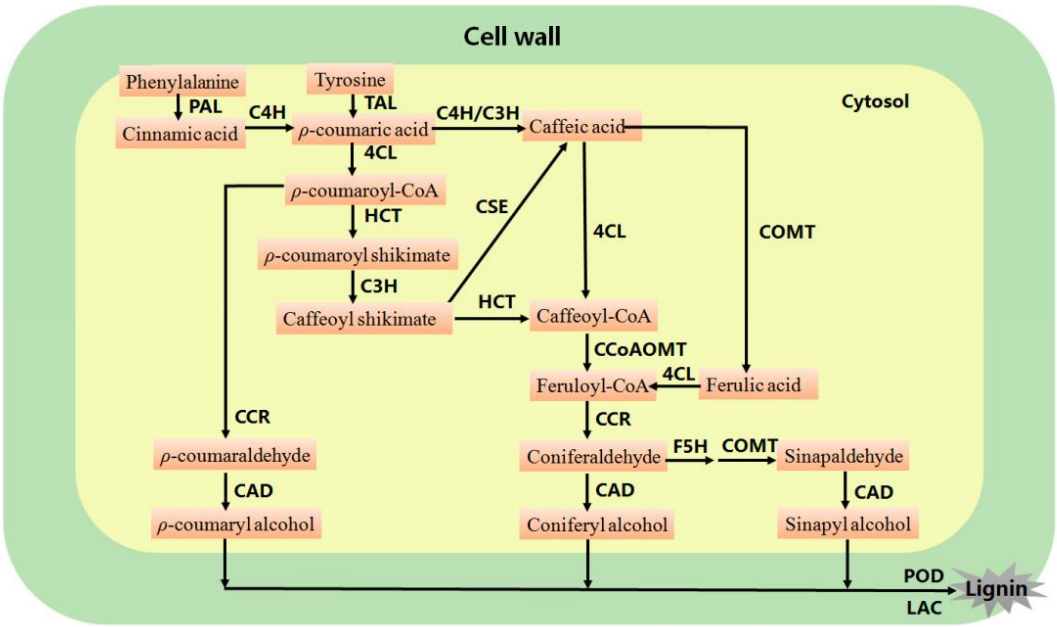

Fig. 1.2-1 Schematic pathway of lignin biosynthesis in plant (Liu *et al.*, 2018). PAL, phenylalanine

ammonia-lyase; TAL, tyrosine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate: CoA ligase; CCR, cinnamoyl-CoA reductase; HCT, hydroxycinnamoyl-CoA shikimate/Quinatehydroxycinnamoyltransferase; C3H, *p*-coumarate 3-hydroxylase; CCoAOMT, caffeoyl-CoA O-methyltransferase; F5H, ferulate 5-hydroxylase; CSE, caffeoyl shikimate esterase; COMT, caffeic acid O-methyltransferase; CAD, cinnamyl alcohol dehydrogenase; LAC, laccase; POD, peroxidase.

Even though the phenylpropanoid pathway is studied in great detail, there are still open questions. Firstly, new enzymes were found to be involved in the lignin biosynthetic pathway. A study on caffeoyl shikimate esterase (CSE) in *Arabidopsis thaliana* revealed that it is an enzyme central to the lignin biosynthetic pathway, hydrolyzing caffeoyl shikimate into caffeate (Vanholme *et al.*, 2013). Barros et al. tested the biochemical characteristics of 8 ammonia-lyases in the model grass species *Brachypodium distachyon* and one of them was found to be bifunctional (Barros *et al.*, 2016). Secondly, the entry steps of lignin biosynthesis still need to be elucidated among different kinds of plants. Unlike the general assumption that most monolignols are synthesized from L-phenylalanine, half of the total lignin in *Brachypodium* was found to be produced via L-tyrosine with fewer steps (Barros *et al.*, 2016; Maeda, 2016). The results indicate that although the monolignol biosynthesis pathways are conserved across the plant kingdom (Chen & Dixon, 2007), function of enzymes involved in the pathway might be multiple in grasses such as *Brachypodium* and *Miscanthus*.

Compared with the detailed biosynthetic pathways of lignin monomers, the current knowledge about polymerization of lignin precursors is limited (Liu, 2012). Hypothesis of lignin polymerization is based on the free-radical mechanism catalyzed by peroxidases and/or laccases (Chen *et al.*, 2002; Wang, J *et al.*, 2015). Whereas peroxidases need hydrogen peroxide for oxidation, laccases use molecular oxygen as the terminal electron acceptor. The lignin polymerization cascade is originated from radicalization of phenol monomers and radical coupling to form a dimer. The dimer is then dehydrogenated again and coupled with another radicalized monolignol, thus forming a growing polymer (Vanholme *et al.*, 2010). In poplar, the radical chain reaction will produce linear lignin chains with an average length between 13 and 20 units (Stewart *et al.*, 2009).

1.2.2 Transcriptional regulation of lignin biosynthesis.

Transcription factors play important roles by regulating the expression of key genes involved in plant growth and development. Lignification happens during the process of secondary cell wall thickening in plants. Several transcription factor families are known as regulators of secondary cell wall synthesis, providing great potential in altering lignin contents in plants, therefore improving the quality of biomass production. Among them, the NAC family and MYB family transcription factors act as master switches of secondary cell wall biosynthesis, while there are more layers of transcription factors involved in downstream regulation of monolignol biosynthesis (Aida *et al.*, 1997; Dubos *et al.*, 2010; Mottiar *et al.*, 2016).

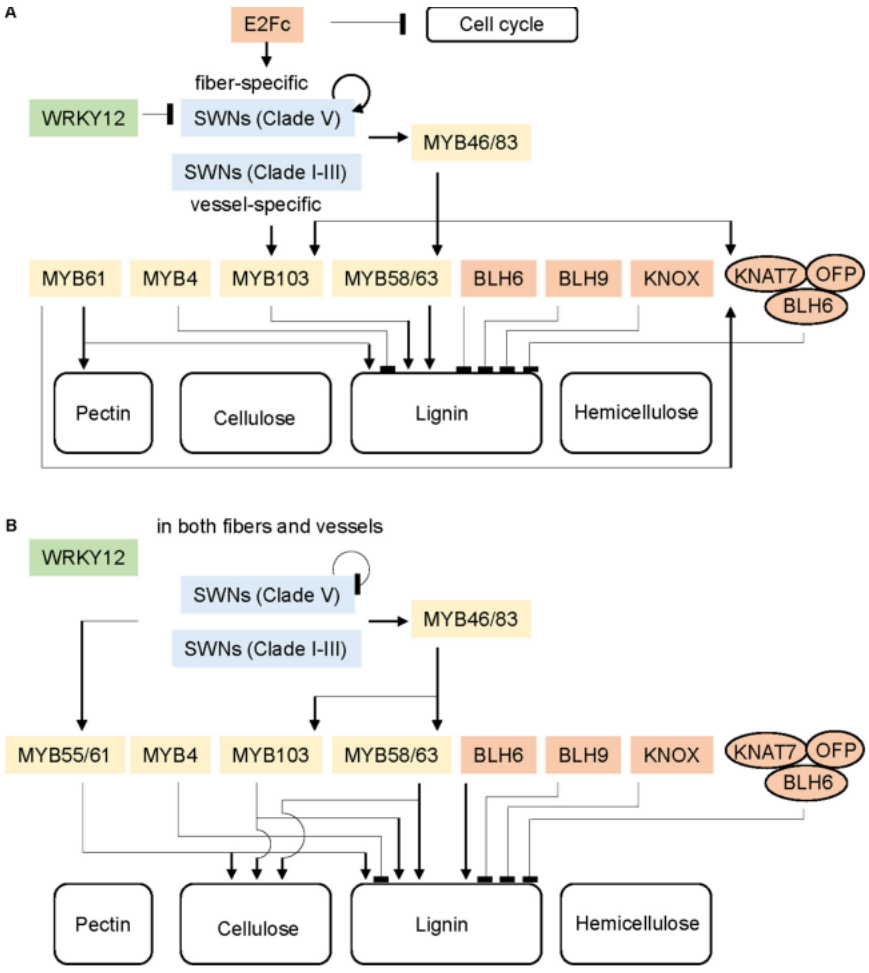

Fig. 1.2-2 Schematic view of the transcriptional regulation network for secondary cell wall formation in *Arabidopsis* and grasses (Rao & Dixon, 2018).

Figure 1.2-2 shows an example of the transcriptional regulation network for biomass

production in model plant *Arabidopsis* and grasses (Zhong & Ye, 2014a; Zhao, 2016; Rao & Dixon, 2018). Some NAC members like SND1 act upstream of the secondary cell wall biosynthesis regulatory network, thus performing as master switches (Wang & Dixon, 2012). They have rather general effects on the development of lignified tissues (Zhong & Ye, 2014b). These NAC transcription factors compose the first layer of the hierarchical transcription factor network (Kubo *et al.*, 2005; Mitsuda *et al.*, 2005). Several MYB transcription factors have been shown to act as regulators of secondary cell wall biosynthesis (Nakano *et al.*, 2015; Taylor-Teeples *et al.*, 2015; Kumar *et al.*, 2016). For example, MYB46 and MYB83 are direct targets of SND1 and work redundantly as a second layer in the TF hierarchy not only to control secondary cell wall formation, but also regulate several downstream MYB factors, i.e. MYB58, MYB63 and MYB85, which in turn specifically control lignin biosynthesis in different tissues and developmental stages (Zhong *et al.*, 2007; McCarthy *et al.*, 2009). Modifying the expression of the NAC transcription factors may lead to severe morphological changes of cells, causing abnormal structures at whole plant level. The downstream transcription factors may provide potential for altering lignin content as they regulate lignin biosynthesis genes in certain plant tissues, raising the possibility to engineer biomass quality. Orthologs of the NACs and MYBs from rice, maize, switchgrass and *Miscanthus* have been demonstrated to regulate secondary wall biosynthesis. Shen et al. overexpressed PvMYB4, a repressor of lignin biosynthetic pathway in switchgrass. PvMYB4 overexpression significantly reduces lignin content, alters cell wall phenolic content, and reduces recalcitrance in transgenic switchgrass (Shen *et al.*, 2012). PvMYB4 has close relationship with ZmMYB42 and ZmMYB31, and overexpression of ZmMYB42 in transgenic *Arabidopsis* changed the contents of monolignols to a decreased S : G ratio by reducing the S units and enriching G and H units (Sonbol *et al.*, 2009). Overexpression of several PvSWNs (secondary wall NACs) in *Arabidopsis* causes similar ectopic deposition, expression of PvSWNs in the *Arabidopsis snd1*/*nst1* double mutant rescued the pendent stem phenotype, indicating the functional ortholog and the evolutionary conservation of the lignin transcriptional network in plant (Zhong *et al.*, 2015). In *Miscanthus*, MsSND1 also

works as a master switch for the regulation of secondary cell wall formation (Golfier *et al.*, 2017).

1.2.3 Genetic engineering of lignification – enzyme downregulation.

Since several enzymes are known to have crucial functions in the biosynthesis of lignin, attempts have been made to individually modulate their expression to affect total lignin content (Table. 1.2-1). Thus, the S/G ratio is increased by suppression of C4H, CAD and F5H with induced production of G unit. The knockdown of these key enzymes in the phenylpropanoid pathway and down regulation of Cytochrome P450 monooxygenases except F5H significantly reduces lignin content in *Brachypodium* and Alfalfa, respectively. However, a reduced amount of lignin is generally accompanied with reduced growth and increased susceptibility towards pathogens (Reddy *et al.*, 2005; Cass *et al.*, 2015). In comparison, down-regulation of CCR, CAD and COMT does not compromise growth of plants, however, the impact on lignin content is comparatively low (Tu *et al.*, 2010; Fu *et al.*, 2011; Tamasloukht *et al.*, 2011). In general, massive down-regulating of lignin biosynthesis enzymes may reduce the need for biomass pretreatment before the enzymatic cellulose digestion (Sticklen, 2008), but may also lead to negative effect on the production of biomass.

Enzyme	Plant	Lignin reduction	Monolignols			Citation
			H	G	S	
PAL	*Brachypodium*	43%	↑	—	↑	(Cass *et al.*, 2015)
C4H	*Medicago sativa*	40%	↑	↑	↓	(Reddy *et al.*, 2005)
4CL	*Sorghum bicolor*	20%	S/G ratio was significantly higher in mutant*			(Saballos *et al.*, 2012)
HCT	*Medicago sativa*	50%	↑	↓	↓	(Shadle *et al.*, 2007)
C3'H	*Zea mays*	Up to 23%*	↑	—	↓	(Fornalé *et al.*, 2015)
	Medicago sativa	30%	↑	↓	↓	(Reddy *et al.*, 2005)
CCoAOMT	*Zea mays*	22.4%	57.08% higher S/G ratio			(Li *et al.*, 2013)
CCR	*Zea mays*	10%	↓	↓	↑	(Tamasloukht *et al.*, 2011)
	Paspalum dilatatum	Up to 20%	—	↓	—	(Giordano *et al.*, 2014)
CAD	*Panicum*	14% - 22%	S/G ratio was reduced			(Fu *et al.*, 2011)

	virgatum		in most of the transgenics			
	Brachypodium	20% - 26%	—	↑	↓	(Bouvier D'Yvoire *et al.*, 2013)
COMT	*Lolium perenne*	6%	↓	↓	↓	(Tu *et al.*, 2010)
F5H	*Medicago sativa*	—	—	↑	↓	(Reddy *et al.*, 2005)

*Calculated or concluded from data in the reference.

Table 1.2-1 Down-regulation of lignin biosynthesis enzymes and their effect on lignin content and composition.

Model plants like *Arabidopsis*, rice and poplar have provided broad genetic resources for deciphering the hierarchical network of lignin regulation in other species, including bioeconomy crops like *Miscanthus* (Zhong *et al.*, 2011; Hirano *et al.*, 2013; Lin *et al.*, 2013). For instance, Lin and Li et al. established a genome-wide high-throughput system to investigate the SND1-directed hierarchical gene regulatory networks (hGRNs) in wood formation by using poplar stem differentiating xylem protoplasts (Lin *et al.*, 2013). This method may be particularly useful to study hGRNs in complex processes in plant species resistant to stable genetic transformation and where mutants are not available, such as *Miscanthus*. Hirano et al. identified 123 TFs as candidate secondary cell wall regulators in rice by using co-expression network analysis. Despite the similar phenotypes of dwarfism in plants overexpressing these TFs, they did not observe any phenotypes related to reduced mechanical strength in the TF knockdown rice plants, which is extensively reported in *Arabidopsis*. These results show the different phenotypes upon downregulation of TFs involved in secondary cell wall formation, indicating the possibility to alter lignin content in grass without obvious effect on plant structure (Hirano *et al.*, 2013). In addition, Agarwal et al demonstrate that the MYB31/42 syntelogs in three monocots maize, sorghum and rice have divergent regulation targets of phenylpropanoid genes along the pathway, reflecting a sub-functionalization of the regulatory genes following gene duplication, which is of great value for engineering of the flux of lignin metabolites in promising biomass sources like *Miscanthus* (Agarwal *et al.*, 2016).

In addition to total lignin content, lignin structure may strongly influence the quality of biomass. Therefore, with the goal to improve biomass degradability, attempts to modify monolignol polymerization by “design” are certainly worth trying. As stated

above, radicalization of monolignols involves peroxidases and laccases (Zhao *et al.*, 2013). Both enzymes comprise large isoenzyme families which seem to exhibit a certain degree of redundancy in lignin polymerization (McCaig *et al.*, 2005). Recent studies on laccases in *Arabidopsis* have revealed that disruption of LAC17 expression affected both lignin content and the deposition of G lignin units in interfascicular fibers (Berthet *et al.*, 2011), however, only lignin content could be complemented by sugarcane laccase SofLAC (Cesarino *et al.*, 2013). The *lac4/lac17* double mutant showed reduced lignin content (-40%), whereas in the *lac4/lac11/lac17* triple mutant lignin deposition was completely abolished (Berthet *et al.*, 2011), resulting in a severe plant growth defect (Zhao *et al.*, 2013). Similar studies in a wide range of biomass crops are needed to confirm the usefulness of downregulating laccases for energy plant improvement. Lignin engineering through laccase modification appeared to be more flexible and tissue specific (Wang, J *et al.*, 2015), and thus may provide a promising way to improve the biomass quality in energy plants like *Miscanthus*.

1.3 Plant laccase.

Laccase belongs to the multicopper oxidase family, a group of enzymes that is widespread in numerous fungi, plants, and bacteria (Mayer & Staples, 2002). The function of laccases varies greatly between different types of organisms. Few bacterial laccases were described with putative role in encystment and melanogenesis (Diamantidis *et al.*, 2000). Laccases in fungi are well-studied, playing an essential role for lignin biodegradation and in other biological functions (Viswanath *et al.*, 2014). After the laccase gene family of *Arabidobsis* was analysed (McCaig *et al.*, 2005), the first genetic evidence for the role of laccase in lignin synthesis was reported (Liang *et al.*, 2006), elucidating the involvement of plant laccases in lignification.

1.3.1 Family of laccase genes

Laccases always exist as a large family in plants, 17 laccase genes were found in

Arabidopsis thaliana (Turlapati *et al.*, 2011), 30 in *Oryza sativa* (Cho *et al.*, 2014), 29 in *Brachypodium distachyon* (Wang, Y *et al.*, 2015) and at least 25 in *Lolium multiflorum* (Gavnholt *et al.*, 2002).

Compared to dicot species, laccase families in monocots appear to be larger (Cesarino *et al.*, 2013; Wang, Y *et al.*, 2015). Previous studies in *Arabidopsis* suggested a division of the laccase family into six subgroups (Fig. 1.3-1) (Turlapati *et al.*, 2011), but fewer subgroups have been described for monocot species. For example, in *Brachypodium*, phylogenetic analysis revealed four subgroups (Wang, Y *et al.*, 2015), while sugarcane contains five subgroups (Cesarino *et al.*, 2013).

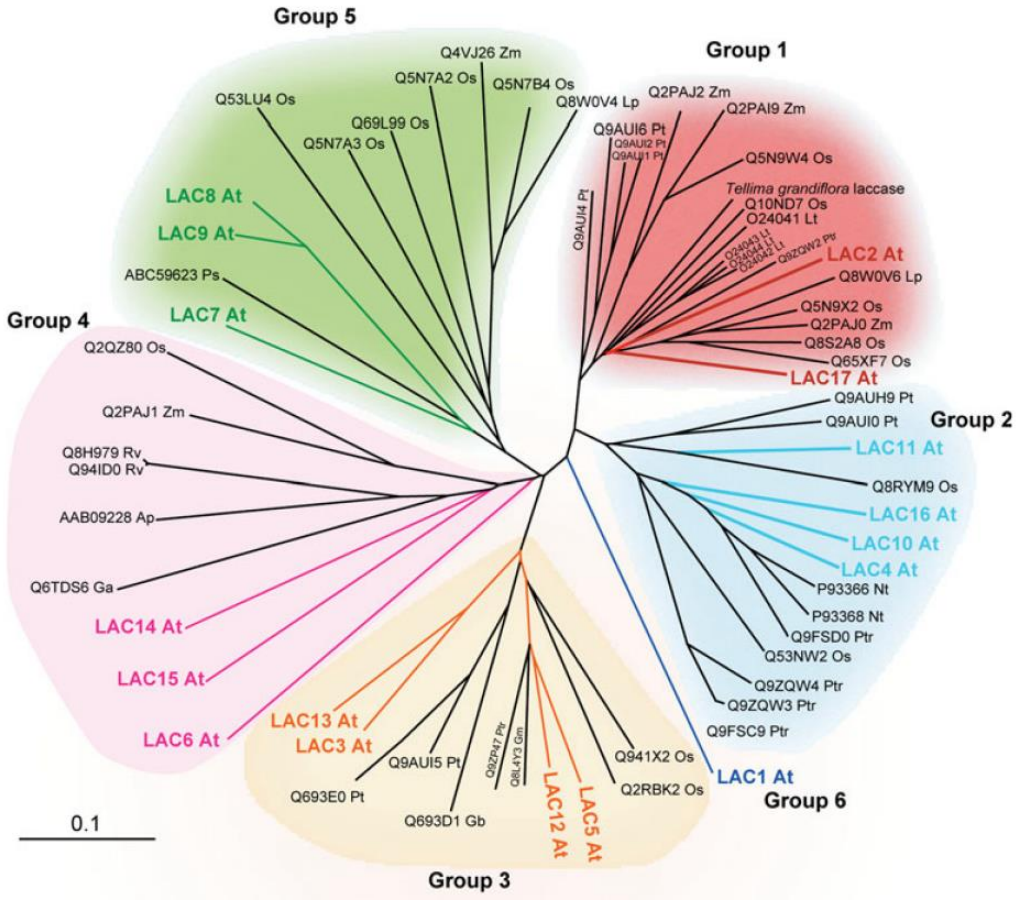

Fig. 1.3-1 Neighbor-joining phylogenetic tree of laccases from different plant species (Turlapati *et al.*, 2011). Ap, *Acer pseudoplatanus*; At, *Arabidopsis thaliana*; Ga, *Gossypium arboretum*; Gb *Ginkgo biloba*; Gm *Glycine max*; Lt *Liriodendron tulipifera*; Lp *Lolium perenne*; Nt *Nicotiana tabaccum*; Os *Oryza sativa*; Ps *Pisum sativum*; Pt *Pinus taeda*; Ptr *Populus trichocarpa*; Rv *Rhus vernicifera*; Zm *Zea mays*.

1.3.2 Expression and localization of plant laccases

The first laccases gene was cloned from sycamore maple in 1995 (LaFayette *et al.*, 1995), since then, laccases genes from different species were isolated and characterized (Kiefer-Meyer *et al.*, 1996; LaFayette *et al.*, 1999; Ranocha *et al.*, 1999; Sato *et al.*, 2001; Gavnholt *et al.*, 2002; McCaig *et al.*, 2005; Caparrós-Ruiz *et al.*, 2006). The expression of laccases showed tissue specificity, resulting in unique spatial and temporal expression pattern for different isoforms. In the model plant *Arabidopsis*, Promoter-GUS analyses revealed the unique cell type-specific expression of some laccases. More specifically, LAC4 was uniquely expressed in stem tissue interfascicular fiber, anther walls, and seed coat columella. By contrast, LAC7 was uniquely expressed in root hairs of early germinating seeds and hydathodes, whereas LAC8 was uniquely noted in leaf primordia, phloem of inflorescence stem tissues and in pollen grains of loral organs (Turlapati *et al.*, 2011). In sugarcane, a lignin-related laccase SofLAC is localized in sclerenchymatic bundle sheaths detected by *in situ* hybridization (Cesarino *et al.*, 2013). Based on RiceXPro database, the spatiotemporal expression of rice laccases was analyzed and most of them were highly expressed in the roots and stems (Liu *et al.*, 2017). Notably, the expression of most of laccase genes is observed in lignifying tissues, suggesting a potential role in cell wall formation.

Localization of laccases significantly affects the function of the enzyme. Study of subcellular localization revealed that most of plant laccases are extracellular proteins. The polymerization of monolignols occurs in the cell wall matrix, thus cell wall-localized enzymes are required (Vanholme *et al.*, 2012). To be more specific, the vacuole-localized AtLAC15 is involved in the polymerization of proanthocyanidins (Pang *et al.*, 2013), whereas the cell wall-associated AtLAC4 is localized throughout the secondary cell wall layers (Yi Chou *et al.*, 2018).

To detect functional genes involved in secondary cell wall biosynthesis, co-expression analysis has been widely performed (Ruprecht & Persson, 2012; Cesarino *et al.*, 2013). Studies in C4 grass *B. distachyon* showed that there is a gradient of increasing

lignification in the internodes along with maturity (Wang, Y *et al.*, 2015), and leaf differentiation in *Miscanthus* follows a linear pattern from the leaf sheath toward the leaf tip with a continuous lignification gradient (Golfier *et al.*, 2017). Co-expression analysis of genes involved in secondary cell wall biosynthesis in *Miscanthus* clearly showed the correlation between laccases and monolignol biosynthesis genes (Hu *et al.*, 2017). In this case, understanding the expression pattern of laccases is a good tool to study the specific function of individual laccase.

1.3.3 Molecular properties of plant laccase

Typical laccases contain 4 copper ions, including one type-1 (T1) copper, one type-2 (T2) and two type-3 (T3) copper ions, classified according to their spectroscopic and ESR parameters (Mayer & Staples, 2002). The T2 and T3 centers are close together and form a trinuclear cluster. T1 is the site where substrate oxidation takes place. Electrons from substrates are transmitted from T1 to the trinuclear cluster along a pathway containing cysteine and histidine residues, where oxygen reduction reaction occurred. To understand the differences between fungal and plant laccases, molecular docking analyses were conducted and revealed a favorable protein structure for plant laccase to synthesis lignin polymer (Fig. 1.3-2) (Awasthi *et al.*, 2015).

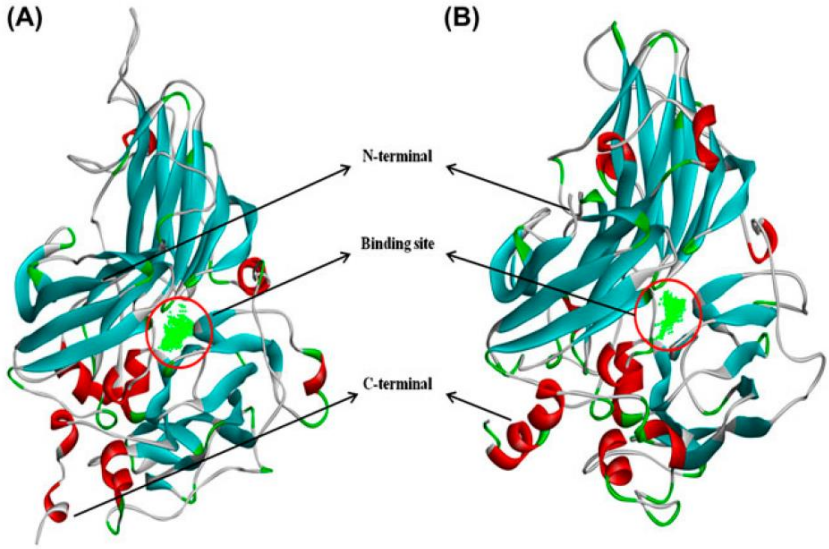

Fig. 1.3-2 3D structures of (A) plant and (B) fungal laccases generated by homology modeling (Awasthi *et al.*, 2015).

Plant laccases are glycoproteins with higher carbohydrate content (20–45 %) than fungal laccases (10–25 %). As a result, the molecular weight of plant laccases (60-130 kDa) can be much higher than the calculated size (*ca.* 60 kDa) (Wang, J *et al.*, 2015). Accordingly, 9-20 N-glycosylation sites can be found in plant laccase sequences (ESAKA *et al.*, 1990) binding carbohydrates including hexoseamine, glucose, mannose, galactose, fucose, and arabinose (Yaropolov *et al.*, 1994).

As a multifunctional oxidase, laccase has a wide range of phenolic substrates, including monolignols, monophenols with amino or methoxy substitutions, bisphenol, etc. Radical-based coupling of phenolic substrates provided the possibility to polymerize monolignols into lignin. The monolignol radicals formed by laccase can subsequently react with each other to form larger polymers (Gavnholt & Larsen, 2002). Furthermore, the properties of laccase make it particularly useful to conduct oxidative processes required in various biotechnological applications (Jeon *et al.*, 2012).

1.3.4 Function of plant laccases in lignification

In this decade, with the in-depth study of plant genomes and the development of transgenic technologies, researches on plant laccase functions and its regulation have been greatly promoted. However, due to the genetic redundancy among those isoenzymes, functional definition of individual isoforms is still unclear. In *Arabidopsis*, 17 laccase isoforms were found and those enzymes were considered to be involved in various metabolic processes (Cai *et al.*, 2006), including biosynthesis of lignin, proanthocyanidin or tannin (Zhang *et al.*, 2013).

In the early 1990's, characterization of the Laccase purified from loblolly pine xylem was shown to produce dehydrogenation polymers (DHPs) from coniferyl alcohol which promoted a reevaluation of the role this enzyme might have in lignification (Bao *et al.*, 1993). Study of *Arabidopsis* laccase gene mutants provided a valuable option to directly characterize those enzymes. Accordingly, *AtLAC4*, *AtLAC11*, *AtLAC17* and *AtLAC15* were reported to participate in lignification in different tissues

based on their localization (Liang *et al.*, 2006; Zhao *et al.*, 2013). Disruption of *AtLAC4* and *AtLAC17* results in reduced lignin content and altered S/G ratio in *Arabidopsis* stems (Berthet *et al.*, 2011).

In other plant species, it is also reported that laccases are involved in lignification. Studies in cotton showed a positive relationship between lignin content and laccase activity (Balasubramanian *et al.*, 2016). In *Brachypodium*, *BdLac5* is necessary for lignification of culm (Wang, Y *et al.*, 2015), mutation of *BdLac5* results in 10% reduction of lignin content. The rice laccase *OsLAC10* also plays an important role in lignin biosynthesis, increasing lignin accumulation in *Arabidopsis* roots if over-expressed (Liu *et al.*, 2017). Research in *Populus*, *Picea*, *Pyrus* and *Saccharum* also suggested a potential function of laccases in lignification (Cesarino *et al.*, 2013; Koutaniemi *et al.*, 2015; Bryan *et al.*, 2016; Cheng *et al.*, 2019).

However, the function of laccases on lignification is non-redundant to peroxidases. The *Arabidopsis* LAC4 localized throughout the secondary cell wall while one lignin-related peroxidase (PRX64) localized to the middle lamella (Yi Chou *et al.*, 2018). Considering the fact that lignification of middle lamella is still detectable in *Arabidopsis lac4-2 lac17* double mutant (Berthet *et al.*, 2011), function of laccases seems to be independent and unique. The spatial control of lignification in plant cell wall by different enzymes still requires more reasons. However, the coordination of enzyme localization and substrate availability is likely to be important for the regulation of spatio-temporal lignification pattern (Tobimatsu & Schuetz, 2019).

2. Aims

Understanding lignin biosynthesis and composition is of central importance for sustainable bioenergy and biomaterials production. Although in *Arabidopsis* it is confirmed that some laccases are playing important role in lignification, knowledge about *Miscanthus* laccases is still limited. Furthermore, the genetic redundancy among plant laccase isoforms in the multigene family makes the characterization of specific plant laccase a challenge. This thesis aims to identify and characterize some laccase isoforms in *Miscanthus*, elucidating the function of laccases in lignin polymerization and monolignol distribution, and try to understand the differences of *in vivo* functions between laccase isoforms.

To elucidate the function of laccases in lignin biosynthesis, multiple experimental strategies including molecular, biochemical, and histochemical analyses were performed. This work consists of the following project parts:

(1) Identification of *Miscanthus* laccase contigs and bioinformatic characterization of five cloned laccase sequences.

(2) Co-expression studies (qRT-PCR analysis) of laccases with genes involved in lignin biosynthesis (monolignol biosynthesis genes and lignin-related TFs).

(3) Understanding the localization of *Miscanthus* laccases by histochemical staining and tobacco transient expression with fluorescence tags.

(4) Revealing the regulation of Miscanthus laccase promoters by secondary cell wall related NAC and MYB factors.

(5) Determining the *in vitro* function of *Miscanthus* laccase in monolignol polymerization using recombinant laccase protein expressed in *P. pastoris*.

(6) Determining the *in vivo* function of *Miscanthus* laccases in lignification by expressing them with native AtLAC17 promoter in *lac4-2 lac17* or with CaMV 35S promoter in *Col-0*.

Based on the fact that lignin can be engineered through laccase modification, we sought to identify the *Miscanthus* laccase genes as promising breeding targets.

3. Results

3.1 Laccases exist as a large gene family in Miscanthus

3.1.1 Staining and activity assay revealed the first evidence connecting laccases and lignification in *Miscanthus*

The localization of laccase enzyme activity in plant tissue is possible by histo-enzymology studies using specific laccase substrates. Adams et al. (2014) detected laccase activity on dead, lignified cells undergoing depolymerization with the presence of fungal hyphae, thus declaring the function of fungal laccases in lignin degradation. Using 3,3'-diaminobenzidine as substrate, laccase activity was also detected in fresh cross section of hemp stem, showing a stronger signal in secondary xylem than fibers (Behr *et al.*, 2018). In our study, ABTS was used for staining of laccases, showing bright blue signal after oxidizing. Cross sections were incubated with catalase to remove H_2O_2, thus exclude the influence of peroxidases. The same tissue was used for lignin staining to make a comparison.

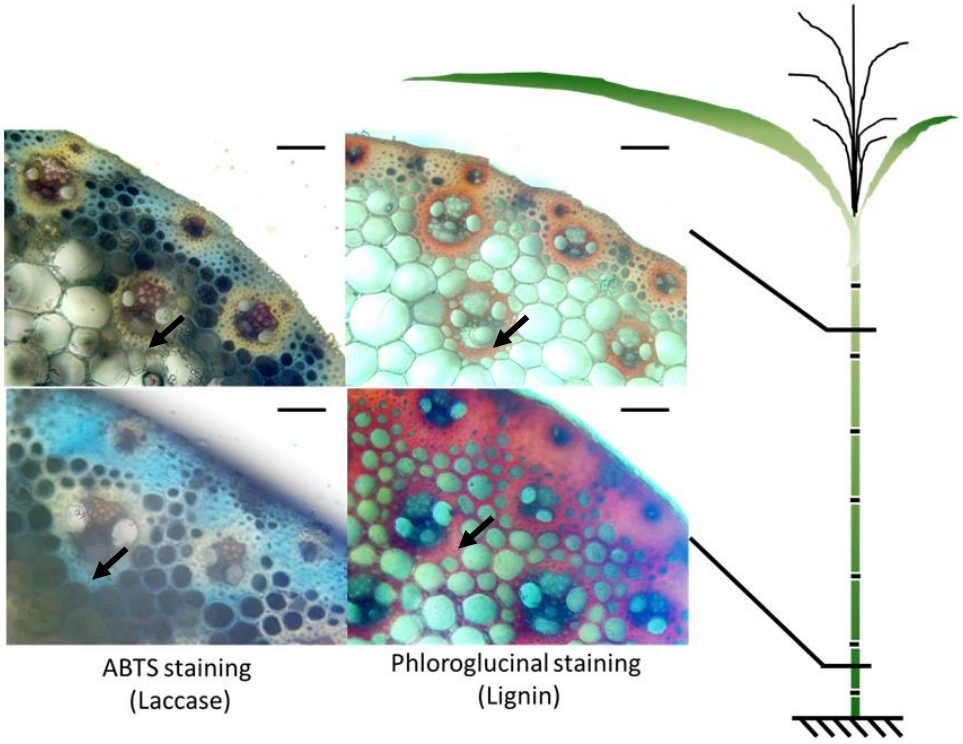

Fig 3.1-1 Staining for laccase activity and lignin in cross sections of second and seventh internode

of mature *Miscanthus* plant. Arrows indicate the thickened fiber cells surrounding vascular bundle. Bar = 100μm.

After staining with ABTS, a bright blue signal indicating laccase activity was detected only in lignified cell walls, including sclerenchyma fiber cells in both, young and old tissues, as well as the thickened fiber cells of vascular bundles in fully developed stem (Fig 3.1-1). Phloroglucinol staining was carried out to visualize lignin in sections of two different internodes. In the monocot plant switchgrass the lignin content changes as the plant grows (Shen *et al.*, 2009). In mature *Miscanthus* plants, the staining of lignin showed increased lignification from the top to the bottom of a stem; similarly, stronger laccase activity signal was detected in the second than in the seventh internode. However, laccase activity was not detected in vascular bundles, although they were highly lignified. By comparing lignification in three segments of an elongating internode (2nd from top) in *Miscanthus*, Hu et al. (2017) has found significantly expanded vascular bundles, probably due to the the thickened fiber cells (Hu *et al.*, 2017), which is exactly where we have found the activity of laccases. Our data had clearly shown that there was a strong correlation between lignification and laccase localization/activity in *Miscanthus*. Thus, we've hypothesized that laccase may play an important role in lignin biosynthesis in *Miscanthus*.

3.1.2 *Miscanthus* has complex laccase zymogram

In higher plants, the analysis of completed genome sequences revealed that laccases are widely distributed as multigene families (McCaig *et al.*, 2005; Liu *et al.*, 2017), encoding isoforms with distinct properties. Because of the diverse molecular weights and isoelectric points (pI) of laccases, isoforms can be detected as single bands on native-page gel (He *et al.*, 2015a). Compared with laccases in *Arabidopsis*, although the total laccase activity does not differ much, the zymogram of *Miscanthus* is more complex since bands were detected both on acidic and basic gels (Fig S1). Using *Miscanthus* leaf gradient as an efficient candidate to evaluate the expression of genes in developmental level (Golfier *et al.*, 2017), we found that along the development gradient of the leaf, both the composition of zymogram and total laccase activity

against ABTS changed continuously (Fig 3.1-2b). More bands were shown on the acidic gel, meaning that majority of laccases in *Miscanthus* could be basic proteins with high pI.

Plant laccases are glycoproteins with high carbohydrate content (20–45%), making the molecular weight of plant laccases (60-130 kDa) much higher than calculated (~60kDa) (Wang, Y *et al.*, 2015). Proteins extracted from leaf sheath (gradients 1-3) and blade (gradients 4-10) were loaded onto gel penetration column, separately, and the laccase activity in fractions was tested to measure the molecular weight of corresponding laccase(s). The apparent weight ranges from 62kDa to 233kDa (Fig 3.1-2a). In addition to glycosylation, dimer or multimer may also be formed thus contributing to the extremely large size of laccase in *Miscanthus* (Jaiswal *et al.*, 2015).

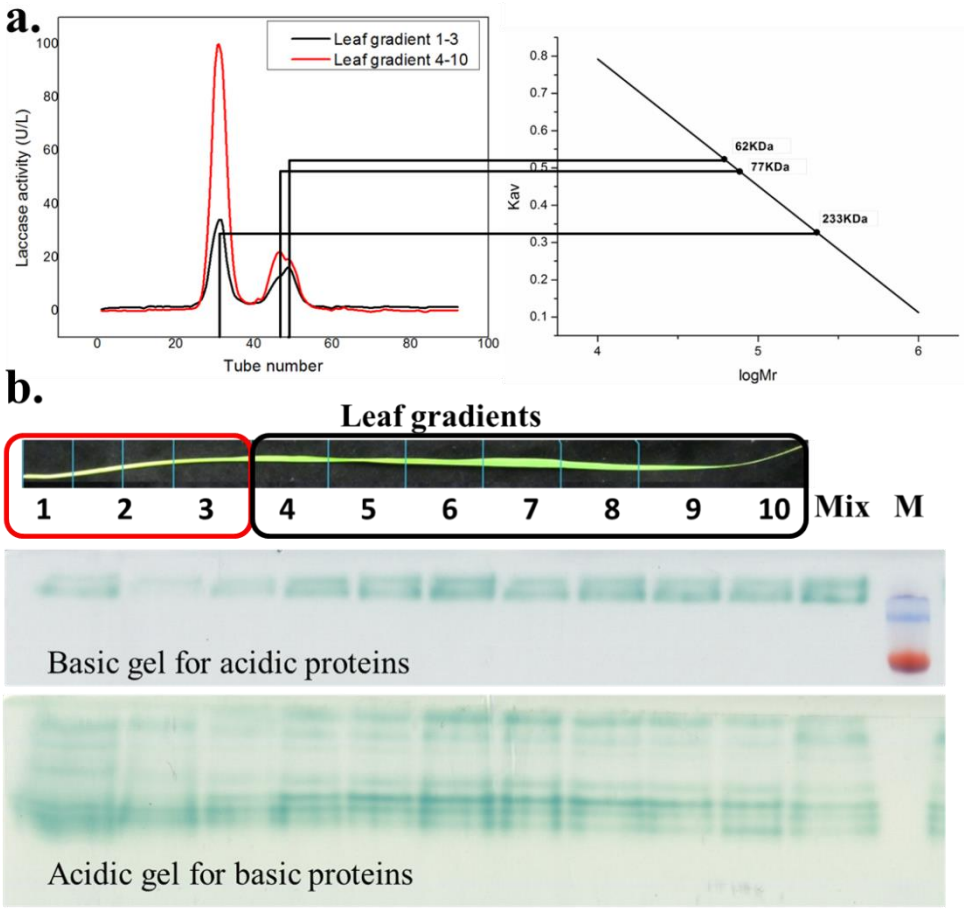

Fig 3.1-2 (a) Analysis of laccase molecular weight by gel penetration chromatography (GPC). (b) Zymography of laccases with protein extracted from different leaf gradients. Activity of laccase was measured in each tube after elution. To calculate the molecular weight of picks with high laccase

activity, a standard curve based on Ribonuclease A (13.7 kDa), carbonic anhydrase (29 kDa), conalbumin (75 kDa), aldolase (158 kDa) and ferritin (440 kDa) was used. Same samples were loaded on basic and acidic gels to separate acidic and basic laccases. Gels were stained with ABTS after 1h incubation with catalase.

3.1.3 Identification of laccase isoforms in Miscanthus

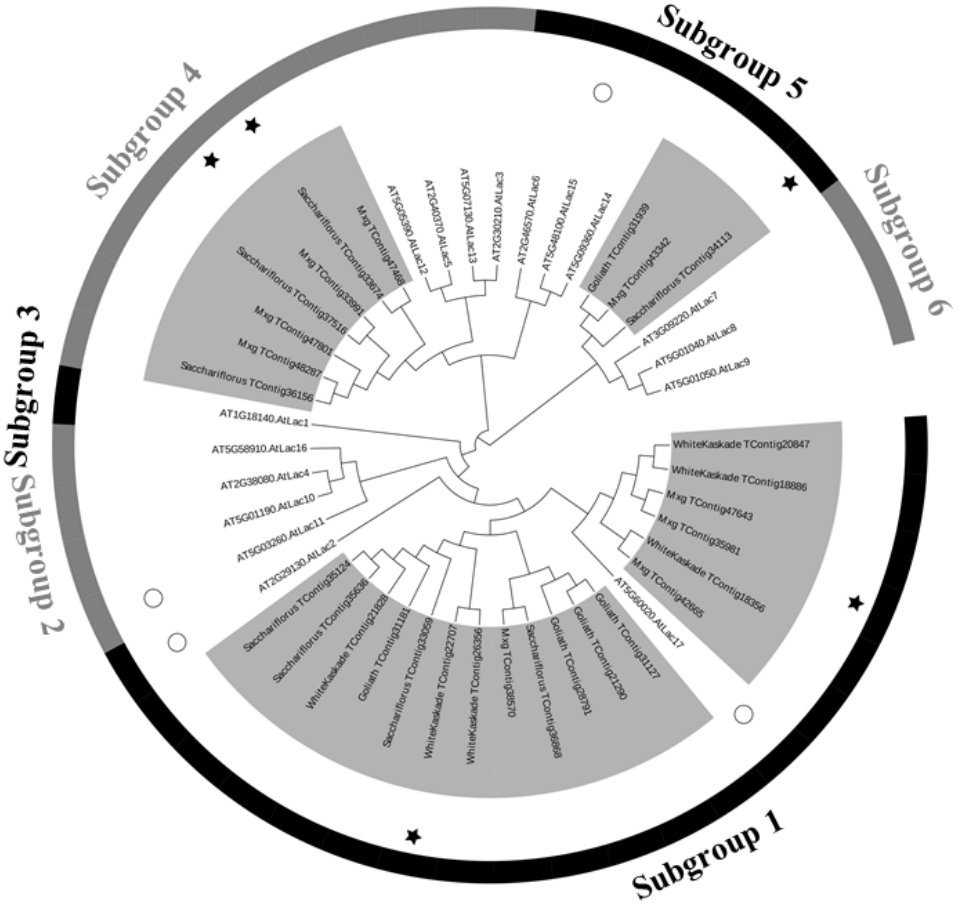

Fig 3.1-3 Neighbor-joining phylogenetic tree of *Miscanthus* and *Arabidopsis* laccases. All the *Miscanthus* laccase sequences were marked with grey background. The stars indicate the five laccases successfully cloned. The circles indicate the *Arabidopsis* laccases involved in lignin biosynthesis.

Laccases are widespread in different plant species. For example, 17 laccase isoforms were found in *Arabidopsis thaliana* (Turlapati *et al.*, 2011), 25 in *Sorghum bicolor* (Rai *et al.*, 2016), and 29 in *Brachypodium distachyon* (Wang, Y *et al.*, 2015). Compared with dicot plant laccase family, the number of laccase isoforms in monocots appears to be larger (Cesarino *et al.*, 2013; Wang, Y *et al.*, 2015). By using those published laccase sequences, we performed tBLASTn analysis of the

Miscanthus transcriptome. In total, 95 laccase-like contigs were identified, including 28 contigs containing complete putative laccase coding sequences. To better understand laccase sequence divergence and similarities among *Miscanthus* laccase family and other plants, a phylogenetic analysis was performed to classify those contigs (only the ones with complete ORFs) against *Arabidopsis* and *Brachypodium* laccases (Fig. 1.1-3).

As reported in previous studies, *Arabidopsis* laccases can be divided into six arbitrary subgroups (Turlapati *et al.*, 2011), while less subgroups were found in monocot plants like *Brachypodium* and sugarcane. Specifically, four subgroups were classified in *Brachypodium* (Wang, Y *et al.*, 2015) while sugarcane laccase family was divided into five subgroups (Cesarino *et al.*, 2013). Consistent with those reports, laccases in *Miscanthus* can be categorized into only three out of six *Arabidopsis* subgroups (Subgroup 1, 3, 5), the most of them belonging to the phylogenetic group 1. The putative *Miscanthus* laccases were generally clustered separately from the analyzed dicot laccases, indicating relatively low sequence homology between those species at least in part.

3.1.4 Laccase cDNA cloning and sequence comparisons

LAC1	100%									
LAC2	66.97%	100%								
LAC3	47.96%	47.02%	100%							
LAC4	37.61%	38.55%	35.42%	100%						
LAC5	51.41%	52.19%	47.96%	48.11%	100%					
35981	81.97%	64.10%	47.17%	38.40%	51.41%	100%				
22707	66.30%	76.64%	45.45%	36.05%	50.47%	68.65%	100%			
34113	48.11%	47.17%	99.05%	35.42%	48.11%	47.33%	45.76%	100%		
33674	37.77%	38.40%	35.42%	96.55%	49.05%	38.24%	36.20%	35.42%	100%	
33991	52.11%	53.67%	48.35%	48.20%	81.37%	52.26%	51.64%	48.35%	48.98%	100%
	LAC1	**LAC2**	**LAC3**	**LAC4**	**LAC5**	**35981**	**22707**	**34113**	**33674**	**33991**

Table. 3.1-1 Sequence identity matrix of *Miscanthus* laccase amino acid sequences. 35981, 22797, 34113, 33674, 33991 are short for Mxg_TContig35981, WhiteKaskade_TContig22707, Sacchariflorus_TContig34113, Sacchariflorus_TContig33674, and Mxg_TContig33991, respectively.

Based on the phylogenetic analysis, sequence information of Mxg_TContig35981,

WhiteKaskade_TContig22707, Sacchariflorus_TContig34113, Sacchariflorus_TContig33674, Mxg_TContig33991 (Fig. 1.1-1, with star) belonging to different subgroups were used as templates, and by using cDNA of *Miscanthus sinensis* (identification number: Sin-13) five laccase were cloned and named MsLAC1-5. Sequences cloned exhibit high identity with their templates (Table. 1.1-1, dark green) although they originate from different cultivars. However, the identities between those laccases are very low, although they belong to the same subgroup according to the phylogenetic analysis. Specifically, MsLAC1 and MsLAC2 share the highest identity of 67%, while the lowest identity of only 35% was found between MsLAC3 and MsLAC4. These results indicated high sequence homology between different Miscanthus species, whereas the low identities among individual laccases may indicate diverse of functions of *Miscanthus* laccases.

Laccases	Base pairs	Amino acids	N-glycosylation sites	Signal peptide	isoelectric point	Molecular weight/kDa
MsLAC1	1752	583	15	32	5.95	63.0
MsLAC2	1737	578	12	31	9.08	63.0
MsLAC3	1794	597	8	32	6.55	64.7
MsLAC4	1791	596	6	27	8.00	64.6
MsLAC5	1740	579	10	30	5.95	62.3
AtLAC4	1677	558	12	24	9.31	61.5
AtLAC17	1734	577	14	22	9.29	64.0

Table. 3.1-2 Characterization of 5 laccases cloned from *Miscanthus* together with two *Arabidopsis* laccases.

Accordingly, MsLAC1-5 have similar size (~60kDa) and N-terminal cleavable signal peptide is predicted on all sequences, targeting them to the secretory pathway (Table. 1.1-2). Analysis of five *Miscanthus* laccases predicted that all the laccases we cloned contain putative N-glycosylation sites, but the potential number of sites vary extensively, ranging from 6 in MsLAC4 to 15 in MsLAC1. All laccases from *Arabidopsis* are predicted to be basic protein and most of them exhibit pI higher than 9 (McCaig *et al.*, 2005). In contrary, the calculated pI of *Miscanthus* laccases can not only basic but also acidic. MsLAC1 and MsLAC5 only have pI at around pH 6.0.

To evaluate the relationship between *Miscanthus* laccases and other laccases that were

reported to be involved in lignification, phylogenetic analyses and multiple sequence alignments were carried out with laccase proteins from *Brachypodium distachyon* (BdLAC5, (Wang, Y *et al.*, 2015)), Sugarcane (SofLAC, (Cesarino *et al.*, 2013)), Poplar (PtLAC3, (Bryan *et al.*, 2016)) and *Arabidopsis* (AtLAC4 and AtLAC17, (Berthet *et al.*, 2011)). As shown in Fig. 1.1-4, MsLAC1 and MsLAC2 turned out to be the close homologous to AtLAC17, which is essential for lignification and distribution of G-units in *Arabidopsis* (Berthet *et al.*, 2011). The studies on other two closely related laccases, SofLAC and BdLac5 (Cesarino *et al.*, 2013; Wang, Y *et al.*, 2015)have shown that those proteins are also involved in lignin biosynthesis. Altogether, those reports have strengthened our hypothesis that MsLAC1 and MsLAC2 may have the lignin-related functions as well.

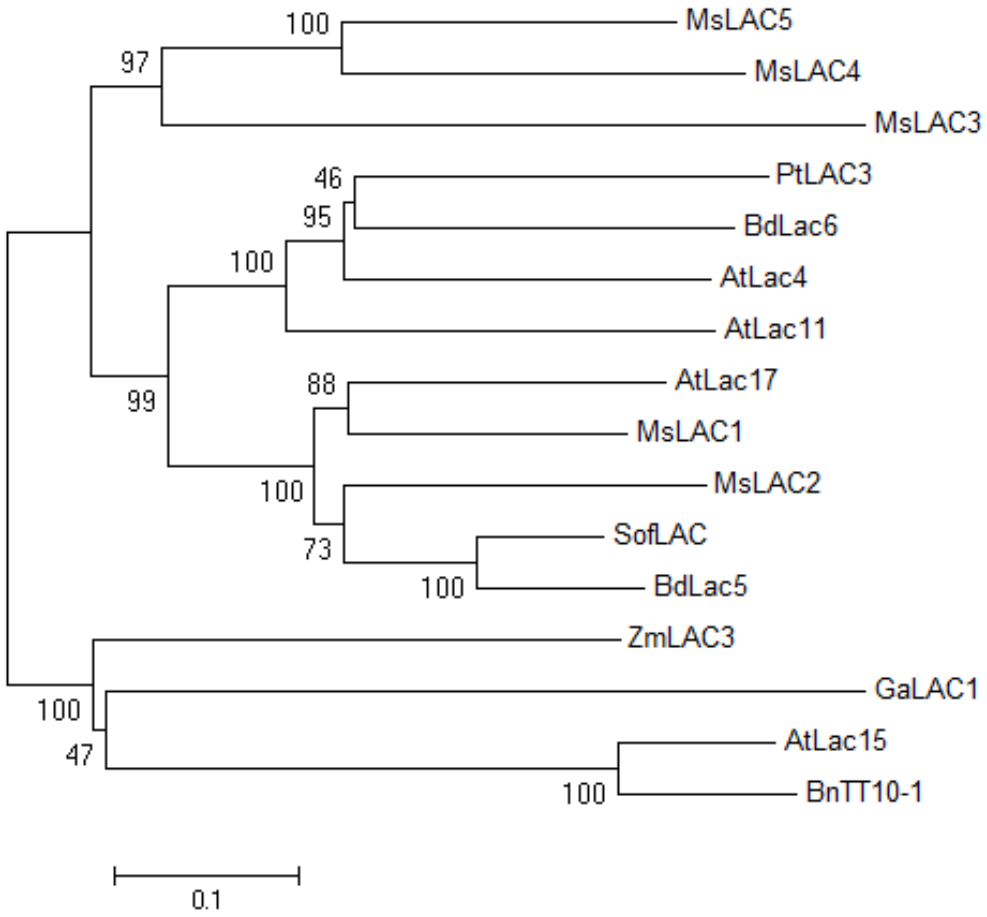

Fig 3.1-4 Phylogenetic tree of five laccases isolated from *Miscanthus* and other lignin-related lacases. Amino acid sequences of *Miscanthus* (MsLAC1-5), *Brachypodium* (BdLAC5), Sugarcane (SofLAC), Poplar (PtLAC3) and *Arabidopsis* (AtLAC4 and AtLAC17) laccases were analyzed with Mega 5 (neighbor-joining method, 1000 bootstraps).

The residues essential for the binding of four copper ions (10 histidines and 1 cysteine) are conserved in nearly all laccases. Alignment of deduced *Miscanthus* laccase amino acid sequences with other lignin-related laccases also showed four conserved copper-binding sites (Fig. 1.1-5). An Asn residue possibly responsible for substrate binding is highly conserved in plant laccases at the position which is frequently occupied by aspartate in fungal laccases, (Madzak *et al.*, 2005; Turlapati *et al.*, 2011). Furthermore, it is reported that laccase redox potential can be influenced by the axial ligand near the T1 Copper binding site, where the Ile or Leu residuaes might lead to high redox potential (Madzak *et al.*, 2005). Accordingly, all *Miscanthus* laccases we cloned and lignin-related laccases from different species are possibly all high redox potential laccases.

The cupredoxin domain is a typical hallmark of laccases, which can be found in all the *Miscanthus* laccases (Fig. 1.1-6a). Using the structure of a laccase from *Rigidoporus Lignosus* (1v10.1.A) (Garavaglia *et al.*, 2004a) as template, the three-dimensional structure for laccases was predicted with SWISS-MODEL (https://swissmodel.expasy.org/). At the monomeric level, the three sequentially arranged cupredoxin-like domains are presented as blue, green and yellow areas (Fig. 1.1-6b), showing very similar putative protein structures. These cupredoxin domains mainly formed by β-barrels (Greek key motif) consisting of β-sheets and β-strands, arranged in sandwich conformation and connected by Short α-helical regions. In addition, the C-terminal portion is always characterized by short α-helix stretch. Despite the low identities among those laccases, they shared a similar structure with a substrate binding pocket, thus providing the structural basis for a similar function.

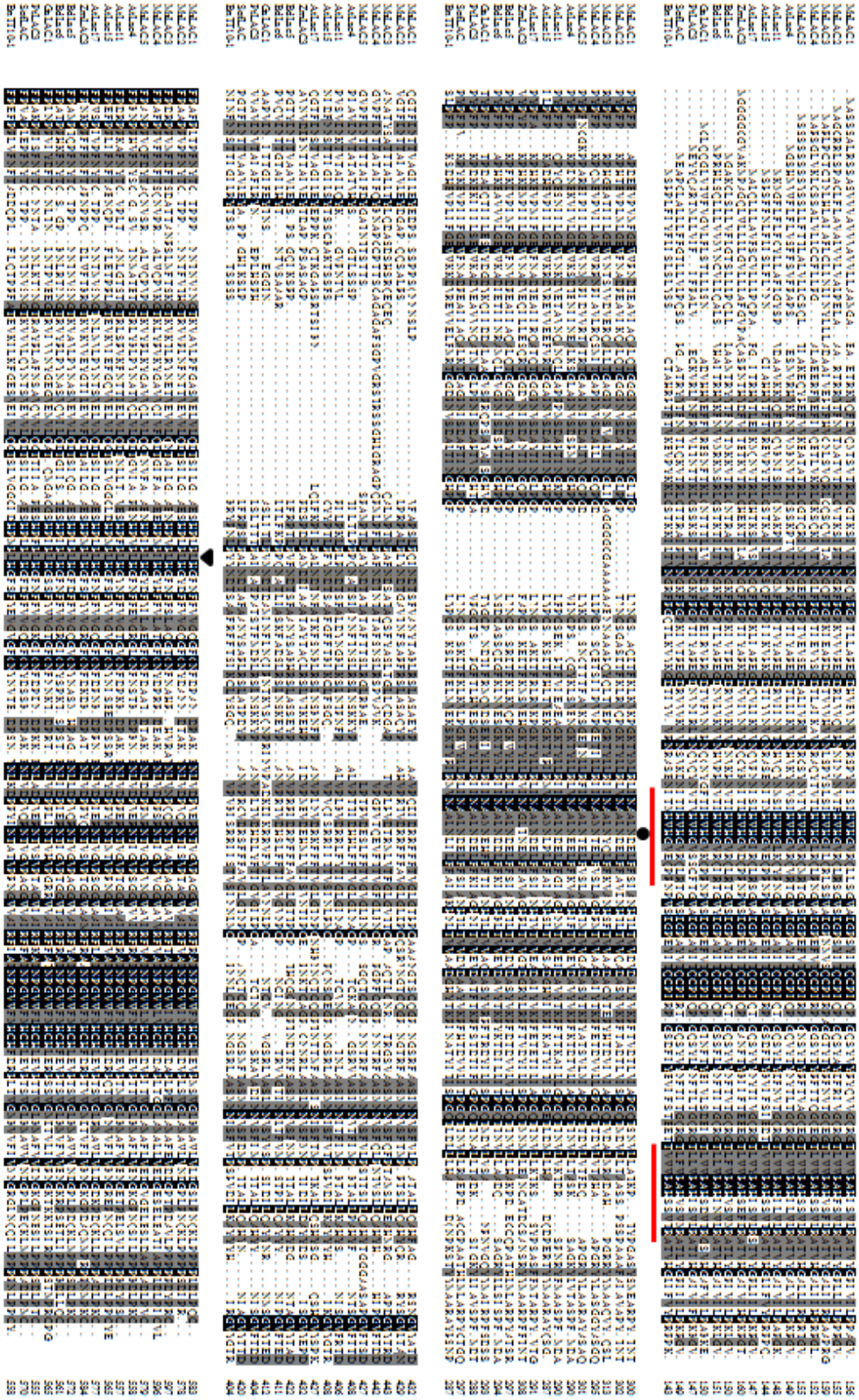

Fig 3.1-5 Multiple alignment of *Miscanthus* laccases with lignin-related laccases using ClustalW.

Amino acid sequences of *Miscanthus* (MsLAC1-5), *Brachypodium* (BdLAC5), Sugarcane (SofLAC), Poplar (PtLAC3) and *Arabidopsis* (AtLAC4 and AtLAC17) laccases were analyzed with ClustalW software. Underlined are the conserved copper binding domains. The typical conserved copper-binding sites are underlined. The triangle represents the amino acid predicted to be responsible for conferring redox potential to T1 Cu, while the Asn residue for pH optimal activity marked by black dot.

In summary, the five sequences cloned from *Miscanthus* showed typical laccase features, but they've also exhibited distinct differences after bioinformatic analysis. Deeper investigation regarding the expression patterns, localization, regulation, enzymatic properties as well as *in vivo* function studies will help us to understand the real function of laccase isoforms in *Miscanthus*.

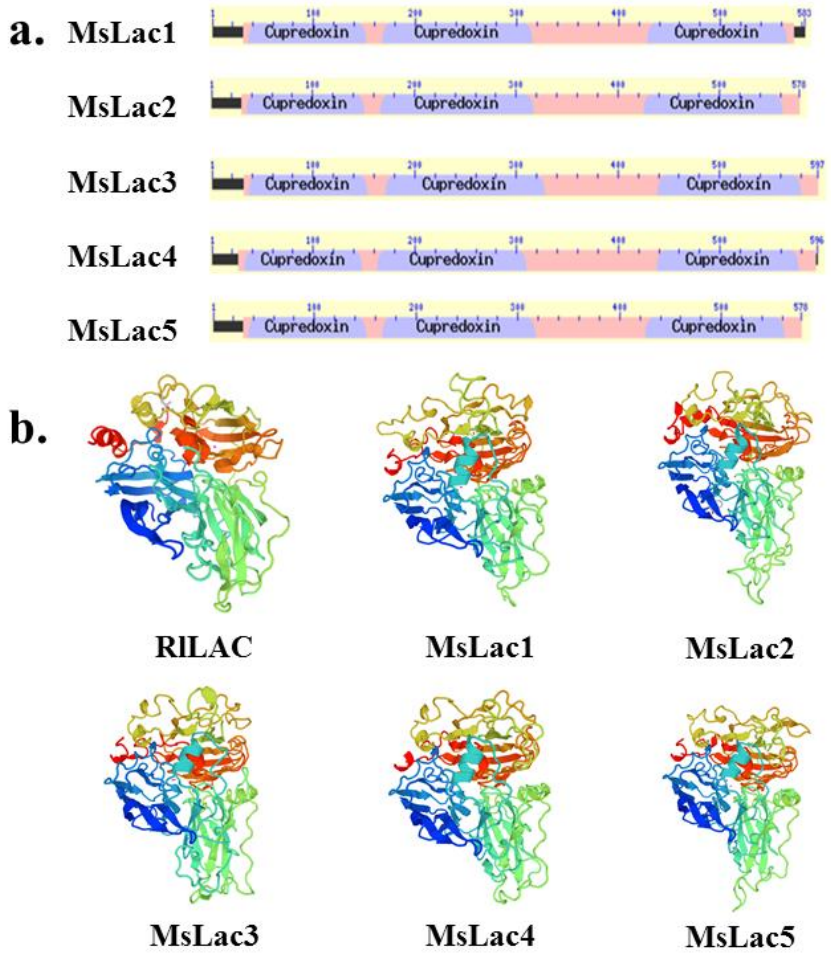

Fig 3.1-6 2-D and 3-D structural analysis of *Miscanthus* laccases. (A) Analysis of 2-D structure of cloned laccase sequences from *Miscanthus*. (B) Ribbon representation of the overall structure of *Miscanthus* laccases and one laccase from rigidoporus lignosus (Garavaglia *et al.*, 2004a).

3.2 Laccases activity and expression are dependent on the growth of *Miscanthus*

The lignin content and composition of grasses varies significantly depending on ecotype and developmental stage (Shen *et al.*, 2009). In principal, the growth of *Miscanthus* can be divided into nine stages, starting from bud development and ending with senescence (Tejera & Heaton, 2017). In our study, *Miscanthus* plants in different developmental stages (bud, leaf development and stem elongation stage) were collected (Fig 3.2-3). Different tissues (leaf, stem, and root) were then separated for further analysis. The cell wall thickening in different parts of internodes differs relating to the maturation of vascular bundles (Hu *et al.*, 2017). The same trend was also detected in fully expanded leaf. Along the development gradient of leaf, the expression of *MsSND1* and its putative targets is concomitant with the onset of vascular development (Tejera & Heaton, 2017). Thus, the entire mature *Miscanthus* plant (~6-month-old, 7 palpable nodes) was segmented; resulting in 7 continues internodes and 5 leaf gradients as indicated in Fig 3.2-2. The laccase activity and the expression level of *MsLAC1-5* as well as lignin-related genes were then determined.

3.2.1 Activity of laccase increased with the maturation of *Miscanthus* plant

Using plant materials from 3-month-old *Miscanthus*, intracellular protein (ICP) and cell wall associated protein (CWP) were extracted separately. As shown in Fig. 3.2-1, laccases activity was detected in each tissue, but with different specific activities.

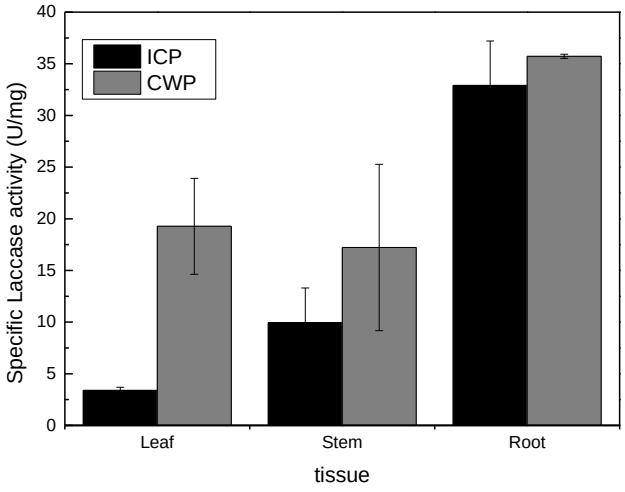

Fig 3.2-1 The specific laccase activity in different tissues. Mixture of five 3-month-old *Miscanthus*

plants was used for protein extraction, and the activity was measured using ABTS as substrate, and then calculated against the protein concentration for specific activity. ICP: Intracellular protein; CWP: Cell wall associated protein.

The highest specific activity was found in root, with a similar activity level between ICP and CWP. However, in leaf and stem tissue, it seems that more laccase activity accumulated in the cell wall. The existence of laccases is very important for lignin biosynthesis and stress tolerance. Lignin is almost undetectable in *Arabidopsis lac11 lac4 lac17* triple-mutant roots, and was also significantly reduced in stem and leaf (Zhao *et al.*, 2013). High level of laccase activity in cell wall probably fulfils the demand of lignin biosynthesis. What's more, the enhanced lignin formation in cell wall and the production of some phenolic polymers as well as flavonoids in cytosol after the overexpression of rice laccase genes in *Arabidopsis* significantly enhanced the tolerance to salt, drought (Cho *et al.*, 2014) and copper stress (Liu *et al.*, 2017). In light of this, it is not surpurising that intracellular laccase activity in root is significantly higher than that in leaf and stem.

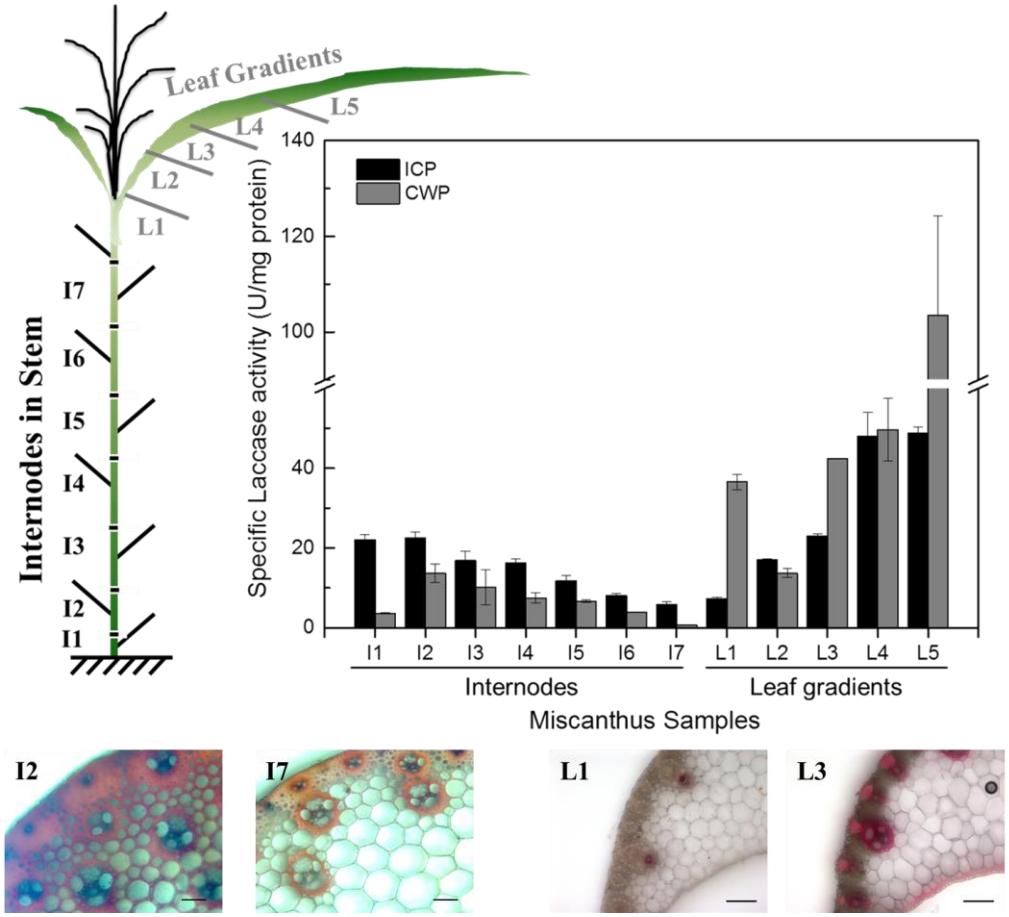

Fig 3.2-2 The specific laccase activity of different segments along the development of *Miscanthus*.

7 internodes and 5 leaf gradients from 6-month-old Miscanthus plant were used for protein extraction, and the activity was measured using ABTS as substrate, and then calculated against the protein concentration for specific activity. I1-I7: First to seventh internode; L1-L5: First to fifth leaf gradient; ICP: Intracellular protein; CWP: Cell wall associated protein. Cross sections of 2nd and 7th stem internodes and 1st and 3rd leaf gradients (Golfier *et al.*, 2017) were stained with HCl-phloroglucinol. Scale bars: 100 µm.

In order to explore the activity of laccase in *Miscanthus* development in more detail, laccase activity was determined along the developmental gradients of the stem and the youngest fully expanded leaf from 6-month-old mature plants. Altogether, seven internodes as well as five leaf sections have been analyzed (Fig 3.2-2). Consistent with the results from histo-enzymology studies (1.1.1), mature tissue showed stronger lignification as well as higher laccase activity.

3.2.2 Co-expression of MsLACs with monolignol biosynthetic genes and their regulators

3.2.2.1 Expression of *Miscanthus* laccases is regulated in both developmental and tissue-specific manner

To explore whether *MsLAC1* is involved in lignification during the growth of Miscanthus, the relative expression levels of *MsLAC1* were studied in different plant organs (leaf, stem and root) at different time points (10 days, 1 month, 2 months and 3 months) by qRT-PCR (Fig 3.2-3). The expression of different genes varies a lot among different tissue types and developmental stages.

As expected, the laccase transcripts were detected in all samples. Generally, the expression level decreased with plant age for most of *Miscanthus* laccases. Among all the laccases, only *MsLAC1* showed the highest expression in stem regardless of the age, while others have highest expression in immature root. In *Arabidopsis*, some laccases were regulated in a developmental or tissue-specific manner, showing strong connection with phylogenetic groups (McCaig *et al.*, 2005). However, *MsLAC2* which can be categorized into same subgroup with *MsLAC1*, showed distinct expression pattern in the early growth stage. Interestingly, the visualized expression pattern

showed that the expression of all laccase genes except *MsLAC1* correspond well with the secondary cell wall related genes in all the tissues (Fig S2).

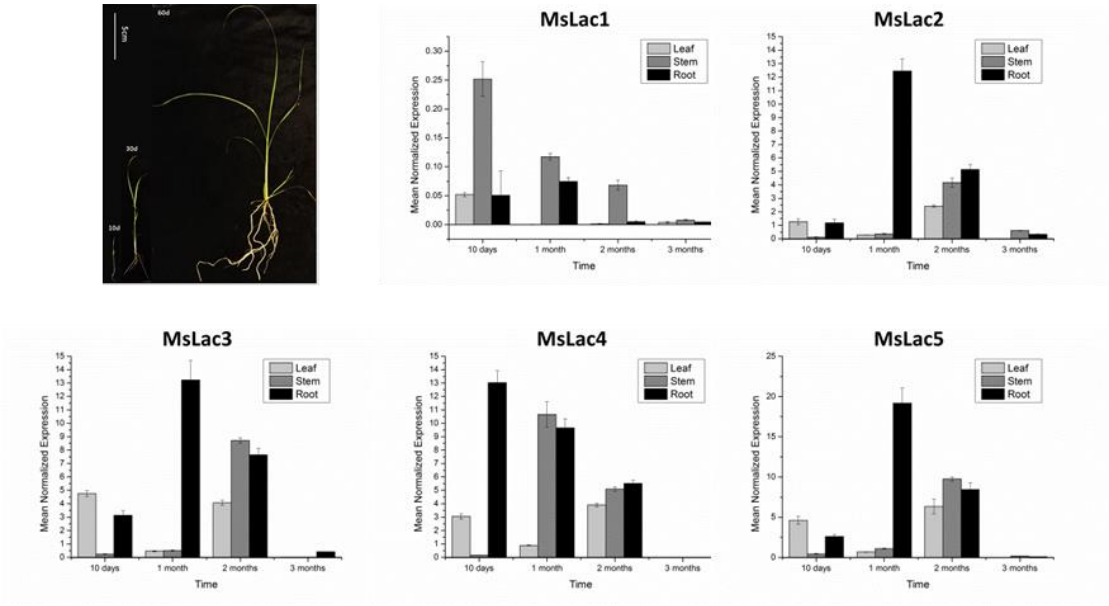

Fig 3.2-3 Expression patterns of Miscanthus genes in various tissues and developmental stages quantified with qRT-PCR. PP2A was used as reference gene.

3.2.2.2 MsLac1 is co-expressed with monolignol biosynthetic genes and their regulators during organ development

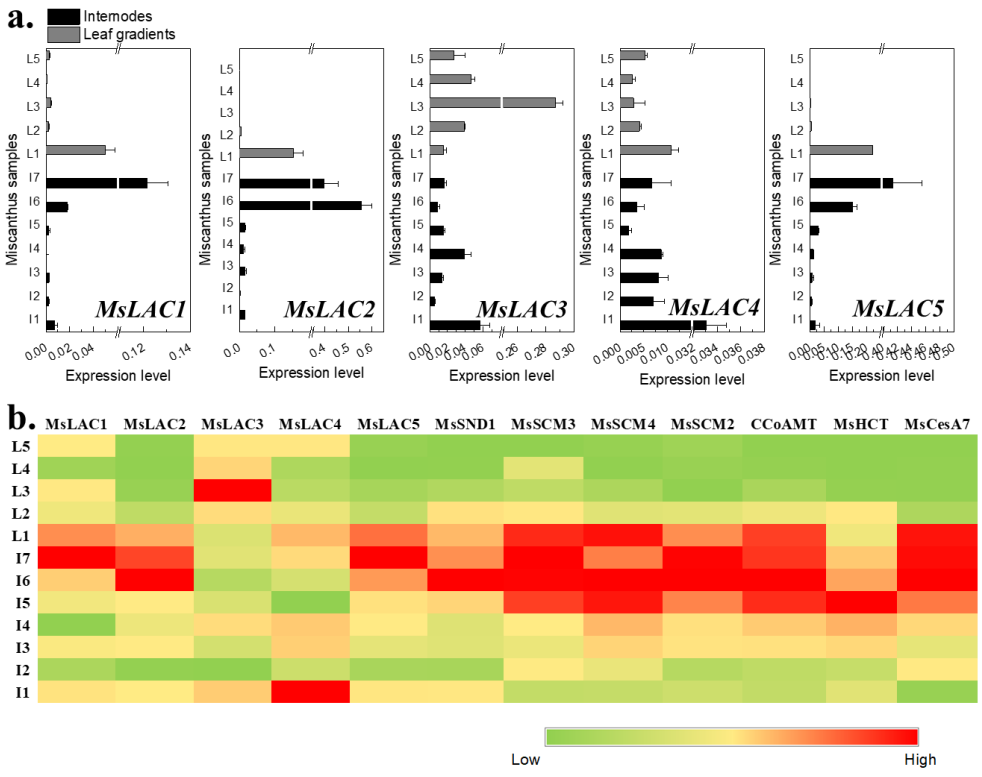

Fig 3.2-4 Expression pattern of *MsLACs* and other secondary cell wall related genes along the

development gradients of 6-month-old *Miscanthus* plant. Data were normalized by *PP2A* in (a) and further visualized in (b). *MsSND1*, *M. sinensis SECONDARY WALL-ASSOCIATED NAC DOMAIN1*; *MsSCM2-4*, *M. sinensis SECONDARY CELL WALL MYBs 2-4*; *MsCcoAOMT*, *M. sinensis Caffeoyl-CoA O-methyltransferase*; *MsHCT*, *M. sinensis Hydroxycinnamoyltransferase*; *MsCesA7*, *M. sinensis Cellulose synthase A catalytic subunit 7*.

The biosynthesis of lignin starts with monolignol formation pathway which requires different enzymes, including Caffeoyl-CoA O-methyltransferase (CcoAOMT) and hydroxycinnamoyltransferase (HCT) (Wang & Balint-Kurti, 2016). The biosynthesis pathway is intensively regulated by lignin-related transcription factors, functioning on those key enzymes (Golfier *et al.*, 2017). Those lignin-related genes were found to be strongly co-expressed. Thus, co-expression analysis of putative cell wall-related genes is a powerful way to investigate the functional network of laccases. For instance, in *Brachypodium*, two laccases (*BdLAC5* and *BdLAC6*) were co-expressed with genes involved in monolignol biosynthesis, and further confirmed to be important for lignification (Wang, Y *et al.*, 2015).

Using the same section method described in Fig 3.2-2, the expression level of five putative laccases, four transcription factors (*MsSND1*, *MsSCM2-4*), two monolignol biosynthesis genes (*MsCcoAOMT*, *MsHCT*) and a cellulose synthase (*MsCesA7*) were compared. As shown in Fig 3.2-4, individual *Miscanthus* laccase genes were also differentially regulated during organ development. Generally, all selected genes displayed higher expression levels in the stem compared to the leaf. In leaf, all laccases had highest expression in leaf sheath except MsLAC3, which is highly expressed at the base of leaf blade, right after the collar. In addition, the expression levels of laccases are all along a developmental gradient in the inflorescence stem. More specifically, the highest expression of *MsLAC1*, *MsLAC2* and *MsLAC5* were detected in the tip of stem, while in the basal for *MsLAC3* and *MsLAC4*.

Interestingly, the highest expression in stem internodes was detected for all lignin-related genes in the last three stem segments while the expression levels in the first two segments were consistently observed to be the lowest. To better understand the correlation of expression between *Miscanthus* laccases and other selected genes, the results were first normalized and then graphically visualized using a heat map (Fig.

1.2-4b). Conspicuously, most studied genes exhibit higher expression levels in younger plant tissues, while *MsLAC3* and *MsLAC4* showed distinct difference. These results indicate that *MsLAC1*, *MsLAC2* and *MsLAC5* are likely involved in lignification processes while *MsLAC3* and *MsLAC4* are not.

3.3 *Miscanthus* laccases are regulated by lignin-related transcription factors (TFs)

3.3.1 Promoters of *Miscanthus* laccases have putative binding sites of secondary wall biosynthesis NAC and MYB factors

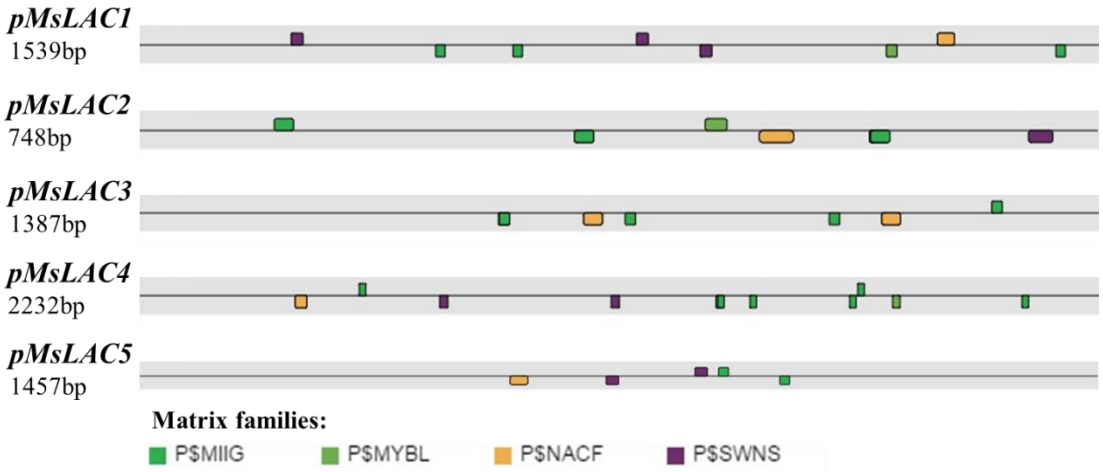

Fig 3.3-1 Identification of putative transcription factor binding sites in *Miscanthus* laccase promoters using Genomatix MatInspector software. P$MIIG, MYB IIG-type binding sites; P$NACF, Secondary wall NACS; P$SWNS, Plant specific NAC; P$MYBL, MYB-like proteins.

The expression analysis showed co-expression profile among some laccases and some lignin-related TFs, indicating the potential function of those TFs on some laccase candidates. The newly identified *Miscanthus* laccase sequences were used as query to find their regulatory region in a self-generated partial genome sequence database of *Miscanthus*. Regions upstream of the start codon of *Miscanthus* laccases were isolated from genomic DNA, with various lengths because of incompleteness of the partial genome database. The cloned promoter sequences were then analyzed with the Genomatix MatInspector software (Cartharius *et al.*, 2005) to identify potential

binding sites of TFs known to regulate secondary cell wall biosynthesis, using a core similarity of >1. Both MYB and NAC binding sites were predicted on all the sequences (Fig 3.3-1), with a total number of 9, 10, 10, 12, 6 on promoter of MsLAC1-5, respectively.

Secondary wall NAC binding elements (SNBEs) and secondary wall MYB responsive elements (SMREs) are important cis-elements involved in the regulation of secondary wall biosynthesis (Zhong *et al.*, 2015). Using the MatInspector software, SMRE was detected on all the promoters but SNBE does not exist on promoter of MsLAC3 (Table 1.3-1).

Matrix	Detailed Matrix Information	Number of matches					Ref.
		pMsLAC1	pMsLAC2	pMsLAC3	pMsLAC4	pMsLAC5	
SNBE	Secondary wall NAC binding elements	3	1	0	2	2	(Zhong *et al.*, 2010)
SMRE	Secondary wall MYB-responsive element	2	1	2	3	1	(Zhong & Ye, 2011)

Table 3.3-1 Number of SNBE and SMRE on promoter of *Miscanthus* laccases.

3.3.2 Promoter of *Miscanthus* laccases can be regulated by lignin-related TFs.

The regulation of cell wall lignification is controlled via a complex network, including a number of well-characterized upstream and downstream regulators, e.g. MYB factors (Zhao & Dixon, 2011). To establish this network in *Miscanthus*, we cloned *MsSND1* and *MsVND7* , *MsSCM2*, *3*, *4*, *MsMYB52*, *MsMYB31* and *MsMYB42* as putative orthologues of the *Arabidopsis* secondary wall synthesis regulators *AtSND1*, *AtVND7*, *AtMYB85*, *AtMYB43*, *AtMYB58/63*, *AtMYB52*, *ZmMYB31* and *ZmMYB42* (Zhong & Ye, 2014c; Agarwal *et al.*, 2016b; Golfier *et al.*, 2017). To confirm the relationship between those TF and laccase candidates, we cloned promoters of all the five laccases and determined the induction/repression by Dual Luciferase Assay (DLA). The cloned TFs were used as effectors in transactivation assays, where the

promoters were fused to the firefly luciferase reporter gene, and co-bombarded with the respective effectors into prepared grapevine suspension cells (Fig. 1.3-2).

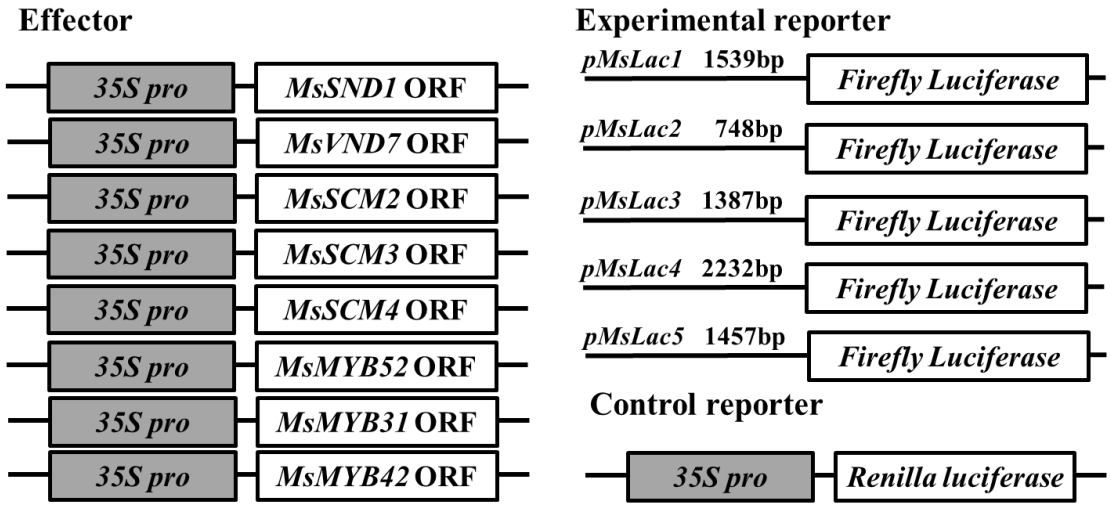

Fig 3.3-2 Constructions for Dual luciferase assay. In the reporter construct, the promoter regions of *Miscanthus* laccases are individually fused upstream of a firefly luciferase gene. pRluc (Renilla luciferase gene fused with 35s promoter) was included as the control for normalization after measurement.

3.3.2.1 Promoters of *Miscanthus* laccases can be differentially activated by lignin-related MYB and NAC factors.

In congruence with our hypothesis, promoters from *Miscanthus* laccases can be activated by both NAC and MYB TFs (Fig. 1.3-3). The studied MYB TFs have been described previously as lower-tier TFs in the regulation cascade, while AtSND1 and its close homologues were considered as master switches of the secondary wall biosynthetic program (Zhong & Ye, 2009). All the promoters can be activated by MYB factors (MsSCM3 and MsSCM4), but only pMsLAC1 and pMsLAC3 can be slightly activated by a NAC domain TF, MsSND1. Surprisingly, another NAC TF, MsVND7, seemingly couldn't activate the tested laccase promoters.

Compared with the data of pMsLAC1 and pMsLAC2, the activation of other promoters was lower with less significance. Generally, the tested MYB factors show stronger activation of laccase promoters when compared to NAC factors. For instance, with promoter of MsLAC1, the highest induction by MsSCM4 was 35-fold higher than the control, and 10-fold higher than that of MsSND1. As reported previously,

AtMYB58 and AtMYB63 (homologue of MsSCM4) act as the direct transcriptional activators of lignin biosynthesis and are downstream targets of AtSND1 (Zhou *et al.*, 2009). Our previous results suggested that MsSND1 directly induces the expression of MYB46 and MYB83 in *Arabidopsis* mCherry-GR-MsSND1 seedlings some MYB factors, however, downstream pathway genes including *AtCCoAOMT1* and *AtLAC4* were only indirectly activated in inducible *Arabidopsis* lines (Golfier *et al.*, 2017). In the presented study, promoters of laccases appear to be a direct target of MsSCM4 and might only be indirectly activated by MsSND1. Thus MsSCM4 could be an interesting target for optimization of lignin content and/or composition in *Miscanthus*.

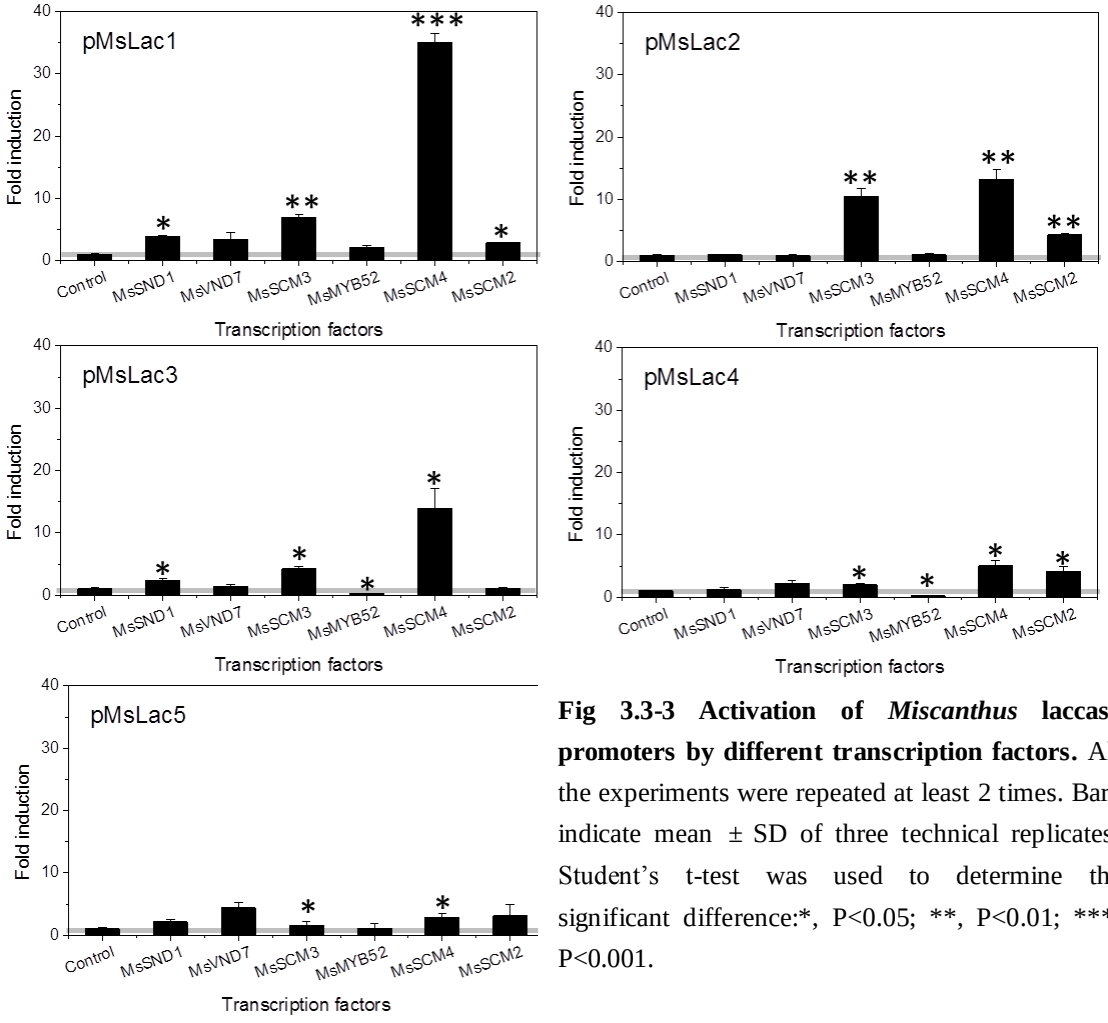

Fig 3.3-3 Activation of *Miscanthus* laccase promoters by different transcription factors. All the experiments were repeated at least 2 times. Bars indicate mean ± SD of three technical replicates. Student's t-test was used to determine the significant difference:*, P<0.05; **, P<0.01; ***, P<0.001.

3.3.2.2 Repressors of phenylalanine pathway also negatively regulated the expression of *Miscanthus* laccases.

The regulation of lignin biosynthesis includes both positive and negative regulators. ZmMYB31 and ZmMYB42 are R2R3-MYB transcription factors in maize, negatively regulating lignin biosynthesis. The regulatory function of these two repressors is conserved in other monocots including sorghum and rice (Agarwal *et al.*, 2016b). The homologue genes in *Miscanthus*, *MsMYB31* and *MsMYB42*, were also cloned in our lab, repressing the promoters of some genes in phenylalanine pathway (ongoing project of my colleague). Using those repressors as effectors, the potential function was examined via DLA.

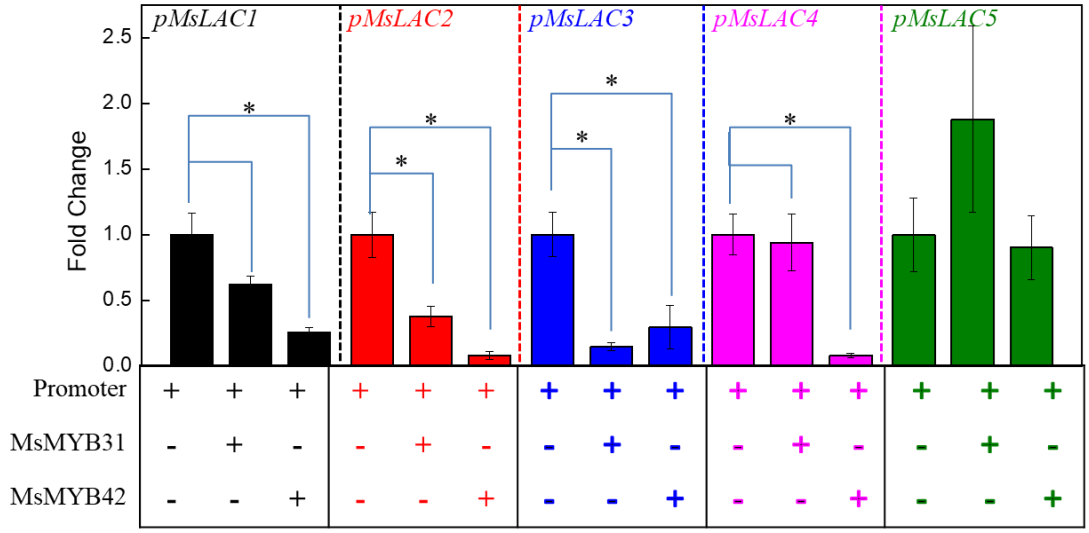

Fig 3.3-4 Repression of *Miscanthus* laccase promoters by MsMYB31 and MsMYB42. All the experiments were repeated at least 2 times. Bars indicate mean ± SD of three technical replicates. Student's t-test was used to determine the significant difference: *, P<0.05; **, P<0.01.

As shown in Fig 3.3-4, the activity of MsLAC1-3 promoters was significantly repressed when MsMYB31 or MsMYB42 was co-bombarded into grapevine suspension cells. However, promoter of MsLAC4 was only repressed by MYB42, while MsLAC5 promoter was not affected by any of the TFs. To further understand the function of MYB31 and MYB42 on *Miscanthus* laccases in the regulatory network, a powerful activator, MsSCM4, which showed induction on all of the promoters was co-bombarded together with repressors.

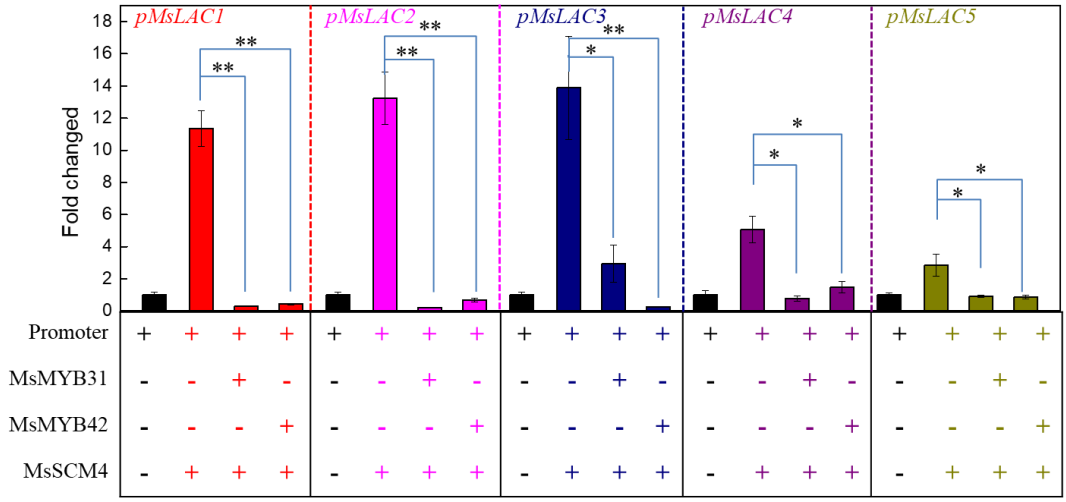

Fig 3.3-5 Repression of *Miscanthus* laccase promoters by MsMYB31 and MsMYB42 in the presence of MsSCM4. All the experiments were repeated at least 2 times. Bars indicate mean ± SD of three technical replicates. Student's t-test was used to determine the significant difference: *, P<0.05; **, P<0.01.

As compared to the control, MsSCM4 increased the expression of reporters fused with all tested promoters. Our results showed that MsMYB31 and MsMYB42 significantly inhibited luciferase expression in the mixed system (Fig 3.3-5). Surprisingly, the MsSCM4-induced expression of pMsLAC4::LUC and pMsLAC5 was repressed upon co-bombardment with inhibitors, although their activity *per se* was not affected by MsMYB31 or, in the case of LAC5, by both inhibitors. This finding indicates that there might be interaction between repressors and activators. As we know, a repressor can inhibit the expression of target gene either directly, by binding on the promoter, or indirectly, by interacting with activators and interfering with the induction. MsMYB31 and MsMYB42 can directly inhibit the activity of laccases promoter (Fig 3.3-4) but also indirectly repress the induction of activators. To our knowledge, it is the first report revealing that the repressors can also regulate the potential enzymes involved in lignin polymerization in both, direct and indirect ways. Our results indicate a complex regulation network of laccases in *Miscanthus*, which is similar to the regulation of monolignol biosynthesis genes.

3.4 *Miscanthus* laccases can be secreted into cell wall

3.4.1 Transient expression of *Miscanthus* laccases in *Nicotinana benthamiana* increased laccase activity but the recombinant protein cannot be detected by Strep II antibody.

The lignification of secondary cell walls starts with biosynthesis of monolignol in cytosol, followed with transportation of monolignol and related enzymes to the cell wall matrix, and then lignin complex is polymerized in the cell wall with sufficient substrates and proper enzymes (Barros *et al.*, 2016). Two *Arabidopsis* laccases which were reported to be involved in lignification were labeled with mCherry and constitutively expressed in transgenic *Arabidopsis* plants, and the fluorescent signal can be detected throughout the secondary cell wall layer without mobility (Berthet *et al.*, 2011; Yi Chou *et al.*, 2018). To understand whether *Miscanthus* laccases can be specifically localized into cell wall, we assessed subcellular localization of MsLAC1 by tracking of fluorescent protein-fusions after transient expression in tobacco leaves. The cDNA coding regions (CDS) of *MsLAC1*, *MsLAC2*, *MsLAC3* and *MsLAC5* were cloned into Greengate C-module as described by (Lampropoulos *et al.*, 2013). In the final construct (Fig S4a), the CDS was driven by 35s promoter and had Strep II tag fused to the C-terminus of the protein. After transformation into Agrobacteria, freshly prepared bacteria cells were infiltrated into tobacco leaves. Crude protein was extracted two days later from infiltrated leaf samples. As shown in Fig 3.4-1a, the infiltrated leaf tissue had higher ABTS-oxidizing activity when compared with the control, except for *MsLAC3::strep*. However, the accumulation of recombinant laccase could not be detected either on SDS-PAGE, or on Western blot stained with Strep II antibody (Fig 3.4-1b), in any of the candidates. I also attempted to purify the protein extracts using Strep-Tactin column (IBA Lifesciences), but the elution did not show specific Strep II band either (Data not shown). The same situation happened when researchers tried to express *Arabidopsis* laccases with Strep tag system in plants (Chaibang, 2014), suggesting us that the Strep tag system might not be a good tool for

plant laccases expression.

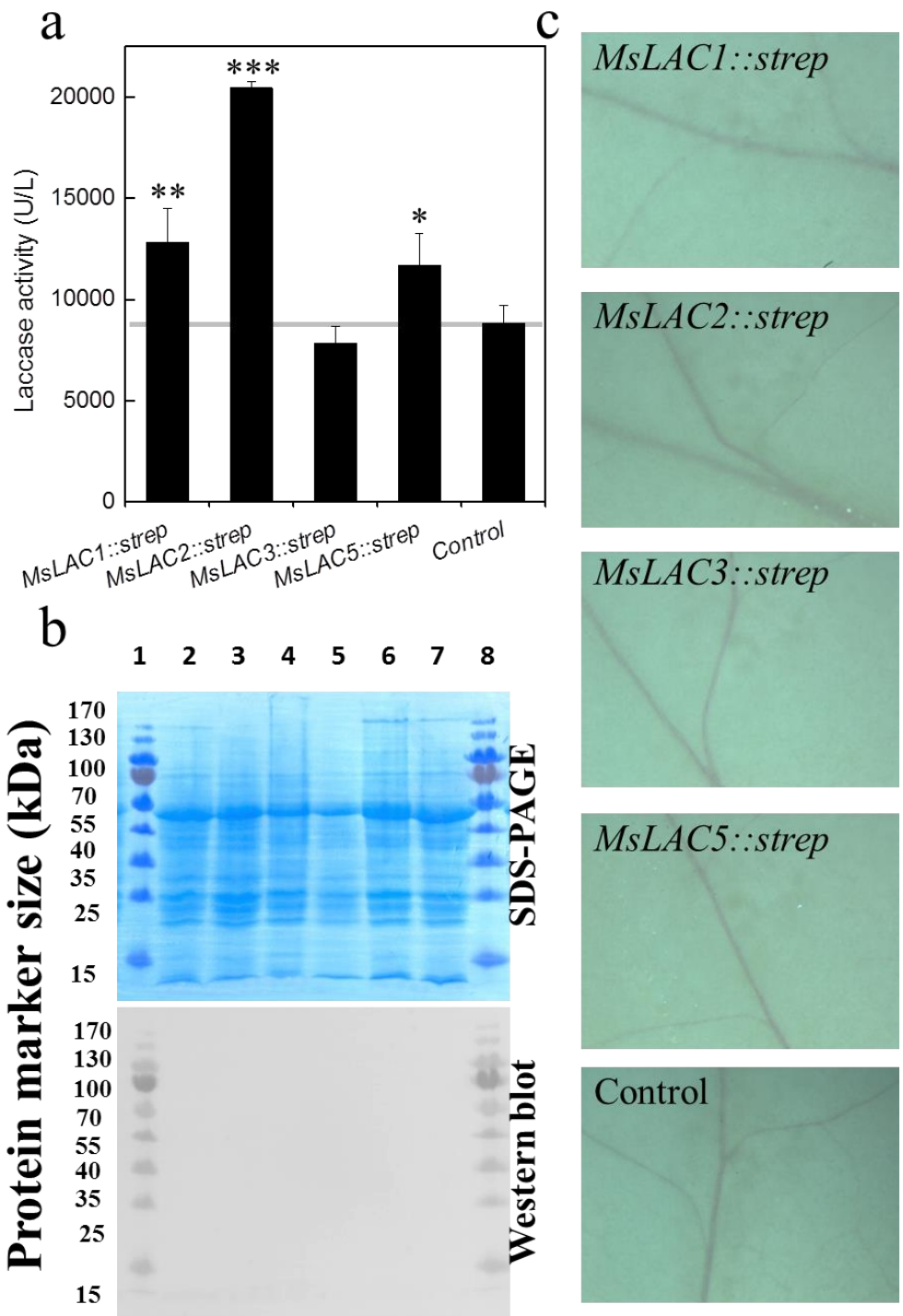

Fig 3.4-1 Transient expression of *Miscanthus* laccases in tobacco leaf. (a) Activity of laccase in tobacco leaves overexpressing *Miscanthus* laccases. Construct containing a short dummy sequence replacing laccase CDS was used as control. Bars indicate mean ± SD of three technical replicates. Student's t-test was used to determine the significant difference: *, P<0.05; **, P<0.01; ***, P<0.001. (b) SDS-PAGE and western blot stained with Strep II antibody of protein samples extracted from tobacco leaf infiltrated with 2-5: *MsLAC1*, *2*, *3*, *5::strep*; 6, control construct; 7, P14 cells only. 1, 8: Prestained protein marker. (c) Tobacco leaf stained with HCl-phloroglucinol after decolourization with methanol overnight. Lignin is stained red and clearly showed the veins.

3.4.2 Overexpression of *Miscanthus* laccase in *N. benthamiana* leaf did not cause lignification in primary cell wall of epidermal leaf cells.

The overexpression of lignin-related TFs or enzymes usually leads to ectopic lignification in plants (Zhang *et al.*, 2015; Zhong *et al.*, 2015; Golfier *et al.*, 2017). After incubation of stably transformed *A. thaliana* lines overexpressing AtLAC4 or AtLAC17, with NBD-tagged coniferyl alcohol (NBD-CA), primary cell walls in cotyledon epidermal cells had strong fluorescence, which indicated lignification (Schuetz *et al.*, 2014). We confirmed the function of NBD-CA as a marker for lignification with cross section of *Arabidopsis* inflorescence stem (Fig S3). In my study, the infiltrated leaves were collected and distained with methanol overnight. However, after lignin staining with HCl-phloroglucinol, lignification was only detected in veins (Fig 3.4-1c). Even after incubation with NBD-CA, when compared with the negative control, no significant difference could be found.

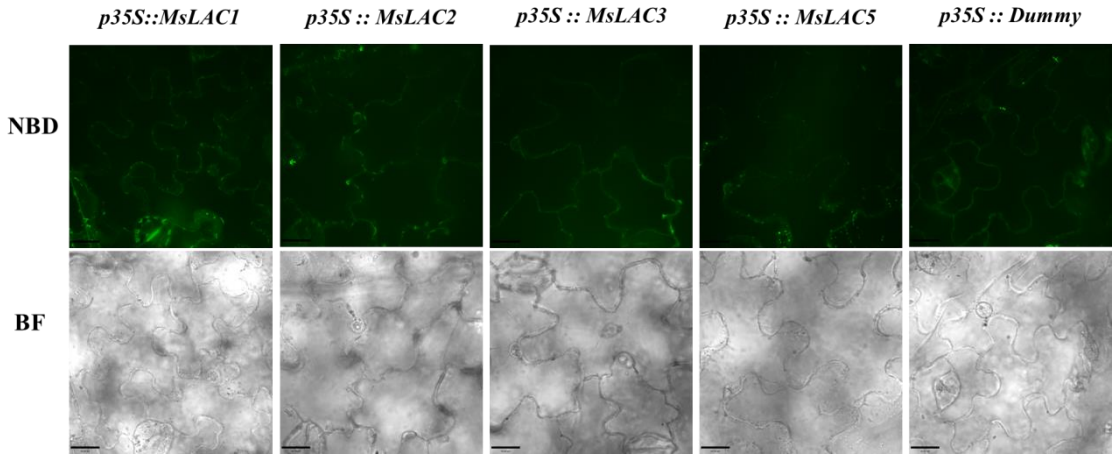

Fig 3.4-2 Microscopy of infiltrated tobacco leaf after incubation with NBD-CA. Images were taken using the Perkin-Elmer UltraView VoX spinning disk confocal mounted on a Leica DM16000 inverted microscope with a Hamamatsu 9100-02 CCD camera. The GFP filter (excitation 488nm, emission 525nm) was used to image NBD-CA. Bar=16µm.

Thus, the overexpression of *Miscanthus* laccases in tobacco leaf is apparently not sufficient to lignify the primary cell wall of epidermal cells, even with external supply of monolignols as substrates.

3.4.3 All *Miscanthus* laccases infiltrated are secreted proteins but have different localization.

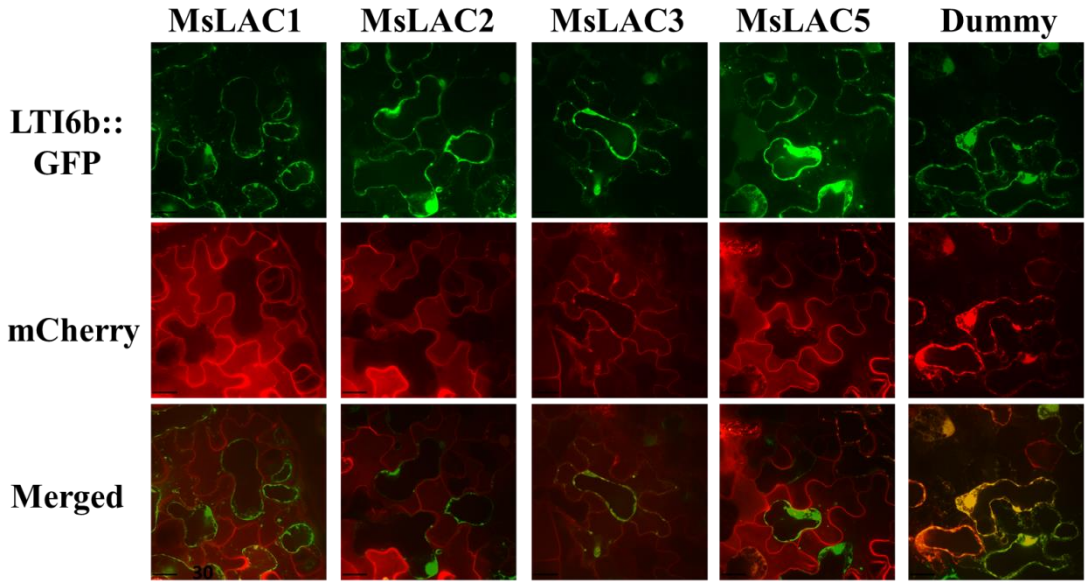

Fig 3.4-3 Localization of *Miscanthus* laccases in cell wall after transient expression in *N. benthamiana* leaf. Images were taken using the Perkin-Elmer UltraView VoX spinning disk confocal mounted on a Leica DM16000 inverted microscope with a Hamamatsu 9100-02 CCD camera. The GFP filter (excitation 488nm, emission 525nm) was used to image GFP. The RFP filter (excitation 561nm, emission 595-625nm) was used to image mCherry-tagged constructs. Samples were mounted in water and imaged using a Leica oil immersion20x or63×objective. All images were processed using Volocity image analysis software (Improvision). Bar=16μm.

The analysis of the *Miscanthus* laccases that I cloned predicted N-terminal signal peptide in all the sequences (section 3.1.4), indicating that those laccases can be secreted outside the cell. Considering the acidic environment in cell wall matrix, we used the pH-stable mCherry-tag fused to C-terminal of laccase CDS (Fig S4b). To distinguish clearly between the plasma membrane and the cell wall, the fluorescence-tagged protein LTI6b was used as a fluorescent marker of the plasma membrane (Cutler *et al.*, 2000). When *LTI6b::GFP* was co-expressed with free mCherry (*Dummy::mCherry*), both GFP and RFP had overlapping fluorescent signals, however, the mCherry-tagged MsLAC1 showed a distinct fluorescence pattern (Fig. S5).

After plasmolysis (Fig 3.4-3), the GFP signal remained associated with the retracting

plasma membrane, while mCherry-derived fluorescence was observed in the primary cell walls of the epidermal cells for all the laccases, thus indicating the localization of *Miscanthus* laccases to the cell wall matrix. None of those laccases were localized in the vacuole, but RFP signals of *MsLAC3::mCherry* and *MsLAC5::mCherry* were also found intracellular. Taken together, the cell wall localization ensures the accessibility of those enzymes to monolignols transported from the cytosol, but the difference in spatial distribution among those laccases may also reflect more diverse functions.

3.5 Recombinant MsLAC1 can oxidize monolignols in vitro

3.5.1 Attempts for heterologous expression of *Miscanthus* laccases

The most direct way to understand the property of enzyme is to obtain the purified functional protein. Fungal laccases have been successfully expressed in different hosts including *E. coli*, yeast and filamentous fungus (Zhuo *et al.*, 2015), thus being intensively studied. But the attempts of expressing plant laccases always failed, limiting the understanding and application of plant laccases. Researchers claimed to see detectable ABTS-activity of laccase using plant expression system, but failed to release the functional protein from the cell wall (LaFayette *et al.*, 1999). Attempts of expressing laccase from *A. thaliana* in *E. coli*, *P. pastoris*, or *N. benthamiana* all failed with no functional protein purified (Chaibang, 2014).

In order to express laccases from *Miscanthus*, I tried *E. coli*, *P. pastoris*, *N. benthamiana* and also insect expression system using Sf9 cells. All five Laccase CDSs were cloned into pETG10A/30A with or without their own signal peptides and then transformed into *E. coli* Rosetta cells. However, all the proteins were expressed as insoluble protein no matter which temperature or IPTG concentration I used. As described in section 3.4.1, transient expression of MsLAC1, 2, 3 and 5 in *N. benthamiana* increased the total laccase activity but could not be detected with Strep II antibody. However, MsLAC5 was successfully expressed in insect expression system. The purified rMsLAC5 has a molecular weight around 80 kDa (Fig. S6), but the purified protein showed no activity against typical laccase substrates (ABTS,

2,6-DMP, guaiacol, hydroquinone) or monolignols (coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol).

Four out of five laccases were successfully cloned into pPICZαA, with or without signal peptide, and then transformed into *Pichia pastoris* X33. After screening, only the yeast stain containing pPICZαA_MsLAC1_sp+ showed significantly higher laccase activity (Fig 3.5-1). So I focused on this strain and tried to study the function after purification.

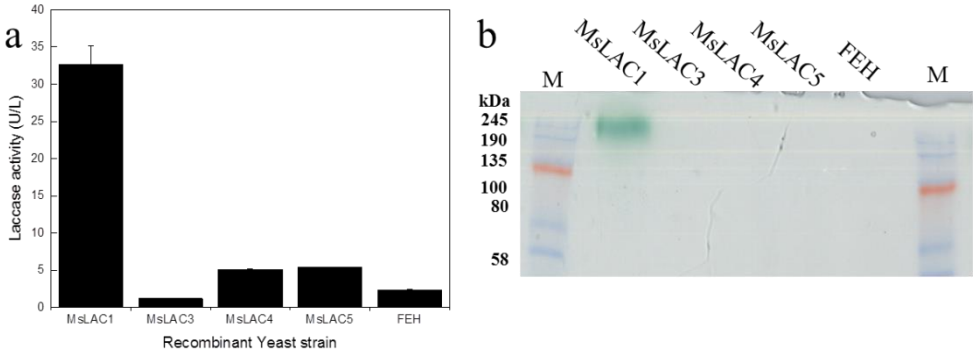

Fig 3.5-1 Expression trials for *Miscanthus* laccases in *Pichia pastrois*. (a) Laccase activity of yeast strains expressing different proteins. FEH was used as a negative control. (b) Native PAGE stained with ABTS for supernatant of yeast culture expressing MsLAC1.

3.5.2 Production of rMsLAC1 in *Pichia pastoris*

Zymograms using the substrate ABTS confirmed the presence of laccase activity in the culture medium. The expression of rMsLAC1 increased gradually after methanol induction and reached the maximum on the eighth day. The activity of the tested laccase using ABTS as a substrate remained consistent, and increased correlatively to the amount of protein over the course of the experiment (top panel, Fig. 3.5-2). Western Blot analysis using anti-MYC antibody confirmed the increasing production of recombinant protein amounts with a signal at ~150kDa (bottom panel, Fig. 3.5-2). The secreted rMsLAC1 has a comparable mobility to the protein extracted from *Miscanthus* development gradients (Fig 3.1-2). As mentioned in section 3.1.4, 15 possible N-glycosylation sites were predicted in the MsLAC1 protein sequence. The main difference between the calculated molecular mass of MsLAC1 (63 kDa) and rMsLAC1 (150 kDa) is possibly due to increased N-glycosylation and additional

O-glycosylation or other post-translational modifications (Coll *et al.*, 1993; Campos *et al.*, 2016; Bronikowski *et al.*, 2017).

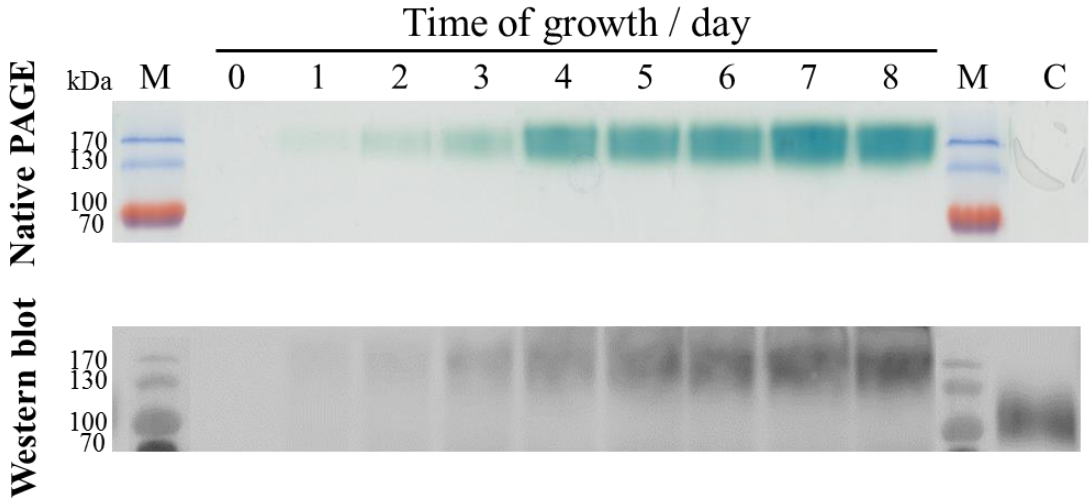

Fig 3.5-2 Zymogram analysis and western blot of l recombinant MsLAC1. Samples were collected every day after induction by centrifugation, and 10 µl supernatant was filtered and loaded. The zymogram was stained with 10mM ABTS and western blot was revealed with anti-MYC antibody. M: PageRular Prestained Protein Ladder, 10-170 kDa. C: Crude extract from fermentation broth of *P. pastoris* transformed with pPICZ α AFEH, 8 days after induction.

3.5.3 Purification of the heterologous laccase rMsLAC1

I briefly optimized the culture condition (methanol, pH, $CuSO_4$ and temperature), and after 8 days fermentation in the optimized condition, rMsLAC1 was purified by using Ni-NTA Columns. The specific activity of laccases increased around 5-fold after purification (Fig 3.5-3c). A specific band showing laccase activity could have been detected in both, crude broth and purified elution fraction after ABTS staining, but not in the flow through fraction (Fig 3.5-3a). The purified rMsLAC1 showed a smeared band between 135 and 190kDa on SDS PAGE (Fig 3.5-3b), indicating diverse degrees of glycosylation of the expressed protein in *P. pastrois*.

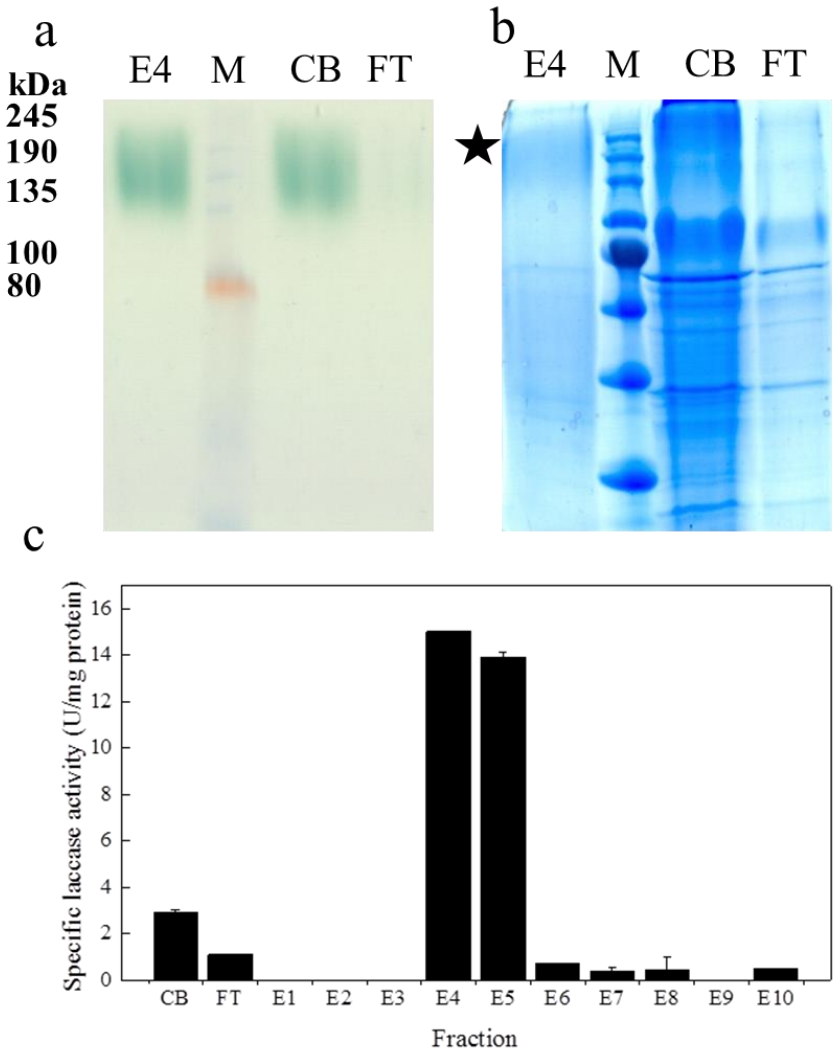

Fig 3.5-3 Purification of rMsLAC1 after fermentation. (a) and (b) Native and SDS PAGE of samples before and after purification. The star indicates the possible smeared band stands for rMsLAC1. (c) Specific laccase activity of different samples in the purification process. CB, crude broth; FT, flow through; E1-E10, elution fraction 1 to 10.

3.5.4 Characterization of the heterologous laccase rMsLAC1

Although laccases are presented throughout the plant kingdom, the large family of laccase created difficulties for purification because of similar protein properties (Ranocha *et al.*, 1999b). Attempts for heterologous expression of plant laccases are also challenging (Chaibang, 2014), thus the detailed characterization of plant laccases is still limited. Some laccases from spruce and pine were successfully expressed and purified, but for those laccases only optimum pH had been measured (Sato & Whetten, 2006; Koutaniemi *et al.*, 2015a). As described in Fig 3.5-4, the optimum temperature

and pH for rMsLAc1 is 3 and 32℃, respectively. In contrast to fungal laccases (Mayer & Staples, 2002), the recombinant protein had high activity within a narrow arrange of pH and temperature. To be more specific, the enzyme retained more than 50% of the maximum activity only between 20-40°C and at pH 3-4. When rMsLAC1 was incubated in buffers with different pH, the highest stability was detected at pH 3 and pH 4. The pH and temperature profiles for rMsLAC1 were significantly different from those observed for fungal laccases (Campos *et al.*, 2016), indicating distinctions between plant and fungal laccases.

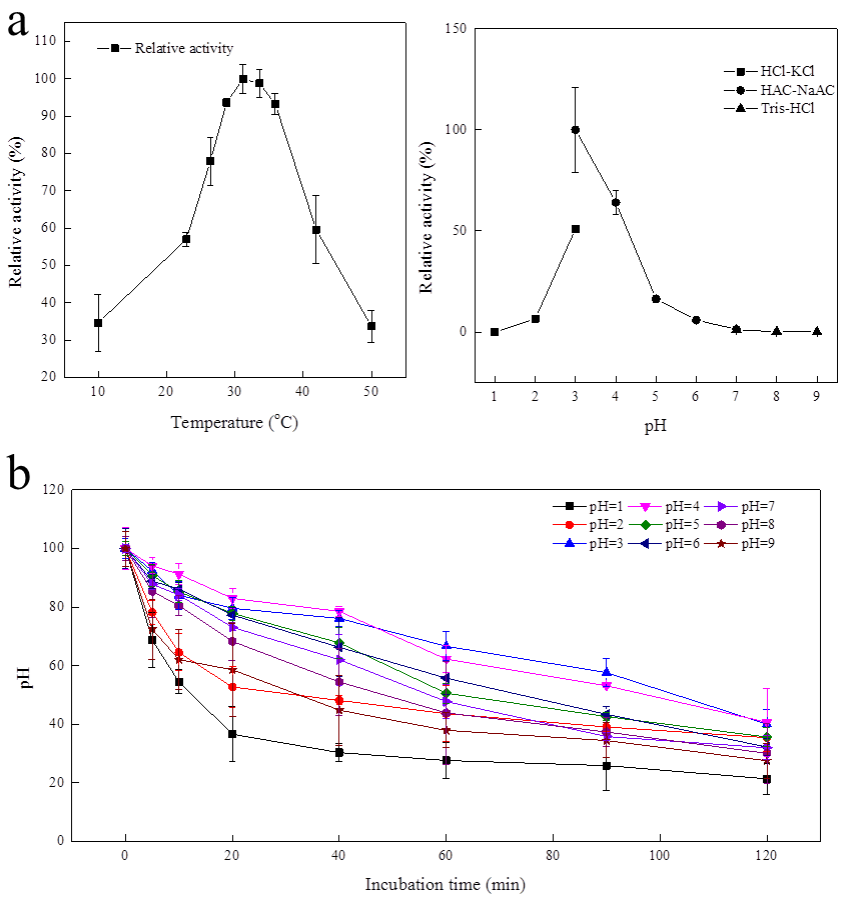

Fig 3.5-4 pH and temperature profile of rMsLAC1. (a) Optimum pH and temperature of rMsLAC1. Relative activity was calculated against the highest activity in the profile. (b) Stability of rMsLAC1 in different pH. Activity was measured after incubation of the enzyme in buffers with different pH for different time, and relative activity was calculated against the non-incubated enzyme.

As described in Fig 3.5-5a, the effects of various enzyme inhibitors on the laccase activity of rMsLAC1 showed different degree of inhibition. Sodium azide, an inhibitor of metal-enzymes (Jordaan *et al.*, 2004), noncompetitively inhibited rMsLAC1 activity. EDTA and DTT significantly inhibited the laccase activity while

SDS had little effect at 1mM concentration. The sensibility of laccase to disulfide reducing agent DTT indicates that the thiol groups played significant role for rMsLAC1, probably keeping the three-dimensional conformation (Jaiswal *et al.*, 2014). As a kind of copper chelator, EDTA was reported to remove T1 copper if it is exposed in solvents (Abadulla *et al.*, 2000), which can also cause strong inhibition.

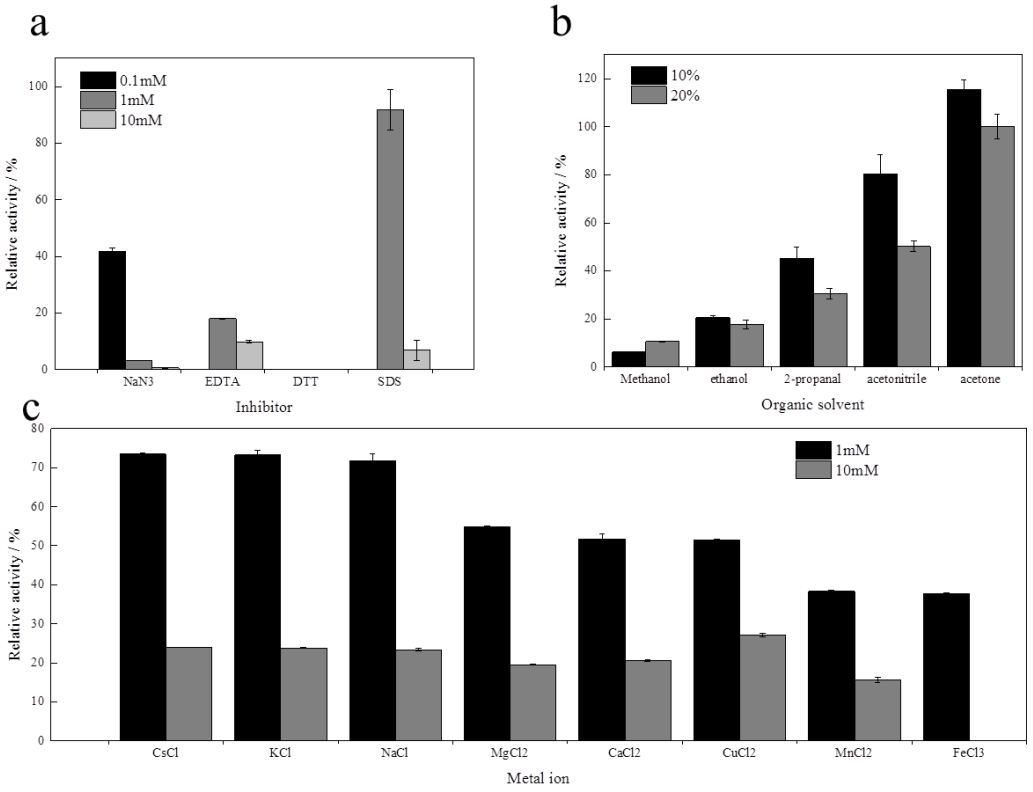

Fig 3.5-5 Tolerance of rMsLAC1 against different enzyme inhibitors, organic solvents and metal ions. ABTS was used as substrate and measurements were carried out in pH 3 50mM HAc-NaAc buffer at 30^{o}C. Relative activity is calculated by compared with the laccase activity of control reaction without any inhibitors. All experiments were repeated at least 3 times and the bar indicates the standard error of repeats.

The effects of different organic solvents (methanol, ethanol, isopropyl alcohol, acetonitrile, and acetone), which are used in a variety of industrial applications, were also evaluated using ABTS as substrate. Organic solvents may alter the enzyme structure due to hydrophobic effects or substitute for water molecules at the active sites, thus causing the instability of laccase in organic solvents (Keum & Li, 2004). As shown in Fig 3.5-5b, rMsLAC1 has strong tolerance against acetone, low concentration of acetone even increased the relative activity by a factor of 20%. Interestingly, my study found that the strength of inhibition increased continuously

with the decrease of carbon atom number of the alcohol, which was right in the opposite way with former report about fungal laccases (He *et al.*, 2015b), athought this laccase was also heterologously expressed in *P. pastrois* (Huang *et al.*, 2011).

With 10 mM of all the metal salts we tested, over 70% of the initial activity was inhibited (Fig 3.5-5c). The structure of laccase active site contains four copper ions (one T1, one T2 and two T3) The catalytic ability of laccase depends on electron transfer between T1 copper and trinuclear cluster (TNC), which contains one T2 and two T3 copper ions (Murugesan *et al.*, 2009). Due to the binding of Fe^{3+} to T1 site, which blocks the access of substrates to laccase or interferes the transfer of electron (Si *et al.*, 2013), laccases are always sensitive to Fe^{3+} (He *et al.*, 2015b). However, Cu^{2+} may activate the laccase oxidation of ABTS by filling T2 copper binding site (Jaiswal *et al.*, 2014), thus Cu^{2+} normally showed activation on laccases activity in fungi. In contrast, 1 mM $CuCl_2$ rather inhibited 50% of the rMsLAC1 activity, showing a strong inhibition.

3.5.5 Oxidation of monolignols by rMsLAC1

I evaluated the ability of the purified rMsLAC1 to oxidize monolignols by using cinnamyl alcohol, *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol as substrates. Interestingly, rMsLAC1 was able to oxidize all of the supplied compounds, particularly sinapyl alcohol, which was oxidized with the highest efficiency, while cinnamyl alcohol oxidation was catalyzed with the lowest efficiency (Fig 3.5-4, Fig S7). The efficiency of rMsLAC1 was tightly connected to the presence of a methoxy substituent, as it has been described before (Barceló *et al.*, 2004). A methoxy substituent at the ortho-position relative to the phenolic OH group can enhance the polymerization rate by laccases (Ramalingam *et al.*, 2017). The obtained results indicates that, with an adequate supply of monolignol, rMsLAC1 is likely capable of promoting the polymerization of lignin monomers into more complex polymers.

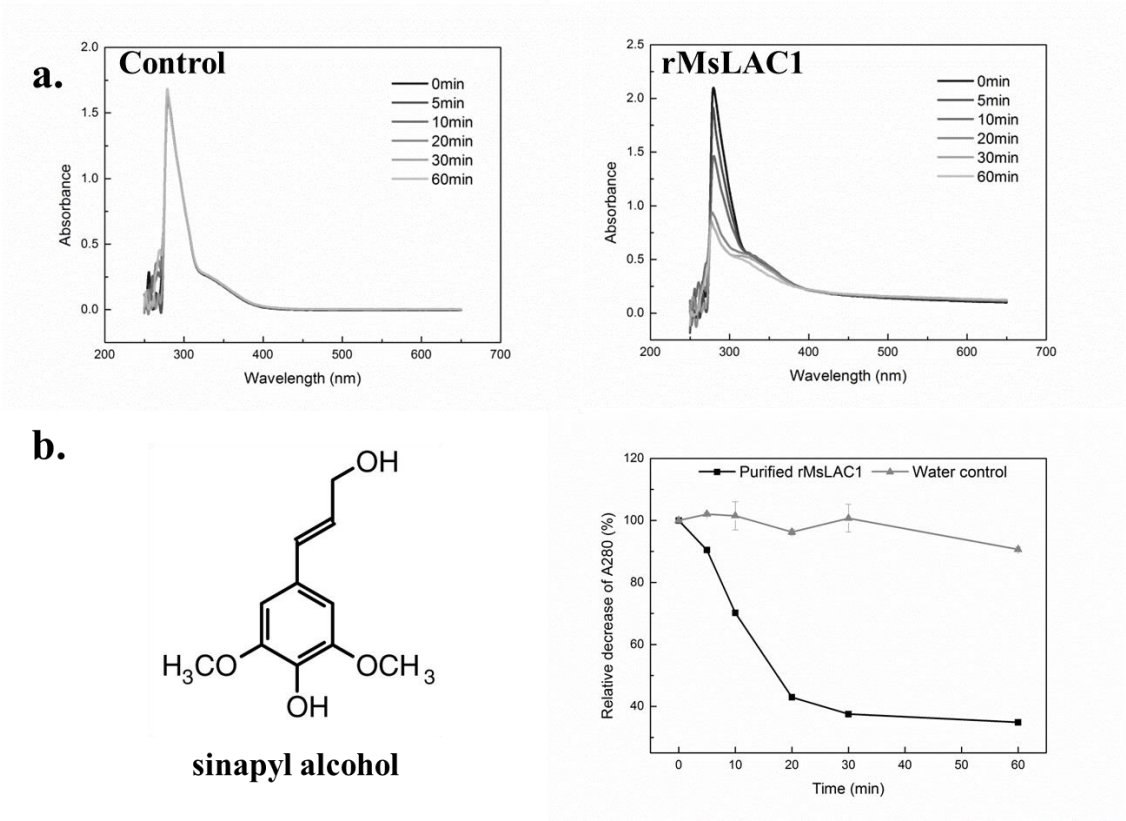

Fig 3.5-6 Spectrum of reaction mixture containing sinapyl alcohol and purified rMsLAC1. Final concentration of 1mM of each substrate in 50mM pH3.0 acetate buffer was mixed with 0.002U rMsLAC1 individually and measured continuously after up to 1 hour. (a) Spectrum between 250 and 650 nm. (b) Chemical structure of sinapyl alcohol and the decrease of absorbance at wavelength of 280 nm compared with the value of 0 h.

3.6 Expression of *Miscanthus* laccases under control of AtLAC17 promoter in *lac4-2 lac17* restored the lignin content but only MsLAC1 and MsLAC2 can complement the phenotypes.

Even though the presented *in vitro* activity assays link MsLAC1 to lignin biosynthesis, transgenic approaches can provide further direct evidence to evaluate the function of the gene. Moreover, the large family of laccase genes in *Miscanthus* indicates the potentially diverse functions of isoforms. To understand the real function of individual *Miscanthus* laccases in lignification, the *Arabidopsis lac4-2/lac17* double mutant was chosen to study the function of *Miscanthus* laccases *via* complementation. This mutant exhibits a semi-dwarf phenotype and irregular xylem cells with divergent

interfascicular fibers when grown under continuous light (Berthet *et al.*, 2011). Independent homozygous lines expressing either *pAtLAC17::MsLACs* or the corresponding control construct (Fig S8a) were obtained and expression of *Miscanthus* laccases was confirmed by qRT-PCR. All the selected lines had significantly higher expression of the corresponding laccases than the wildtype plants or the negative controls (Fig 3.6-1).

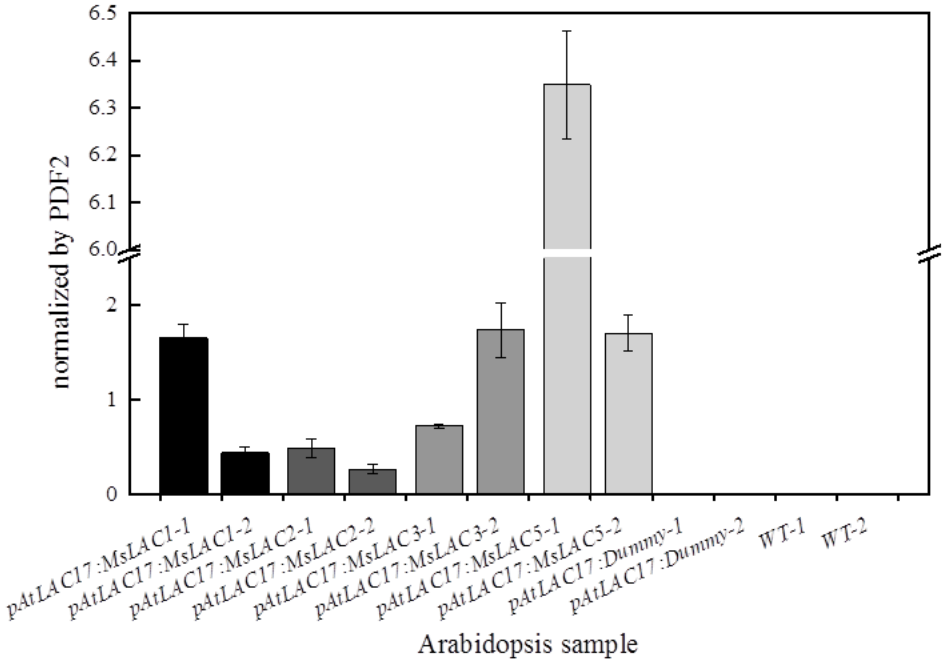

Fig 3.6-1 Expression pattern of *Miscanthus* laccases in different transgenic lines quantified with qRT-PCR. PDF2 was used as a reference gene and the bars stand for the expression level of corresponding overexpressed *Miscanthus* laccases in different lines. Expression level of MsLAC1 was chosen as a sample for WT control and the negative control; the expression of all other *Miscanthus* laccases was not detected in both controls (not shown)

3.6.1 Phenotypical complementation of the *Arabidopsis lac4-2/lac17* double mutant by MsLAC1 and MsLAC2

The semi-dwarf phenotype, which only emerges under constant light conditions, was complemented fully in both pictured lines expressing *pAtLAC17::MsLAC1* and *pAtLAC17::MsLAC2* (Fig 3.6-2a). Using Wiesner staining of stem cross sections, I was also able to show complementation of the irregular xylem phenotypes of the inflorescence stems of the *lac4-2/lac17* double mutant when *pAtLAC17::MsLAC1* or *pAtLAC17::MsLAC2* was expressed (Fig 3.6-3). Additionally, no significant

phenotypical differences of the inflorescence stems were detected between the control lines and the complemented double mutant, thus demonstrating that *MsLAC1* and *MsLAC2* are able to complement the *lac4-2/lac17* double mutant and retained functionality across species.

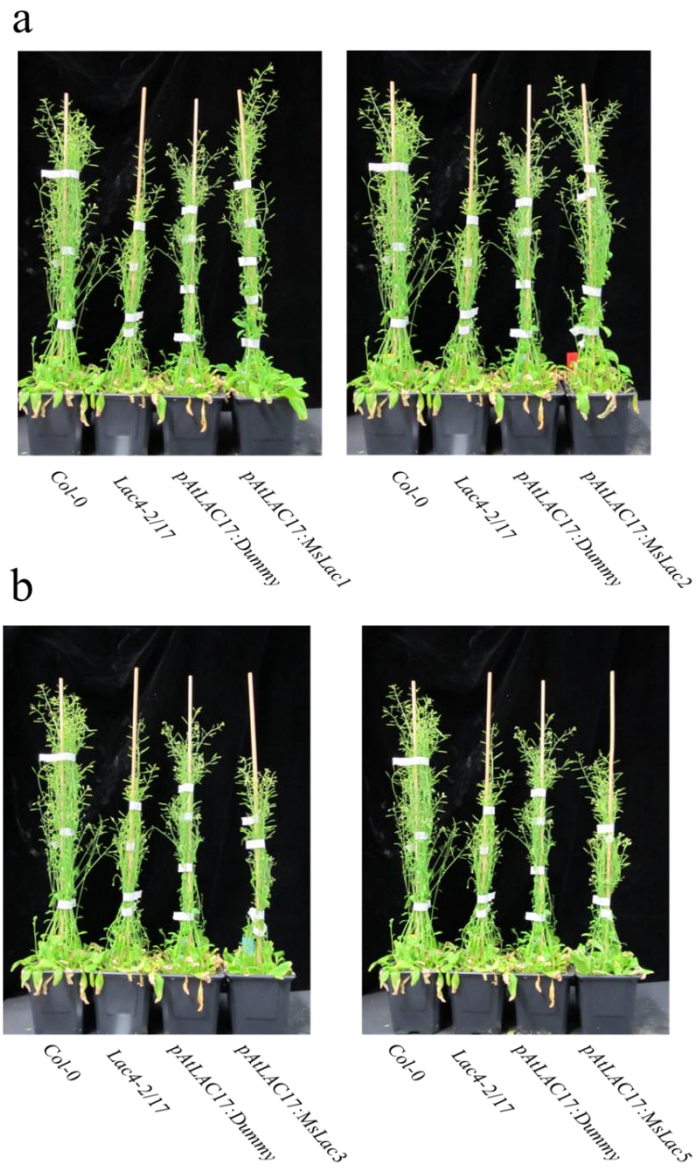

Fig 3.6-2 Transgenic plants grown in growth chamber with constant light after complementation.

3.6.2 Phenotypes of the *Arabidopsis lac4-2/lac17* double mutant cannot be complemented by MsLAC3 and MsLAC5

Unlike MsLAC1 and MsLAC2 which can be categorized into same subgroup with lignin-related laccases from different species (Fig 3.1-4), the promoter activation

study and subcellular localization of MsLAC3 and MsLAC5 revealed that those laccases might have different functions than MsLAC1 and MsLAC2. The selected MsLAC3 and MsLAC5 complementation lines have even higher expression level of corresponding *Miscanthus* laccases (Fig 3.6-1), but under constant light condition, those transgenic plants showed no phenotypical difference to lac4-2/17 mutant and the negative control. The semi-dwarf phenotype (Fig 3.6-2) as well as the irregular xylem cell (Fig 3.6-3) indicates that the complementation failed in plants transformed with *pAtLAC17::MsLAC3* and *pAtLAC17::MsLAC5*.

Therefore, those results suggested that *MsLAC3* and *MsLAC5* had fundamentally different functions than *MsLAC1* and *MsLAC2* since they could not substitute the function of *AtLAC4* and *AtLAC17*. But I cannot conclude that those laccases have a function in the lignification process, since the phenotypes of double mutants are not directly connected to the lignin content (Berthet *et al.*, 2011). Another laccase in *Arabidopsis*, *AtLAC15*, showed function in lignification in seed coat (Liang *et al.*, 2006), but cannot substitute the functions of *AtLAC4* and *AtLAC17* either (Chaibang, 2014). It is worth noting that, in *Miscanthus*, function or the action mode of laccases which are regulated by different TFs, and showed different subcellular localization, can also be not equivalent.

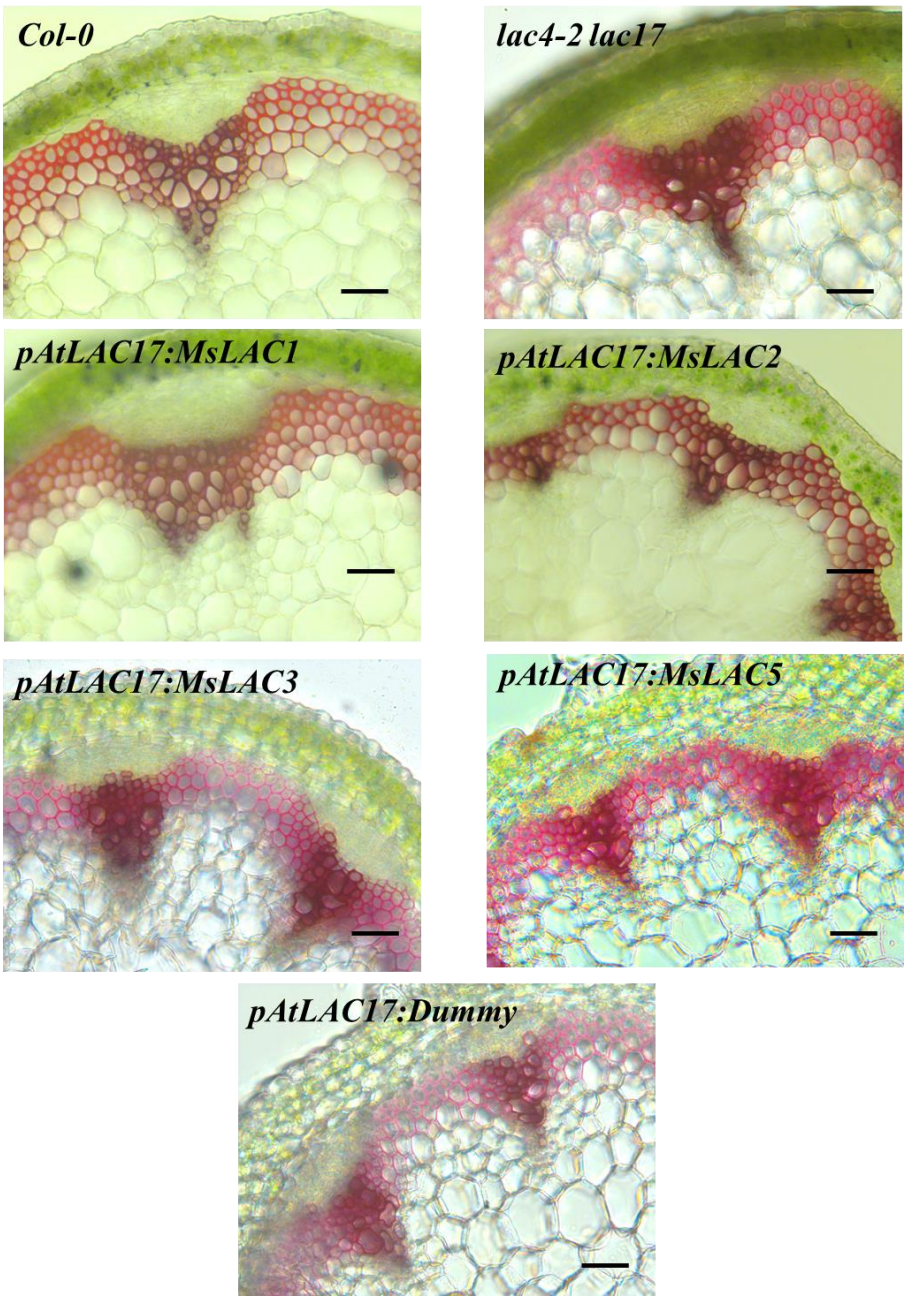

Fig 3.6-3 Genetic complementation of the *Arabidposis lac4-2/lac17* double mutant with *ProAtLAC17:MsLAC1, 2, 3 and 5*. Cross-section of *Arabidposis* stems grown under continuous light stained with HCl-phloroglucinol. Scale bars: 100 µm.

3.6.3 Surprisingly, lignin content is restored in each complementation line

To analyze lignin content, dry stems from the *lac4-2/lac17* double mutant, *Miscanthus* laccase complementation lines, and the corresponding controls were subjected to lignin quantification analysis (Fig 3.6-4). As described before, the *lac4-2/lac17* double mutant has lower lignin content when compared to wild-type

plants (Berthet *et al.*, 2011). Under our conditions, the total klason lignin (KL) content of the wild-type *Col-0* plant was 18.3% including 3.4% acid soluble lignin (ASL) and 14.9% insoluble lignin (IL). The IL amount decreased to around 9% in the *lac4-2 lac17* double mutant while no significant difference was detected in ASL content. In comparison, the negative control containing only the *pAtLAC17::Dummy* construct, representing similar levels as the *lac4-2 lac17* double mutant, contained only ~12% of klason lignin.

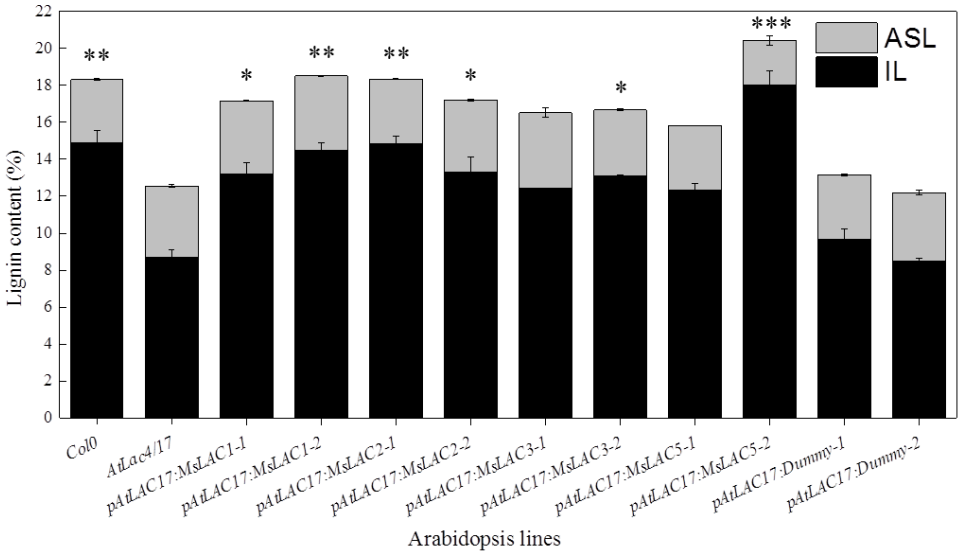

Fig 3.6-4 Lignin content of mature stems from different *Arabidopsis* lines. Insoluble lignin (IL) and acid-soluble lignin (ASL) content are presented as weight percentage in mature stems of different lines. Statistical analysis is based on the total Klason lignin content and the data from *lac4-2/lac17* was used as control. Student's t-test was used to determine the significant difference: *, P<0.05; **, P<0.01; ***, P<0.001.

Two independent lines of plants expressing *pAtLAC17::MsLAC1, 2, 3 and 5* were all able to restore this reduction to different degrees, bringing the content of insoluble lignin back to the level of wildtype plant. All *Miscanthus* laccases can be secreted and localized in the cell wall matrix, enabling them to oxidize monolignols into polymers. The wildtype level of lignin in complemented *Arabidopsis* plants makes a solid proof that all the laccases we studied are functional in lignification, when their expression is regulated by the native promoter of *AtLAC17*. Considering the different phenotypes of plants after complementation, I hypothesized that those laccase isoforms may have distinct preference of substrates, thus affecting the quality of lignin and the

phenotype.

3.6.4 Composition of lignin in transgenic lines was affected by complementation of *Miscanthus* laccases.

Lignin composition was probed using thioacidolysis cleaving β-O-4 bonds. Assayed by gas chromatography, I confirmed that the double mutant also affected the ratio between S and G monolignols in *Arabidposis*, increasing the ratio from 0.47 to 0.82 (Table 3.6-1). The S/G ratio in dicots might not easily be restored by laccases of grasses (Cesarino *et al.*, 2013), since the cell wall differs significantly between grasses and dicot plants. After complementation, *MsLAC3* and the negative control didn't change the composition of S and G lignin in mature stem, while *MsLAC1* and *MsLAC2* partially reduced the ratio to between 0.55 and 0.62.

Arabidopsis lines	H%	G%	S%	S/G
Col-0	0.82 ± 0.11	67.64 ± 1.81	31.54 ± 1.92	**0.47 ± 0.04**
lac4-2/lac17	1.67 ± 0.08	53.99 ± 1.35	44.34 ± 1.27	**0.82 ± 0.04**
pAtLAC17:MsLAC1-1	1.21 ± 0.05	60.92 ± 2.04	37.87 ± 2.08	**0.62 ± 0.06**
pAtLAC17:MsLAC1-2	1.15 ± 0.11	63.14 ± 0.51	35.71 ± 0.40	**0.57 ± 0.01**
pAtLAC17:MsLAC2-1	1.33 ± 0.13	61.56 ± 1.65	37.11 ± 1.78	**0.60 ± 0.05**
pAtLAC17:MsLAC2-2	1.53 ± 0.31	63.48 ± 0.60	34.98 ± 0.79	**0.55 ± 0.02**
pAtLAC17:MsLAC3-1	2.26 ± 0.02	55.21 ± 0.67	42.53 ± 0.66	**0.77 ± 0.02**
pAtLAC17:MsLAC3-2	2.93 ± 0.23	55.06 ± 1.23	42.01 ± 1.15	**0.76 ± 0.04**
pAtLAC17:MsLAC5-1	7.76 ± 0.89	67.79 ± 1.37	24.45 ± 1.80	**0.36 ± 0.03**
pAtLAC17:MsLAC5-2	4.97 ± 0.02	64.85 ± 0.57	30.18 ± 0.56	**0.47 ± 0.01**
pAtLAC17:Dummy-1	1.58 ± 0.26	54.87 ± 1.12	43.55 ± 1.33	**0.79 ± 0.04**
pAtLAC17:Dummy-2	1.56 ± 0.20	55.44 ± 1.33	43.00 ± 1.51	**0.78 ± 0.05**

Table 3.6-1 Lignin composition of different *Arabidopsis* lines as measured by thioacidolysis. The S/G ratio is also calculated and shown on the right.

The synthesis of H lignin is very much upstream in the monolignol biosynthesis pathway, thus it can be affected by the upstream enzymes like Cytochrome P450 Reductase (Sundin *et al.*, 2014), but it is less likely that it would be affected by downstream enzymes in lignin synthesis process like laccases. The proportion of H lignin in different lines is nearly unchanged, except for *MsLAC5*, where it increased 3 and 5 fold in two independent lines. As compared to the double mutant, S lignin decreased significantly by 45% and 32% while G unit increased by 25% and 20% in

MsLAC5-1 and *MsLAC5-2*, respectively. Consequently, those changes resulted in much lower S/G ratio. To understand clearly why the changes in the amount of different units happen, the thioacidolysis yield of monomers was calculated.

The method of lignin composition analysis is based on the cleavage of β-O-4 bonds, thus the total yield can indicate the amount of specific structure in lignin while still reflecting the lignin content in plant material (Robinson & Mansfield, 2009). When grown in the continuous light conditions, the *lac4-2/17* double mutant had a lower thioacidolysis yield on the basis of klason lignin content (Berthet *et al.*, 2011), indicating a lower percentage of the cleavable bond in the mutant. The trend remained un-affected in *MsLAC3*, *MsLAC5*, and the negative control (Table 3.6-2), thus, these two laccases did not affect the frequency of cleavable or condensed bonds in the transgenic lines. In contrast, by expressing *MsLAC1* or *MsLAC2* under the control of *AtLAC17* promoter in the mutants, the relative thioacidolysis yield significantly increased under light stress.

Arabidopsis Line	H lignin		G lignin		S lignin		Total		Relative yield/%	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Col-0	2.0	0.4	145.9	13.9	67.8	2.1	215.7	15.3	100	
lac4-2/lac17	2.7	0.6	63.5	2.9	50.5	3.8	116.7	5.5	79.3	9.2
pAtLAC17:MsLAC1	2.3	0.2	113.5	6.2	70.6	6.4	186.3	10.4	92.6	10.3
pAtLAC17:MsLAC2	3.0	0.3	132.9	13.9	79.9	5.2	215.7	18.5	100.5	15.4
pAtLAC17:MsLAC3	4.6	0.2	86.7	4.9	66.1	2.2	157.5	6.1	80.6	8.9
pAtLAC17:MsLAC5	13.8	1.1	120.7	3.8	43.6	5.2	178.1	8.1	74.2	5.8
pAtLAC17:Dummy	2.7	0.3	61.2	3.3	47.4	0.7	111.4	3.2	77.7	4.4

Table 3.6-2 Characterization of lignin composition in different *Arabidopsis* lines. Yields of H, G and S thioacidolysis monomers released from extract-free samples are presented in unit of μmol per gram sample after acetone extraction. The relative yield is calculated based on total amount of monomer per gram of Klason lignin. Wildtype plant is used as control and the yield is set as 100%.

Generally, regarding to the composition of lignin in stem of different *Arabidopsis* lines after acetone extraction, the amount of S unit did not change much while the amount of G unit varied a lot. In *pAtLAC17:MsLAC3* line, the induced lignin biosynthesis was found to be non-specific to individual monomer, the amount of H/G/S unit increased evenly, without affecting the S/G ratio. *MsLAC1* and *MsLAC2*

complemented the mutant by increasing the amount of both G and S unit, resulting in a lower S/G ratio and partially restored the composition profile to the level in *Col-0*. As shown in the table, it is very clear that *MsLAC5* cannot complement the mutant, although the S/G ratio decreased to normal level. As compared to the double mutant and control, complementation with *MsLAC5* didn't change the amount of S unit, but H unit and G unit increased nearly 6 fold and 2 fold, respectively. This is the first report revealing diverse function of laccase isoforms in altering the lignin composition, but the mechanism beneath is still unknown.

3.6.5 Sugar content can be restored by *MsLAC1* and *MsLAC2*.

After enzymatic hydrolysis, the saccharification of *lac4-2/lac17* double mutant is much more efficient as compared to the wild type plants (Berthet *et al.*, 2011). As shown in Fig 3.6-5, the structural glucose increased from 29% in *Col-0* to 33% in the mutant. Only with the complementation by *MsLAC1* and *MsLAC2*, the content of sugars can be restored to the wild type level. It is claimed that the disruption of the LAC17 gene will induce redirection of the phenylpropanoid pathway (Berthet *et al.*, 2011). In terms of broader metabolic levels, the AtLAC17 homologue in *Miscanthus*, MsLAC1 and MsLAC2 may have the ability to influence the carbon flow.

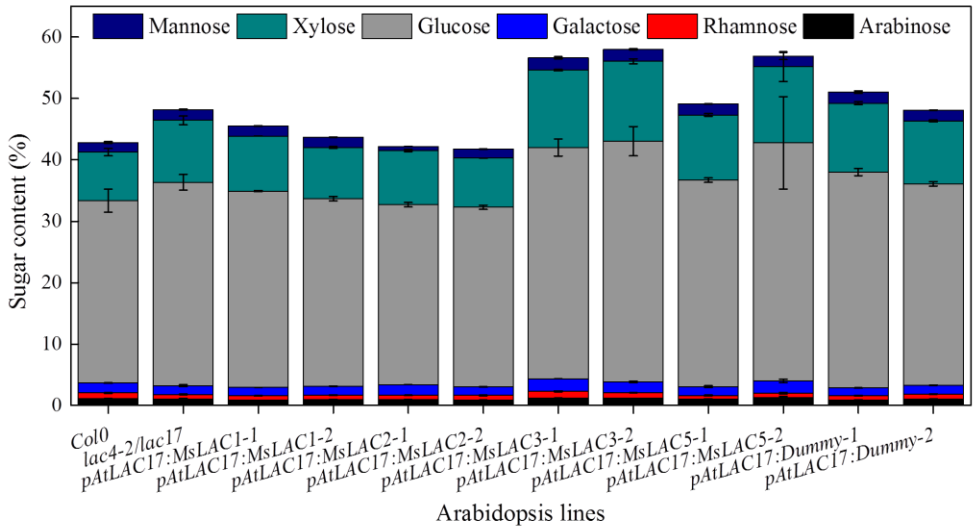

Fig 3.6-5 Composition of structural sugars in different *Arabidopsis* lines.

Those observations demonstrated that by expressing *MsLAC1* or *MsLAC2* with *AtLAC17* promoter in the *lac4-2 lac17* background, not only the phenotypes but also the content of lignin and sugar can be restored. The proportion of G unit in the complementation lines was induced to normal level, which partly restored the high S/G ratio in double mutant, suggesting the similar potential function of MsLAC1 and MsLAC2 in lignification and the deposition of G unit.

Indeed, there are differences on the composition of lignin in different transgenic lines. However, it is hard to attribute the difference to the substrate specificity of individual laccases. rMsLAC1 expressed in yeast showed highest activity against synapyl alcohol but the complementation of *MsLAC1* increased only G lignin and showed no influence on the property of S lignin. Since all other attempts expressing *Msicanthus* laccases failed, the biochemical characterization of each laccase isoforms need to be obtained before this conjecture can be finally confirmed.

3.7 Ectopic expression of *Miscanthus* laccases in *Arabidopsis* leads to altered plant growth and increased lignification.

Over-expression of either *AtLAC4* or *AtLAC17* in *Arabidopsis* will lead to ectopic lignin polymerization even in primary cell walls as long as exogenous monolignols are sufficiently supplied (Schuetz et al., 2014), indicating a potentially higher lignin content in over-expression lines, which in turn could affect the plant development. To evaluate the effect of *Miscanthus* laccase over-expression on the growth of *Arabidopsis*, plants expressing laccases from *Miscanthus* under the control of the CaMV 35S promoter were generated in the *Col-0* background, in addition to the corresponding *p35S::Dummy* control line (Fig S8b). The high expression level of *Miscanthus* laccases was confirmed by qRT-PCR, and two independent lines with distinct high expression levels of MsLAC1 were selected (Fig. S5).

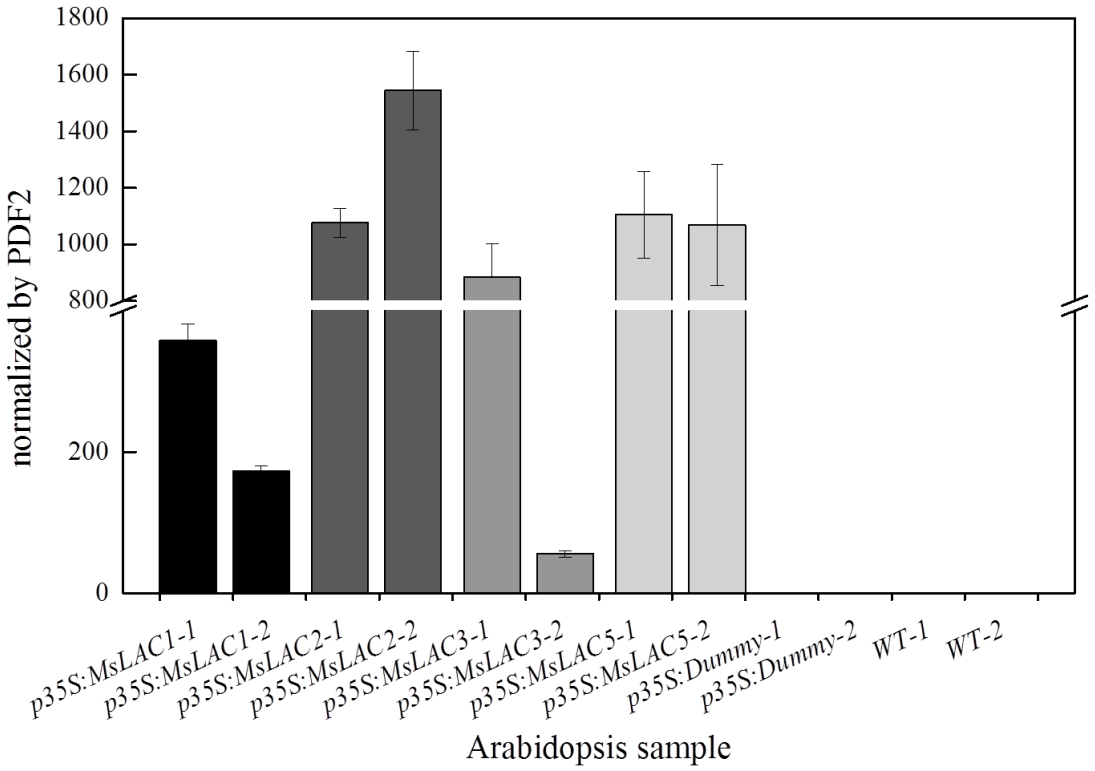

Fig 3.7-1 Expression pattern of *Miscanthus* laccases in different transgenic lines quantified with qRT-PCR. PDF2 was used as reference genes, and the bars stand for the expression level of corresponding overexpressed *Miscanthus* laccases in different lines. Expression level of *MsLAC1* was chosen as a sample for WT control and the negative control; the expression of all other *Miscanthus* laccases was not detected in both controls (not shown).

3.7.1 Phenotyping of lines overexpressing *Miscanthus* laccases.

Although it was not clear if lignin content in transgenic plant overexpressing a laccase gene from yellow poplar had been changed (Dean *et al.*, 1998), a 2.1–19.6% increase in total lignin content of the stem was found in transgenic poplar plants overexpressing a cotton laccase (GaLAC1) (Wang, J *et al.*, 2008a). In the *lac4-2/lac17* double mutant and *lac4/lac11/lac17* triple mutant, changes in lignin content, xylem lignification and stem height were described (Berthet *et al.*, 2011; Zhao *et al.*, 2013). Thus, in my study, I've focused on the height of mature inflorescence stem as well as lignification in transgenic plants overexpressing *Miscanthus* laccases. Those lines were grown in both long-day conditions in glasshouse and in continuous light condition in growth chamber, with different phenotypes observed.

3.7.1.1 Higher inflorescence stem in *pro35S:MsLAC1* lines were observed.

When grown in long-day conditions for eight weeks, the plants overexpressing *MsLAC2*, *MsLAC3* and *MsLAC5* showed similar development characteristics as the wild type and negative controls. However, the overexpression of *MsLAC1* in *Col-0* background significantly promoted the elongation of stem in both independent lines (Fig 3.7-2). In my study, MsLAC1 has been proven to be functional in restoring lignin content of *lac4-2/lac17* mutant. The study of the expression of lignin-related enzymes and the lignin content in transgenic lines will help to explain the phenotype.

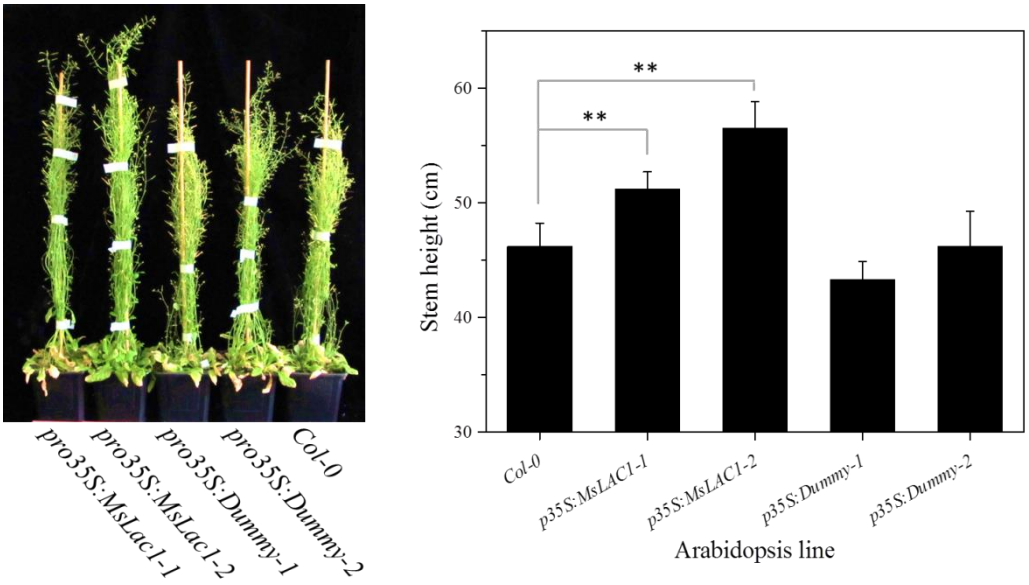

Fig 3.7-2 Transgenic plants grown in glasshouse with long-day conditions. Two independent lines of *pro35S:MsLAC1* and negative control were selected for comparison. Only the longest main stem was measured. Bars indicate mean ± SD of at least ten plants of each line. Student's t-test was used to determine the significant difference: *, P<0.05; **, P<0.01.

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3.7.1.2 Surprisingly, *pro35S:MsLAC1* exhibited a semi-dwarf phenotype under light stress.

The semi-dwarf phenotype of lac4-2/lac17 double mutant growing under constant light was hypothesised to be induced by light stress (Berthet *et al.*, 2011), indicating a possible connection between the stress-induced phenotype and laccases. Consistent with the phenotype under long-day condition, overexpression of *MsLAC3* and *MsLAC5* showed similar stem length as WT (Fig S9). The growth of *pro35S:MsLAC1*

and *pro35S:MsLAC2* under constant light was negatively affected as compared with *Col 0* and *pro35S:Dummy* lines (Fig 3.7-3). It is generally assumed that greater levels of lignification will hinder the development of the plant, as it has been reported for lines over-expressing lignin-related TFs or enzymes (Zhou *et al.*, 2009; Zhang *et al.*, 2015). Thus the lignin content and composition of transgenic plants growing under light stress also need to be measured.

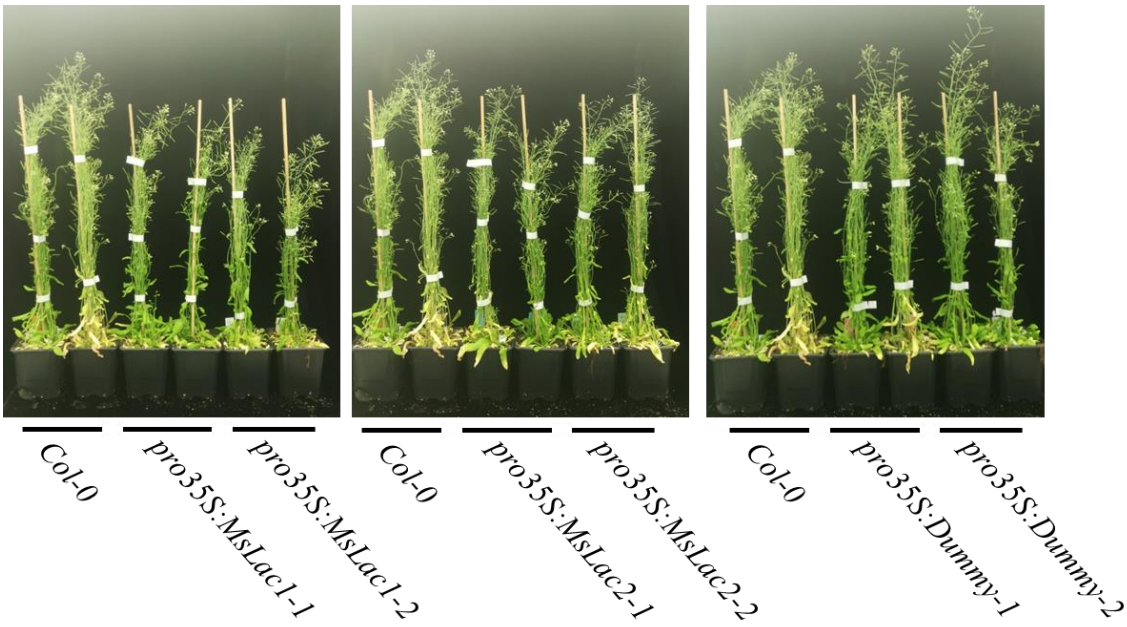

Fig 3.7-3 Plant morphology of 8 weeks old WT and *Miscanthus* laccase transgenic plants under continuous light.

3.7.1.3 Lignin staining of *pro35S:MsLAC1* stem cross section revealed the earlier and stronger lignification in xylem fibers.

Since the plant yield is only changed after over-expression of *MsLAC1*, cross sections of transgenic and wild type plants were stained with HCl-phloroglucinol. There is much stronger lignification in the xylem fibres of mature *p35S:MsLAC1* plants (Fig 3.7-4b), and staining of stem cross sections in early developmental stages showed premature lignification of interfascicular fibres (Fig 3.7-4a). The lack of lignification in the interfascicular fibres of *lac4-2/lac17* mutant plants (Berthet *et al.*, 2011), which can be complemented by MsLAC1, indicates the potential function of MsLAC1 in the development of xylem fibres.

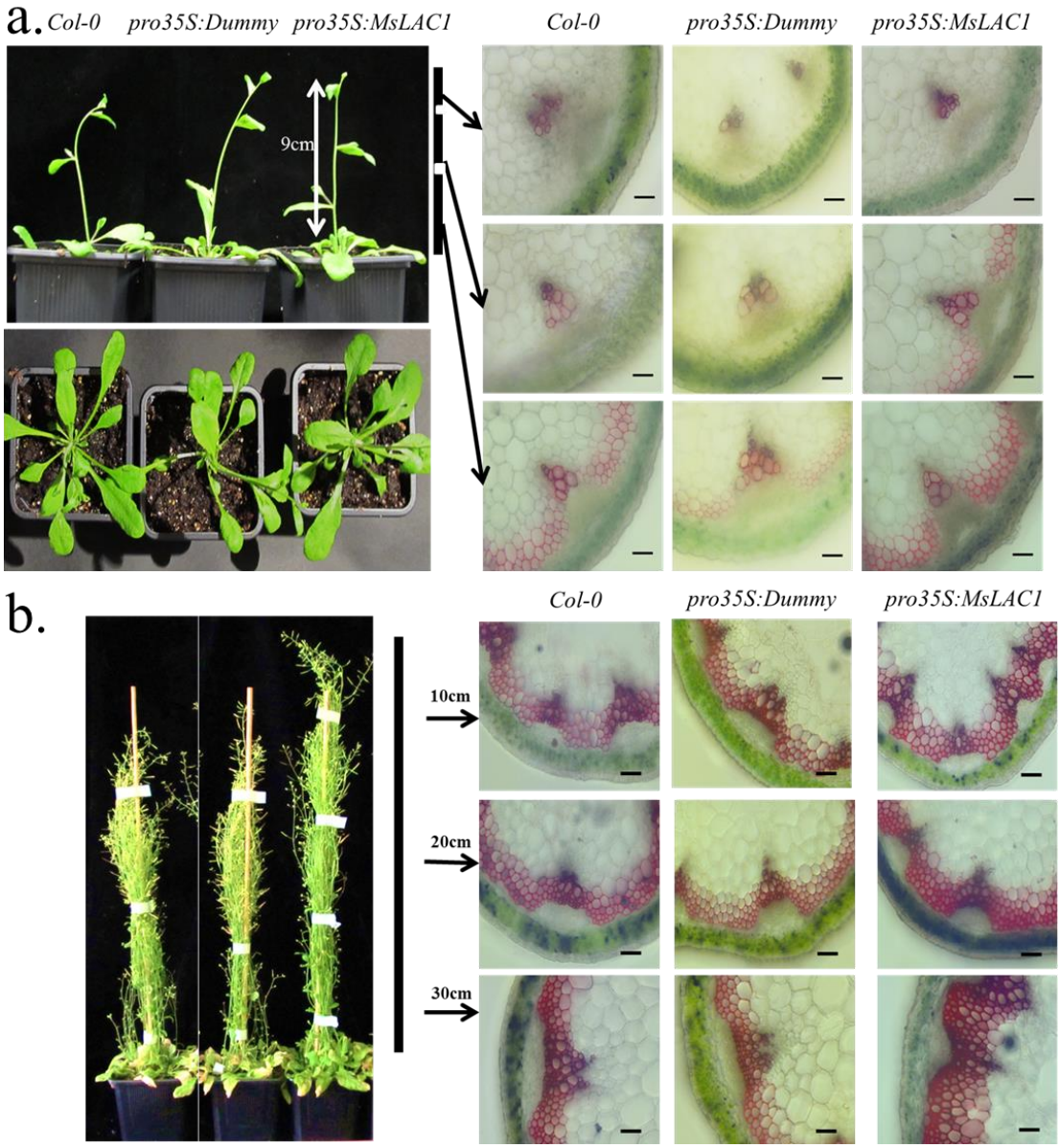

Fig 3.7-4 Wiesner staining of stem cross sections from (a) immature and (b) mature plants. *Col 0*, the negative control and *Arabidopsis* plants overexpressing *MsLAC1* were compared. Scale bars: 100 μ.

3.7.2 Overexpression of *Miscanthus* laccases increased the Lignin content in *Arabidopsis*.

On the basis of the altered growth and the stronger staining of MsLAC1 stem, I hypothesized that the lignin content in the transgenic can be higher than wild type. As shown in Fig 3.7-5, there is significant induction on the synthesis of lignin in *p35S:MsLAC1* in both growth conditions. *MsLAC2* and *MsLAC3* also increased the

klason lignin content in long day and continuous light condition, respectively. However, the significance in other laccase over-expressing lines was narrowly missed because of the unchanged acid soluble lignin. In another word, if we only take insoluble lignin into account, overexpression of all the *Miscanthus* laccases seemed to have positive function on lignin biosynthesis in *Arabidopsis*.

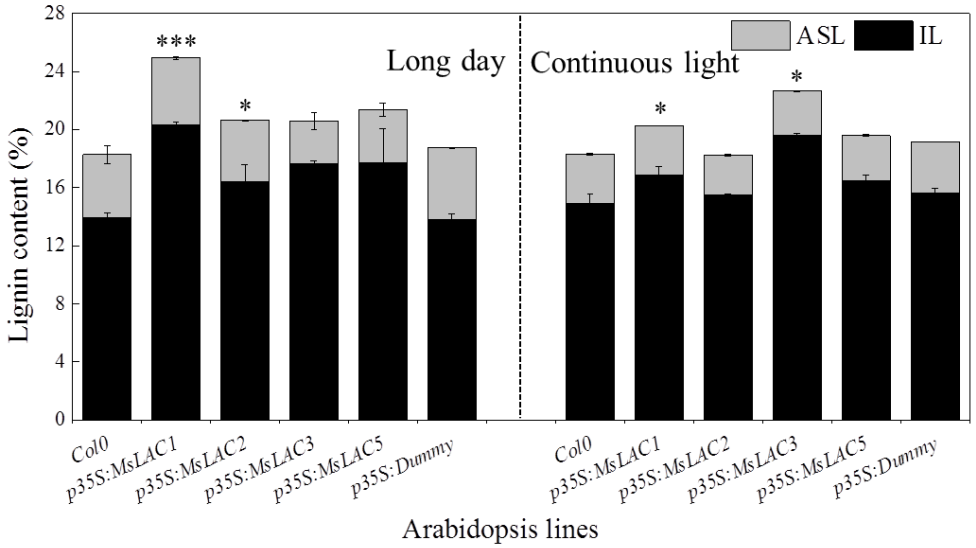

Fig 3.7-5 Lignin content of mature stems from different *Arabidopsis* lines. Insoluble lignin (IL) and acid-soluble lignin (ASL) content are presented as weight percentage in mature stems of different lines. Statistical analysis is based on the total Klason lignin content. Student's t-test was used to determine the significant difference: *, P<0.05; **, P<0.01; ***, P<0.001.

3.7.3 *Miscanthus* laccase isoforms changed lignin composition in *Arabidopsis* transgenic lines with distinct preference.

The results from complementation project already indicate the possible function of laccase isoforms in the composition of lignin (Section 3.6.4). Here, the speculated role of *Miscanthus* laccases is further proofed in the over-expression lines. The growing environment is very important for the structure of lignin in *Arabidopsis*, leading to a higher S/G ratio when the wild type plants were cultured in long-day condition (Table 3.7-1). Genes involved in the lignin biosynthesis pathway exhibits altered expression profile when Arabidopsis plants were submitted to high intensity of light (Kimura *et al.*, 2003), thus lignin content and composition might be altered (Moura *et al.*, 2010). H lignin only make up less than 2% of the released monomers whatever the sample is,

so we will not discuss it here. However, the function of *Miscanthus* laccase isoforms on S/G ratio seems independent of light stress. In both conditions, the ratio decreased 15%-25% in *MsLAC1* and *MsLAC5* over-expressing lines, but slightly increased in *pro35S:MsLAC3*. *MsLAC2* didn't influence the factor significantly, remaining a similar ration with *Col-0* and negative control.

Growth condition	*Arabidopsis* lines	H%	G%	S%	S/G
Long day	*Col-0*	1.92 ± 0.06	62.19 ± 0.54	35.89 ± 0.60	**0.58 ± 0.01**
	Pro35S:MsLAC1	1.96 ± 0.10	68.10 ± 1.98	29.94 ± 2.07	**0.44 ± 0.04**
	Pro35S:MsLAC2	0.83 ± 0.05	61.39 ± 0.58	37.78 ± 0.61	**0.62 ± 0.02**
	Pro35S:MsLAC3	1.87 ± 0.13	59.93 ± 0.51	38.21 ± 0.40	**0.64 ± 0.00**
	Pro35S:MsLAC5	1.83 ± 0.13	65.91 ± 0.53	32.26 ± 0.65	**0.49 ± 0.01**
	Pro35S:Dummy	1.46 ± 0.39	61.81 ± 0.94	36.74 ± 0.90	**0.59 ± 0.02**
Continuous light	*Col-0*	0.82 ± 0.11	67.64 ± 1.81	31.54 ± 1.92	**0.47 ± 0.04**
	Pro35S:MsLAC1	1.19 ± 0.72	70.63 ± 0.84	28.18 ± 1.15	**0.40 ± 0.01**
	Pro35S:MsLAC2	1.27 ± 0.12	64.95 ± 1.39	33.78 ± 1.30	**0.52 ± 0.03**
	Pro35S:MsLAC3	1.44 ± 0.18	62.77 ± 0.98	35.79 ± 0.91	**0.57 ± 0.02**
	Pro35S:MsLAC5	1.04 ± 0.05	71.15 ± 0.26	27.81 ± 0.39	**0.39 ± 0.01**
	Pro35S:Dummy	1.81 ± 0.26	64.36 ± 0.22	33.83 ± 0.47	**0.53 ± 0.01**

Table 3.7-1 Lignin composition of different *Arabidopsis* lines as measured by thioacidolysis. The S/G ratio is also calculated and shown on the right.

The thioacidolysis yield of monomers from most plant materials didn't show significant differences (Table 3.7-2), indicating the unchanged frequency of cleavable or condensed bonds in most transgenic lines. In this case, the total monomer (μmol per gram extract-free cell wall) could reflect the lignin content in different lines, making it clear that 1) light intensity will stimulate the synthesis of lignin and 2) all

the laccases can increase the lignin content when plants were grown without light stress.

Regarding to the relative yield, the only exception is *Pro35S:MsLAC1* in continuous light condition, which is 14.7% higher. The finding supports my speculation that MsLAC1 would preferentially catalyze the coupling of β-O-4 linkage rather than other condensed bonds.

Growth condition	*Arabidopsis* Line	H lignin		G lignin		S lignin		Total		Relative yield/%	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Long day	*Col-0*	3.4	0.1	111.2	2.2	64.2	1.5	178.8	3.1	100	
	Pro35S:MsLAC1	4.7	0.2	161.7	4.5	71.1	5.1	237.5	1.8	97.3	1.3
	Pro35S:MsLAC2	1.7	0.3	123.4	14.4	75.9	7.7	201.0	22.3	99.4	9.9
	Pro35S:MsLAC3	4.1	0.8	129.6	15.1	82.6	9.2	216.2	25.1	107.5	14.1
	Pro35S:MsLAC5	4.2	0.3	150.0	5.9	73.5	4.4	227.7	10.2	108.8	2.9
	Pro35S:Dummy	2.5	0.4	111.2	19.7	66.2	12.7	179.9	32.0	98.0	17.4
Continuous light	*Col-0*	2.0	0.4	145.9	13.9	67.8	2.1	215.7	15.3	100	
	Pro35S:MsLAC1	3.1	1.7	192.1	6.3	76.6	5.2	271.8	10.2	114.7	7.1
	Pro35S:MsLAC2	2.8	0.5	142.0	22.6	73.6	9.0	218.4	31.6	102.7	22.2
	Pro35S:MsLAC3	3.3	0.3	146.1	6.5	83.2	3.0	232.7	8.2	87.5	7.1
	Pro35S:MsLAC5	2.2	0.1	151.3	4.1	59.1	0.9	212.6	5.0	92.3	4.6
	Pro35S:Dummy	4.0	0.8	141.7	9.6	74.4	3.9	220.1	14.3	97.7	7.5

Table 3.7-2 Lignin composition of WT, negative control and plants over-expressing Miscanthus laccases grown in different conditions. Yields of H, G and S thioacidolysis monomers released from extract-free samples are presented in unit of μmol per gram sample. The relative yield is calculated based on total amount of monomer per gram of Klason lignin. Wildtype plant is used as control and the yield is set as 100%.

3.7.4 Sugar content is not affected by most of the *Miscanthus* laccases.

As a supplement, I also measured the structure sugar content of all the transgenic lines. Only *MsLAC5* significantly reduced the total content in both growth conditions. But if we focus on glucose, which is mostly derived from cell wall cellulose, a greater fluctuation can be seen under the long-day condition (Fig 3.7-6). The result somehow compensates the increased lignin content (Table 3.7-2), keeping a balance of total yield after thioacidolysis.

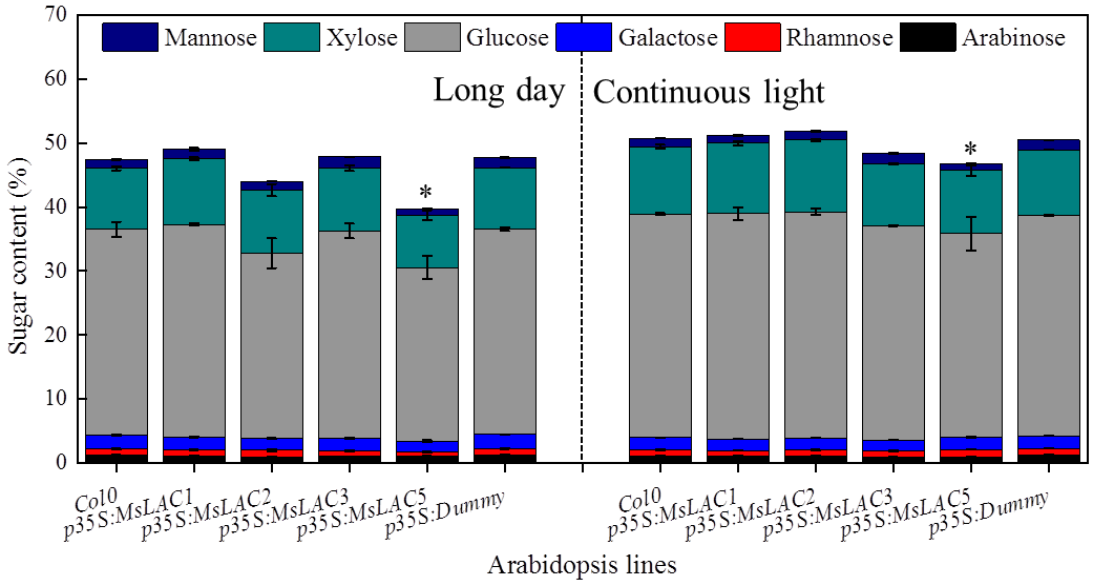

Fig 3.7-6 Composition of structural sugars in different *Arabidopsis* lines. Statistical analysis is based on the total sugar content. Student's t-test was used to determine the significant difference: *, P<0.05; **, P<0.01.

3.7.5 Expression of lignin-related genes and flavonoid synthesis genes are all affected in MsLAC1 overexpression line.

Since earlier lignification of interfascicular fibres in middle part of immature stem was observed in *Pro35S:MsLAC1*, same plant materials were collected and the expression levels of key genes involved in lignin biosynthetic pathway were evaluated by qRT-PCR analysis. The transgenic line showed strong expression of *MsLAC1*, and all the monolignol and lignin biosynthesis genes were upregulated (Fig 3.7-7). However, among all the transcription factors we measured, only MYB63 and MYB85 were significantly upregulated. The overexpression of *OsPRX38* also induced the mRNA transcript level of lignin biosynthetic genes (Kidwai *et al.*, 2019), further increasing the lignin content in transgenic plant. Those preliminary findings revealed the molecular basis for the induction of lignin biosynthesis by overexpressing laccases from *Miscanthus*.

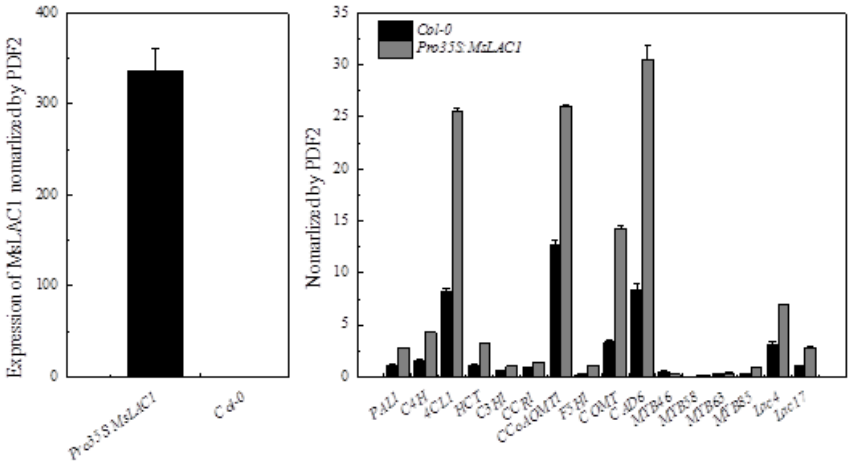

Fig 3.7-7 Expression of *MsLAC1* and lignin-related genes in *Arabidopsis Col-0* and *Pro35S:MsLAC1* lines. Middle part of immature stem where earlier lignification was observed, was collected for mRNA extraction and cDNA obtained afterwards was used as template. PAL1, phenylalanine ammonia lyase 1; C4H, cinnamate 4-hydroxylase; 4CL1, 4-coumarate CoA ligase 1; HCT, hydroxycinnamoyl CoA:shikimate/quinate hydroxycinnamoyltransferase; C3H1, p-coumarate 3-hydroxylase 1; CCoAOMT1, caffeoyl CoA 3-O-methyltransferase 1; CCR1, cinnamoyl CoA reductase 1; F5H1, ferulate 5-hydroxylase 1; COMT, caffeic acid O-methyltransferase; CAD6, cinnamyl alcohol dehydrogenase 6.

Lignin and flavonoid biosynthesis are two main routes of the phenylpropanoid pathway, the metabolic flux is controlled by CHS and HCT activities (Besseau *et al.*, 2007). It has been reported that accumulation of flavonoids repressed in lignin synthesis affects the growth of plant (Besseau *et al.*, 2007), thus the increased lignin content may in turn relieve the flavonoid accumulation and have positive influences on plant growth. So I also evaluated the expression of some flavonoid synthesis genes including chalcone synthase (CHS), chalcone isomerase (CHI) and flavonol synthase (FLS). As shown in Fig 3.7-8, all the genes were highly expressed in inflorescence in wild type and negative control lines, but strongly repressed in both overexpression lines.

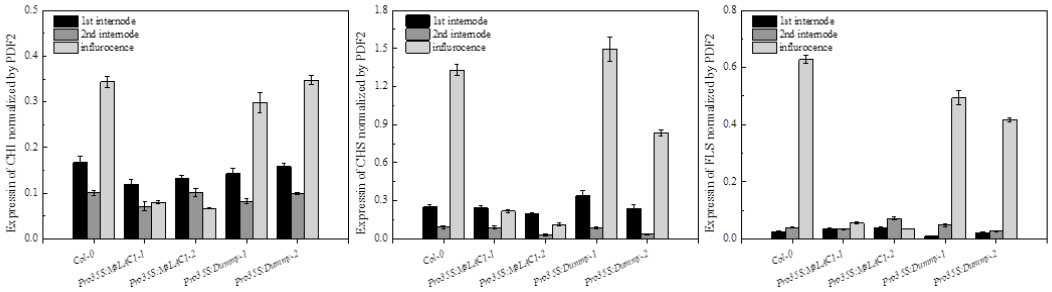

Fig 3.7-8 Expression of flavonoid biosynthesis genes (CHI, CHS and FLS) in different internodes

of immature *Arabidopsis* transgenic and wild type plants.

3.8 Supplementary figures

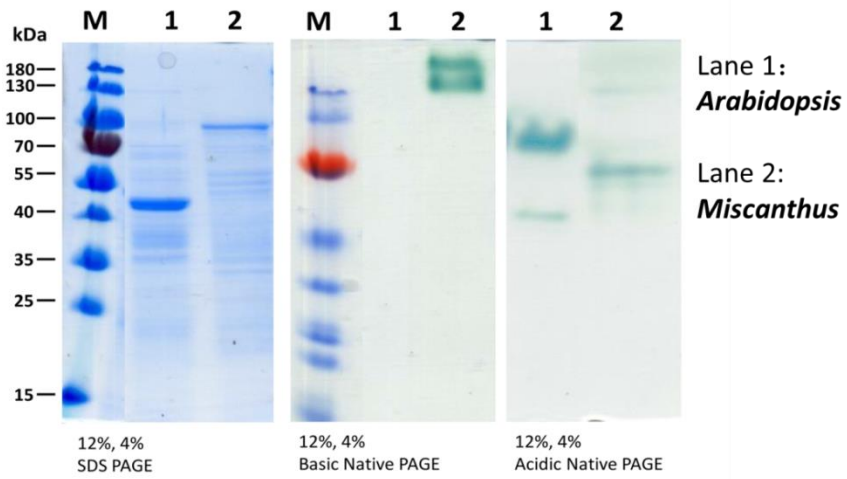

Fig S1 (a) Laccase activity in Arabidopsis and Miscanthus. (b) Zymography of laccases with protein extracted from Arabidopsis and Miscanthus. Specific laccase activity was calculated against the protein concentration. Same samples were loaded on basic and acidic gels to separate acidic and basic laccases. Gels were stained with ABTS after 1h incubation with catalase.

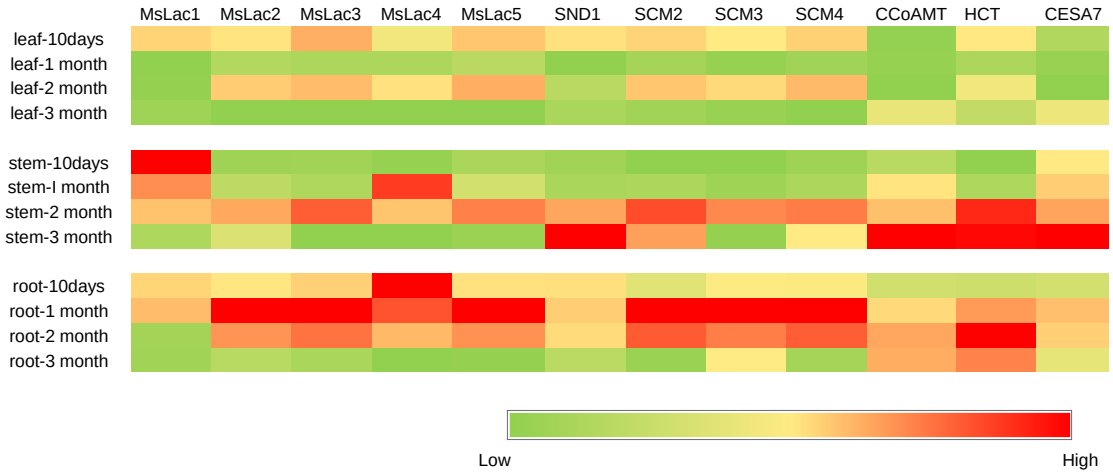

Fig S2 Visualized Expression pattern of MsLACs and other secondary cell wall related genes in different tissue of *Miscanthus* plant, aging 10 days to 3 months. Data were normalized by PP2A. *SND1, M. sinensis SECONDARY WALL-ASSOCIATED NAC DOMAIN1*; *SCM2-4, M. sinensis SECONDARY CELL WALL MYBs 2-4*; *CcoAOMT, M. sinensis Caffeoyl-CoA O-methyltransferase*; HCT, *M. sinensis Hydroxycinnamoyltransferase;* CESA7, *M. sinensis Cellulose synthase A catalytic subunit 7*.

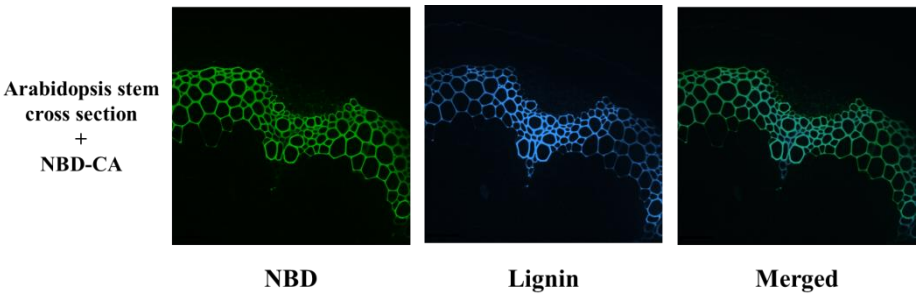

Fig S3 Confirmation of NBD-CA as a marker for lignification.

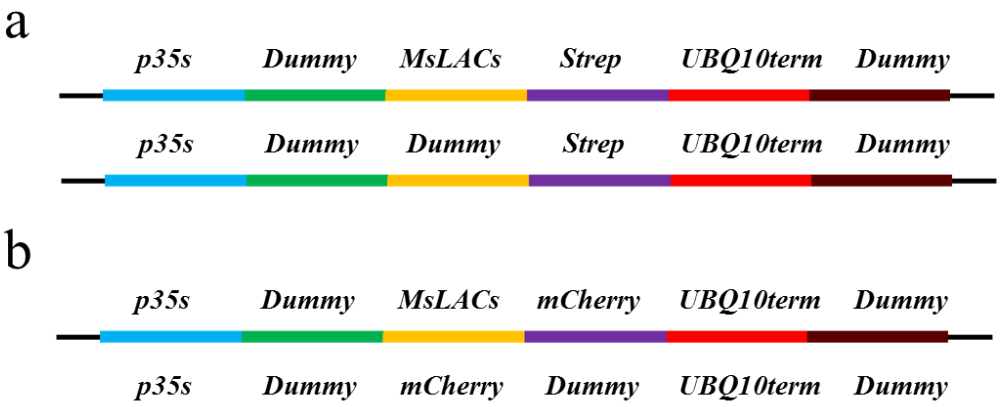

Fig S4 Schematic structure of Greengate constructs for transformation into Agrobacteria. (a) aiming at protein expression; (b) aiming at localization of laccases. Dummy is a short random sequence designed as negative control.

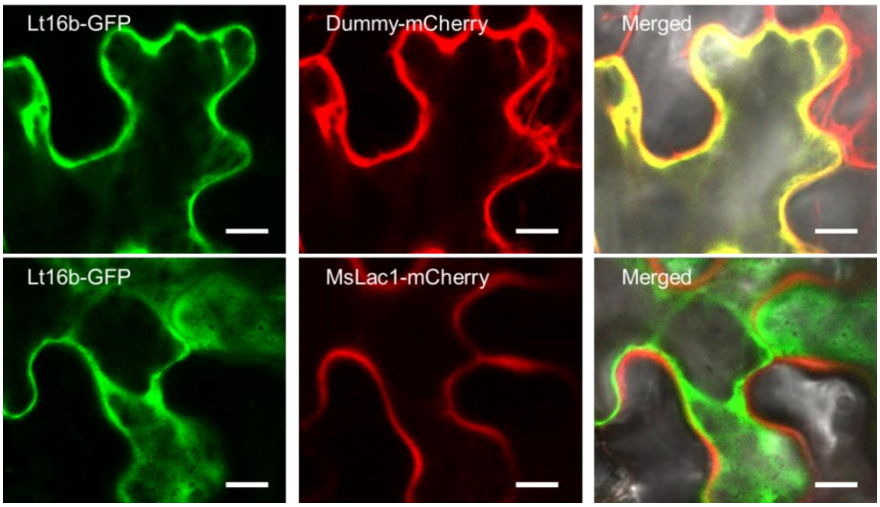

Fig S5 Transient expression of indicated GFP and mCherry fusion proteins in tobacco epidermal leaf cells after co-infiltration of different *Agrobacteria*. Bar=10μm.

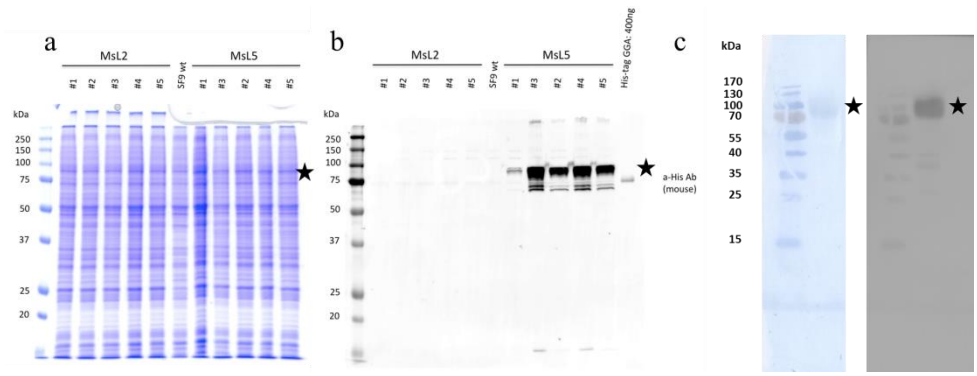

Fig S6 Heterologous expression of MsLAC2 and MsLAC5 in S9 insect cells. (a) and (b): SDS PAGE and Western blot with anti-his tag antibody after P1 expression; (c) SDS PAGE and Western blot after purification. The stars mark the expressed rMsLAC5.

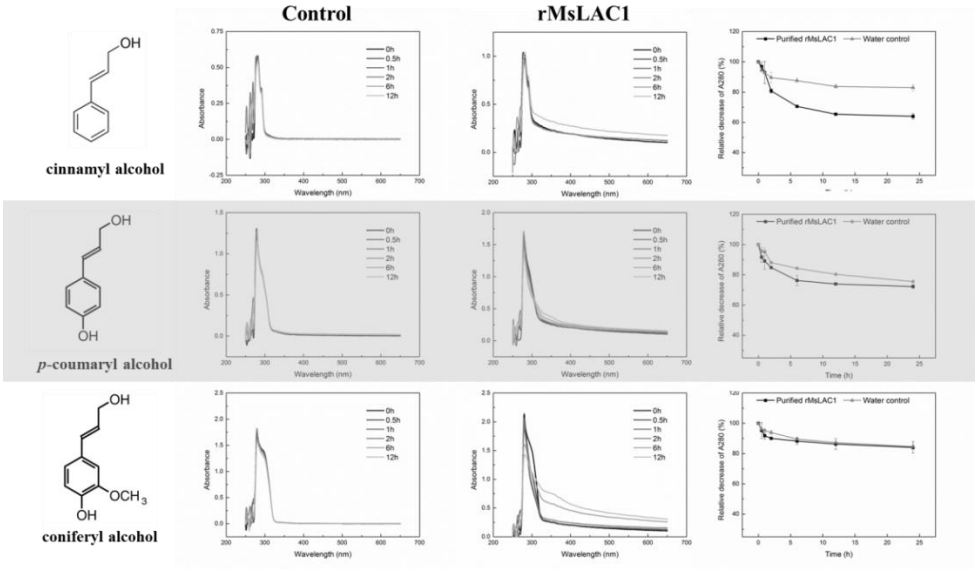

Fig S7 Chemical structures of cinnamyl alchol, p-coumaryl alcohol, cinapyl alcohol and spectrum of reaction mixture containing those monolignol components and purified rMsLAC1. Final concentration of 1mM of each substrate in 50mM pH3.0 acetate buffer was mixed with 0.002U rMsLAC1 individually and measured continuously after up to 12 hours. The decrease of absorbance at wavelength of 280 nm was calculated against the value of 0 h.

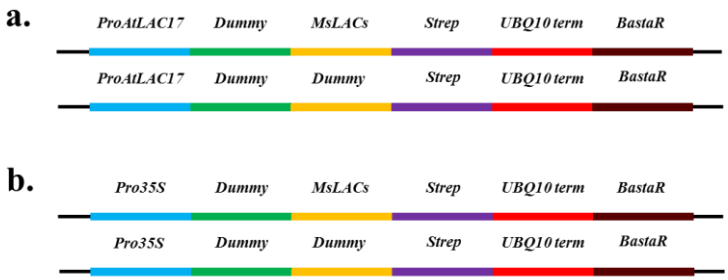

Fig S8 Schematic structure of Greengate constructs for transformation into Arabidopsis. (a) aiming at complementation of *lac4-2/lac17*; (b) aiming at overexpression of laccases in *Col 0*.

Dummy is a short random sequence designed as negative control.

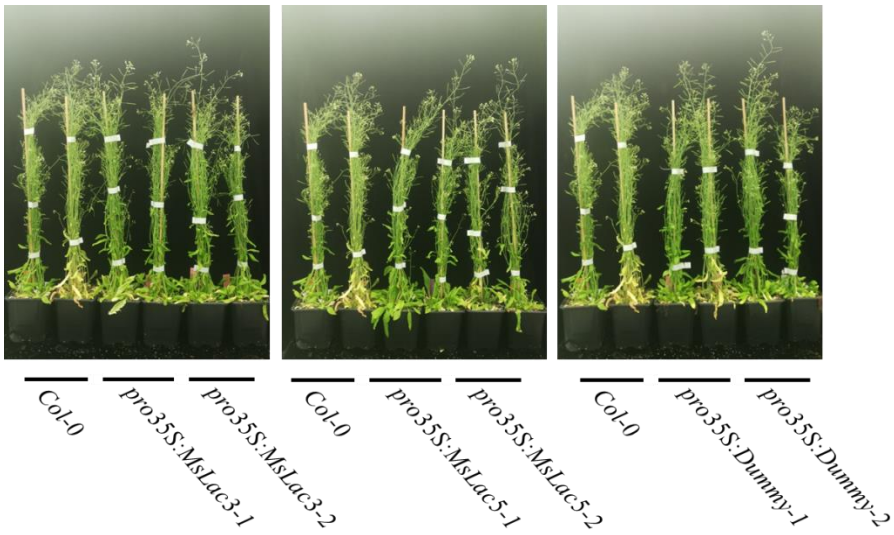

Fig S9 Plant morphology of 8 weeks old WT and Miscanthus laccase transgenic under continuous light.

4. Discussion

Facing the increasing demand of low-cost and environmental-friendly chemical engineering and energy generation systems, the genus *Miscanthus* presents a great potential to provide an alternative resource or act in conjunction with non-renewable resources (Adams *et al.*, 2018). As a highly photosynthetic-efficient C4 grass, *Miscanthus* requires low nutrient input while returning high yields of lignocellulosic feedstocks (Lewandowski *et al.*, 2000). Lignin, one of the major components of lignocellulose, is recalcitrant to enzymatic degradation because of its chemical complexity. Genetic research on various alfalfa lines indicates the relationship between lignin and saccharification, identifying lignin as the major factor in recalcitrance of cell walls to saccharification (Chen & Dixon, 2007). Delignification of *Miscanthus sinensis* significantly improves the initial rate of enzyme hydrolyzation (Yoshida *et al.*, 2008), suggesting that lignin is likely the most significant resistance factor against enzymatic treatment in *Miscanthus*. Moreover, the composition of lignin also highly influences the efficacy of lignocellulosic biomass utilization. To be more specific, the proportion of H/G/S units and linkages between these monomers strongly affects the characteristics of plant cell wall lignin. The presence of methoxy substituents in the monolignol structure reduces available reactive sites, resulting in decreased polymerization access. Thus, it is assumed that S-rich lignin is less condensed and therefore can be more efficiently degraded when compared to G-rich lignin (Ziebell *et al.*, 2010). Although no effect on direct cell wall degradation was found in *Arabidopsis f5h* mutants with complete loss of S lignin (Jung *et al.*, 1999), the composition of lignin somehow affects the pretreatment efficiency. A study comparing the cell wall degradability of *A. thaliana* wildtype plants with a G-rich mutant line (*fah1-2*) and an S-rich (C4H:F5H) genetically modified line showed that the pretreatment efficiency significantly increased in S-rich lignin (Li *et al.*, 2010).

In *Miscanthus*, both lignin content and composition are tightly controlled and

regulated. Previous studies on the lignocellulosic composition of *Miscanthus* reveal variation in lignin content among different genotypes (Lygin *et al.*, 2011) and different tissues (Huyen *et al.*, 2010). Further, both genetic and environmental factors influence its cell wall composition strongly (Hodgson *et al.*, 2010). Additionally, the harvesting season (spring/winter) of *Miscanthus* has been shown to significantly influence lignin composition in cell walls as well as the phenolic acid content in the whole biomass (Huyen *et al.*, 2010). To identify the regulatory network of lignin biosynthesis, the transcriptome of developing *Miscanthus* internodes was studied (Hu *et al.*, 2017). In agreement with the well-described biosynthetic and regulatory network of Arabidopsis (Tobimatsu & Schuetz, 2019), this particular research revealed co-expression patterns of putative lignin-related TFs and genes in *Miscanthus*. However, the current lack of in-depth studies of lignin-related genes in *Miscanthus* is largely limiting the understanding of lignin biosynthesis in this promising bioenergy crop. In this part of my thesis, the detailed expression pattern of lignin-related genes in *Miscanthus* and the regulation of laccase promoters by lignin-related TFs will be discussed, providing a first comprehensive overview of the entire lignification network in *Miscanthus*.

Laccases are phenol-oxidizing enzymes which can convert monolignols into free radicals, leading to the formation of lignin polymers. In plants, a number of laccases have been reported to be involved in lignin polymerization in secondary cell walls and middle lamellas, not only affecting lignin content, but also influencing the composition of lignin monomers. Recent publications in *Arabidopsis* indicate that disrupting LAC17 affects both lignin content and deposition of G lignin units in interfascicular fibers (Berthet *et al.*, 2011). Further, the *lac4-2/lac17* double mutant shows a lignin reduction of up to 40% (Berthet *et al.*, 2011), whereas lignin deposition in the *lac4/lac11/lac17* triple mutant is completely abolished, causing severe plant growth defects (Zhao *et al.*, 2013). Based on these results, laccases present themselves as great candidates for the study of lignin biosynthesis and alteration of lignin quality in *Miscanthus*. However, the genetic redundancy of the

large laccase family restricts functional studies of individual laccases. For example, expression of 16 individual *Arabidopsis* laccases under control of the *AtLAC4* promoter in the *lac4-2/lac17* double mutant, the severe phenotype can be restored completely by AtLAC10, 11, and 17, and partially by AtLAC1, 2, 5, and 12 (Chaibang, 2014), further indicating their redundancy. Moreover, lignin content of the *lac17* mutant can be complemented by the sugarcane laccase SofLAC, while the S/G ratio remains unchanged (Cesarino *et al.*, 2013). To gain a better understanding of the role of *Miscanthus* laccases in the polymerization and modification of lignin, I will discuss here the function of a specific laccase, using a combination of enzymatic approaches and metabolic analyses following genetic complementation and overexpression.

4.1 The large laccase family in *Miscanthus*.

Laccases are ubiquitous enzymes, found in fungi and plants as well as bacteria and insects. The first laccase has been described in the sap of the Japanese lacquer tree (Yoshida, 1883), however laccases from fungi have been studied more intensively. Recently, more and more studies have been published on the isolation and characterization of laccases from rice (Huang *et al.*, 2016; Liu *et al.*, 2017), Norway spruce (Koutaniemi *et al.*, 2015a), papaya (Jaiswal *et al.*, 2015), litchi (Fang *et al.*, 2015), sugarcane (Cesarino *et al.*, 2013), cotton (Wang, J *et al.*, 2008b), and other plants. Genome analyses further revealed the occurrence of large laccase families in the plant kingdom (Cai *et al.*, 2006).

In the *Miscanthus*, analyses done using RNA-Seq libraries including multiple accessions (Rai *et al.*, 2016b), revealed more than 95 laccase-like contigs, with 28 complete sequences which can be categorized into only 3 subgroups (Fig. 3.1-3). Laccase gene families have been analyzed in various plants, including *Arabidopsis* (17 isoforms, 6 subgroups), rice (30 isoforms, 5 subgroups), cotton (42-46 isoforms), maize (5 subgroups), *Sorghum* (25 isoforms), and *Brachypodium* (29 isoforms, 4 subgroups) (Caparrós-Ruiz *et al.*, 2006; Turlapati *et al.*, 2011; Wang, Y *et al.*, 2015;

Balasubramanian *et al.*, 2016; Rai *et al.*, 2016; Liu *et al.*, 2017). The size of the laccase gene family in *Miscanthus* is in agreement with those studies.

To better understand the function of *Miscanthus* laccases, five laccase sequences cloned from cDNA of *Miscanthus* were characterized *in silico*. In agreement with the analyses of *Arabidopsis* and rice laccases (Turlapati *et al.*, 2011; Liu *et al.*, 2017), all of the *Miscanthus* laccases I cloned located to the secretory pathway. These laccases shared high structural similarity (Fig 3.1-6), but also exhibited distinct differences including isoelectric point (Table 3.1-2). The diversity of laccase isoforms leads to the multiple functions of this unique enzyme in plants. In *Arabidopsis*, laccases are involved in lignin biosynthesis (Berthet *et al.*, 2011), pigmentation (Turlapati *et al.*, 2011), and cell elongation (Littoz & McClements, 2008). The overexpression of a rice laccase (OsCHI1) in Arabidopsis increases tolerance to drought and salinity stress (Cho *et al.*, 2014). Plant laccases are also known to be involved in flavonoid oxidation (Pourcel *et al.*, 2007), which is important for plant growth and defense.

Because of genetic redundancy among laccase isoforms in the multigene family, functional characterization of specific plant laccases can be a challenge. Although *Miscanthus* has fewer subfamilies when compared to *Arabidopsis*, the majority of the putative laccases can be classified into subgroup 1 and are phylogenetically closely related to AtLAC17, which is involved in lignification (Fig 3.1-3) (Berthet *et al.*, 2011). These results indicate that the described *Miscanthus* laccases could also be involved in lignin metabolism, which is further supported by transcriptome analysis data (Hu *et al.*, 2017).

4.2 Histochemical staining of laccases connects laccase activity with lignification in *Miscanthus*.

ABTS, which was used as a staining substrate to localize laccase, results in a bright blue staining under microscope. Previously, a histochemical study in *Rhus verniciflua* stokes using *p*-dihydroxybenzene, which can be oxidized to a dark brown product by

laccases, identified the latex canals as the localization of laccases (Li, 1989). Another study using 3,3'-diaminobenzidine revealed orange areas indicating laccases activity in both secondary xylem and bast fibers in cross sections of hemp hypocotyl (Behr *et al.*, 2018). Consistent with these findings, *Miscanthus* laccase activity was also detected in fiber and xylem region (Fig 3.1-1). Along with the maturation of *Miscanthus* stem, stronger laccase activity as well as higher degrees of lignification was found in the cell walls of the basal internodes (Fig 3.2-2). The highest lignin content has been reported at the basal internode of *Miscanthus* (Huyen *et al.*, 2010), thus further supporting the likely correlation between laccase activity and lignification.

However, laccase activity has not been detected in either the non-lignified ground tissue cells or the strongly lignified vessel cells. The absence of laccase activity in those tissues might due to the lack of activate lignification. Developmental analysis of the *Miscanthus* stem revealed more pronounced cell thickening in the basal internode, indicating expansion of the thickening fibers outside the xylem vessel (Hu *et al.*, 2017). Visualizing the existence of laccase in lignifying cells, this finding provides the first direct evidence for the function of *Miscanthus* laccases in lignification *in vivo*.

4.3 Co-expression profiles suggest diverse function of *Miscanthus* laccases.

Co-expression analysis is an efficient tool to predict the function of enzymes, as genes involved in same metabolic pathways tend to be transcriptionally coordinated (Le Roy *et al.*, 2017). In *Arabidopsis*, this strategy has been applied to assign functions to laccase genes (Turlapati *et al.*, 2011). Further, to study the function of genes involved in cell wall formation, co-expression analysis appears to be conserved among different plant species, including monocots (Rai *et al.*, 2016; Hu *et al.*, 2017; Liu *et al.*, 2017). Transcriptional coordination of SCW-related genes revealed that *Miscanthus* laccase genes were also included in this co-expressed list (Hu *et al.*, 2017).

On the developmental level, most laccases are expressed highest in roots (Fig3.2-3), especially in the early stage of growth. In *Arabidopsis*, the expression profiles of all laccase isoforms were studied (Turlapati *et al.*, 2011), and 14 out of 17 laccases were strongly expressed in roots by 3-5 weeks growth, with only 11 isoforms expressed after 8 weeks. In contrast, no more than 9 isoforms were expressed in the stem in all the growth stages. In addition, GUS staining revealed activation of most laccase promoters in roots of germinating seeds or seedlings. It has been proposed that laccases are involved in resistance to environmental stresses in growing plants. In maize, the expression of ZmLAC1 is highly enhanced under high salinity in the primary root (Liang *et al.*, 2006) and the expression level of rice laccase OsCHI1 in root is highly induced during chilling, drought and salinity stresses (Cho *et al.*, 2014). Furthermore, by overexpressing OsCHI1 in *Arabidopsis*, root length increased significantly under dehydration and high-salinity treatments (Cho *et al.*, 2014). The tolerance of environmental stresses is attributed to enhanced lignification in root (Kidwai *et al.*, 2019), strongly connected the function of laccase in stress resistance to its role in lignification.

Analyses on stem and leaf gradients conducted in this study led to a better understanding of *Miscanthus* laccases. The expression patterns of *MsLAC1*, *MsLAC2,* and *MsLAC5* correlate strongly with the patterns of monolignol biosynthesis genes as well as SCW related TFs (Fig 3.2-4). All of the before-mentioned genes are highly expressed in elongating organs of the entire plant, including the two youngest internodes as well as the leaf sheath, where lignification of interfascicular fibers is ongoing. Based on transcriptome information of internodes in *M. lutarioriparius*, co-expression analysis using TFs and SCW biosynthesis-related genes shows a direct connection and reveals a tight network of all lignin-related genes (Hu *et al.*, 2017). Phylogenetic analysis suggests that MsLAC1 and MsLAC2 are closely related to AtLAC17, and can be clustered into the same subgroup with other lignin-related laccases from *Arabidopsis, Sorghum* and *Brachypodium* (Fig 3.1-4). These lignin-related laccases are also co-expressed with genes involved in the

phenylpropanoid pathway. Chemical analysis reveals that SofLAC complements the lignin content in the *Arabidopsis AtLAC17* mutant (Cesarino *et al.*, 2013), while the *Brachypodium BdLAC5* mutant shows a 10% reduction of Klason lignin (Wang, Y *et al.*, 2015). Based on these results, it is reasonable to speculate that at least MsLAC1 and MsLAC2 are enzymes responsible for lignin polymerization in *Miscanthus*.

The highest expression of MsLAC1, 2 and 5 has been detected in the top internode of the *Miscanthus* stem, however, MsLAC3 and 4 are strongly expressed in the basal part of the plant. Similarly, in *Arabidopsis*, a number of laccases are differentially expressed in base of the stem and the mid-stem (Turlapati *et al.*, 2011). Interestingly, AtLAC3, 7, 10 and 12 show higher expression levels at the base stem, but only AtLAC10 could complement the *lac4-2 lac17* double mutant phenotype (Chaibang, 2014). The diverse expression patterns of laccases indicate a redundant function of laccases in *Miscanthus*.

4.4 Regulation of *Miscanthus* laccases is part of a complex network.

In the *Miscanthus* stem/leaf sections expression profile, a coordinated expression between genes of enzymes and transcription factors, including MYB and NAC factors, was found (Fig 3.2-4). In plants, the expression of genes related to secondary cell wall formation is regulated by the NAC-MYB-based transcriptional network (Zhong & Ye, 2014; Nakano *et al.*, 2015). In *Miscanthus*, *MsSND1*, *MsVND7*, *MsSCM2*, *3, 4* and *MsMYB52* were cloned as putative orthologues of SCW TFs in *Arabidopsis* (Golfier *et al.*, 2017). Transient expression of those TFs in *N. benthamiana* leaves lead to ectopic lignification with increased lignin content and altered lignin composition (unpublished data from Dr. Philippe Golfier), indicating a crucial role of those TFs in regulating lignin biosynthesis. It is highly likely that these TFs not only activate monolignol biosynthesis genes, but also genes responsible for lignin polymerization, thus accelerating lignin formation.

The results of dual luciferase assays indicate induction of *Miscanthus* laccase

promoters by some of the above-mentioned TFs (Fig 3.3-3). In *Arabidopsis*, genes encoding laccases can be direct targets of AtMYB58 and AtMYB63 (Zhou *et al.*, 2009). Furthermore, a DNA-protein binding assay indicates that the expression of laccases is regulated directly by AtMYB46/83 (Nakano *et al.*, 2015). In addition, the transcriptional activators AtMYB58 and AtMYB63 induce expression of the whole lignin biosynthetic pathway during the SCW formation, and further induce the lignin-related laccase gene (*AtLAC4*) (Zhou *et al.*, 2009). Consequently, *MsSCM3* and *MsSCM4*, being highly similar to *AtMYB46* and *AtMYB58/63*, respectively, efficiently induce all *Miscanthus* laccases promoters except MsLAC3 (Fig 3.3-3).

However, on the tissue level (Fig S2), all MYB factors are highly expressed both in root and stem while the NAC domain factor SND1 is mainly expressed in stem tissues. Moreover, although all laccase promoters can be significantly induced by MYB factors, the activation by MsSND1 and MsVND7 remains rather low. The differences between NAC and MYB factors indicate that either NAC factors cannot directly activate the promoter of *Miscanthus* laccase or the contribution from MYB factors is much higher. In *Arabidopsis*, *AtNST1*, *AtVND7*, and also their downstream target *AtMYB46*, can regulate the expression of *AtMYB58/63* (Zhou *et al.*, 2009). Meanwhile, downstream genes like *CesA4* and *CesA8* can be directly targeted by both NAC and MYB factors (Zhong *et al.*, 2010), while the effect of MYBs is much stronger (McCarthy *et al.*, 2009b; Zhong & Ye, 2011). With the current data, it is still hard to say whether the induction of MsSND1 on some of the laccase promoters is direct or indirect.

The overexpression of ZmMYB31 or ZmMYB42 in *Arabidopsis* largely decreased the lignin content of transgenic plants (Fornalé *et al.*, 2006). Additionally, MYB31 and MYB42 are conserved in at least three grasses, including maize, *Sorghum* and rice (Agarwal *et al.*, 2016), regulating further genes in lignin biosynthesis pathway. The function of these TFs appears to be conserved in *Miscanthus*, too, as MsMYB31 and MsMYB42 repress the transcription of phenylpropanoid genes (ongoing project

of colleague, Wan Zhang), as well as laccases responsible for lignin polymerization (Fig 3.3-4). Interestingly, lack of repression was observed for the promoters of MsLAC5 (MsMYB31 and MsMYB42) and MsLAC4 (MsMYB31), possibly as a result of redundancy within the laccase gene family. Further, I observed that MsMYB31 and MsMYB42 seem to compete with the transcriptional activator MsSCM4, either through direct competition for binding sites or protein-protein interaction. Both MsMYB31 and MsMYB42 counteracted the activation of laccase promoters by MsSCM4 (Fig 3.3-5). It happens that in tissue specific level, the occupancy of promoter by MYB31 or MYB42 didn't significantly affected the expression level of target genes, raising the possibility that strong repression can only be detected by competing with activators (Agarwal *et al.*, 2016b).

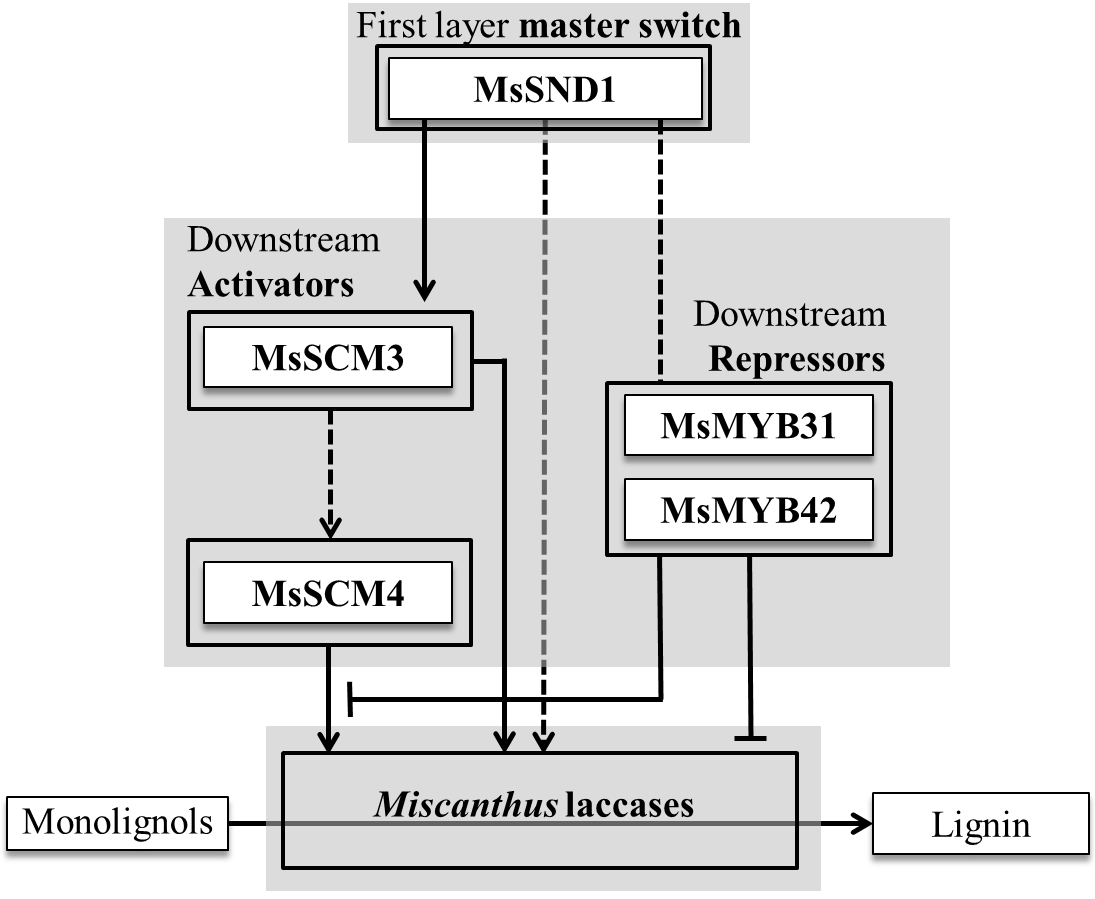

Fig 4.4-1 Transcriptional network regulating laccases involved in lignin biosynthesis in *Miscanthus*. As the master switch of the lignin biosynthesis pathway, MsSND1 induces the expression of downstream MYB factors and thus affected the expression of lignin related laccase genes.

In *Arabidopsis*, the transcriptional regulation of lignin biosynthesis is well studied (Section 1), pointing to a complex network of activators and repressors (Nakano *et al.*, 2015). A similar regulation cascade involving several layers of TFs is described in grasses (Rao & Dixon, 2018), indicating a functional conservation of transcriptional regulators amongst different plants. The AtSND1-orthologue in *Miscanthus*, MsSND1, can rescue the non-lignified phenotype of interfascicular fibers of the *Arabidopsis snd1/nst1* double mutant (Golfier *et al.*, 2017), while the *Arabidopsis myb46/83* double mutant can be complemented by MYB46/83 orthologues of rice, maize and switchgrass (Zhong *et al.*, 2011; Zhong *et al.*, 2015). Based on homologous TFs in *Miscanthus*, the experimental observations of NAC and MYB factors on laccase promoters suggest a comparable transcriptional regulatory network. In conclusion, a simplified scheme of this network controlling the expression of laccases involved in lignin polymerization is deduced (Fig 4.4-1).

4.5 *Miscanthus* laccases localize to cell walls, however, MsLAC3 and MsLAC5 were also detected in the intracellular.

All five laccases cloned from *Miscanthus* are predicted glycoproteins containing signal peptides (Table 3.1-2), and thus to be secreted into cell wall matrix. Histochemical studies on *Miscanthus* stem cross sections confirm the secretion of laccases *via* detection of enzymatic activity in the cell wall and intracellular spaces (Fig 3.1-1). Congruently, the majority of laccases from three cotton species are also predicted to be secretory proteins (Balasubramanian *et al.*, 2016). Immunolocalization additionally confirmed another laccase in the thick lignified cell walls of xylem and fibers of sycamore stem cross sections, and laccase activity was also detected in the culture medium of suspension-cultured cells (Driouich *et al.*, 1992). Based on this data, it is reasonable to assume that *Miscanthus* laccases have the potential to localize to the cell wall.

However, when comparing *Miscanthus* laccase isoforms in cell wall-associated and intracellular protein samples, a distinct difference was detected (Fig S10). It appears

that the isoenzyme associated with cell wall can also be found in cytosol. This is further supported by observations in *Arabidopsis*, where AtLAC15 (TT10) is a vacuole-localized laccase (Pang *et al.*, 2013), while AtLAC4 and AtLAC17 localize throughout the secondary cell wall layers (Yi Chou *et al.*, 2018). Moreover, transient expression of fluorescence-tagged MsLACs in tobacco leaves clearly shows the cell wall localization of *Miscanthus* laccases, while MsLAC3 and MsLAC5 were also found in cytosol (Fig 3.4-3).

The function of plant laccases is directly connected to their subcellular localization. Polymerization of monolignols occurs only in the cell wall matrix, thus cell wall-localized enzymes are required to obtain the necessary level of complexity (Vanholme *et al.*, 2012). In this case, MsLAC1 and MsLAC2 are presumably involved in lignin biosynthesis, however, the function of MsLAC3 and MsLAC5 cannot be inferred from their localization, though it seems likely that cytosol-localized laccases are involved in distinct biochemical reactions. Protein extracted from *Miscanthus* stems (but not root or leaf protein extraction) showed a strong ability to decolorize methyl green (Fig S11), which can be terminated by laccase inhibitor. The results suggested that at least one unique laccase might be expressed in stem but not in other tissues. In *Arabidopsis*, the laccase-like polyphenol oxidase TT10 is confined to the vacuole and thus possible involved in the polymerization of proanthocyanidins (Vanholme *et al.*, 2012; Zhang *et al.*, 2013), whereas the cell wall-associated AtLAC4 and AtLAC17 play an important role in fiber lignification (Berthet *et al.*, 2011; Yi Chou *et al.*, 2018). Taken together, it is highly likely that MsLAC1 and MsLAC2 fulfil the spatial distribution requirements for polymerization of lignin.

Interestingly, transient expression of MsLAC1 or MsLAC2 in tobacco leaves did not lead to ectopic lignification of the primary cell wall of epidermal cells, even after exogenous supply of coniferyl alcohol (Fig 3.4-1, Fig 3.4-2). By contrast, incubation of seedlings overexpressing *AtLAC4* or *AtLAC17* with fluorescence-tagged coniferyl alcohol displayed strong fluorescence in primary epidermal cell walls of the cotyledons (Schuetz *et al.*, 2014). A possible reason might be the temporal and spatial

differences of substrate supply that leads to on-site lignification, which can be marked by the exogenous supply of fluorescence-tagged monolignol. The expression of monolignol biosynthesis genes in leaf is much weaker than that in root and stem in the same developmental stage (Le Roy *et al.*, 2017), but the expression level is quite high in seedlings (Costa *et al.*, 2003). The lignin formation in the primary walls of maize coleoptiles starts at the onset of elongation growth, but is kept at a low level during the period of rapid cell extension (Müse *et al.*, 1997). A higher level of monolignol gene expression may supply sufficient substrates for polymerization in seedling, thus results in ectopic lignification.

4.6 Comparing plant and fungal laccases.

EST database analysis using three *Miscanthus* species revealed that genus *Miscanthus* generally harbors large laccase gene families, in total 95 candidate contigs were found (Fig 3.1-3). In comparison, *Miscanthus* and *Arabidopsis* laccases exhibit distinctly higher isoelectric points (pH 7.0 or higher) than fungal laccases (pH 3.0) (Heinzkill *et al.*, 1998; Baldrian, 2004). Moreover, although all *Miscanthus* laccase isoforms display a comparable 3-D structure to fungal laccases (Fig 3.1-6), differences are found at catalytic sites, indicating functional diversity (Dwivedi *et al.*, 2011).

The molecular weight of laccases in *Miscanthus* varies between family members (Fig 3.1-2). Further, most plant and fungal laccases are present as either dimeric or tetrameric glycoproteins (Dwivedi *et al.*, 2011). Although the calculated molecular weight based on amino acid sequence of both plant and fungal laccases is generally around 60 kDa, higher carbohydrate content in plants can result in larger apparent molecular weights. To be more specific, plant laccases commonly exhibit 22 - 45% glycosylation, while fungal laccases present only 10 - 25% carbohydrate content (Wang, Y *et al.*, 2015). An extreme example would be a laccase purified from papaya, with a molecular weight of around 260kDa (Jaiswal *et al.*, 2015). The MsLAC1 protein sequence has 15 predicted N-glycosylation sites, and accordingly, the recombinant, yeast-expressed rMsLAC1 shows a much larger molecular weight of

~150kDa based on SDS-PAGE. Further post-translational modifications like e.g. O-glycosylation may also contribute to the larger than predicted molecular weight, as it has been previously described for yeast systems (Bronikowski *et al.*, 2017).

rMsLAC1 shows a maximum enzyme activity at pH 3, which is consistent with values found in the literature (He *et al.*, 2015b). However, the optimal temperature of rMsLAC1 is rather low (32 ^{o}C) when compared to other plant and fungal laccases (60 ^{o}C) (Jaiswal *et al.*, 2015; He *et al.*, 2015a). Nevertheless, lower optimal temperatures have been reported previously. Accordingly, the optimum temperature for the purified laccase from the plant *Blumea malcolmii* is 30^{o}C (Kagalkar *et al.*, 2015). Further, laccases from the fungus *Ganoderma lucidum* exhibit optimal temperatures as low as 25^{o}C (Ko *et al.*, 2001). Considering the large gene family of *Miscanthus* laccases, more in-depth studies on the other isoforms are required to reach a better understanding of their catalytic properties.

Both plant and fungal laccases are able to oxidize monolignols, revealing a broad substrate range. Phenoxy radicals are produced by laccases after oxidation of lignin phenolic groups, and then polymerized into lignin (Madhavi & Lele, 2009). However, the efficiency of oxidizing different monolignols by individual laccase varies a lot. rMsLAC1 shows the highest affinity to sinapyl alcohol, and similarly, two laccases isolated from *Pinus* exhibit higher activities when using sinapyl alcohol (Sato & Whetten, 2006). In contrast, a Sycamore Maple laccase displays higher oxidation efficiency when coniferyl alcohol is used as substrate (Sterjiades *et al.*, 1992). According to (Barceló *et al.*, 2004), the methoxyl substitution in monolignol may facilitate the coupling of radicals, thus sinapyl and coniferyl residues are easier to be oxidized and polymerized (Koutaniemi *et al.*, 2015).

To our knowledge, this is the first report of heterologous expression of a laccase from grasses with sufficient enzyme activity for characterization, thus contributing greatly to the understanding of their mode of function. The participation of MsLAC1 in lignin biosynthesis positions this particular enzyme as a potential target for genetic

engineering of lignin in the energy crop *Miscanthus* to improve biomass properties for enhanced applications.

4.7 What is the basis for complemention of the lac4-2/lac17 double mutant?

Evidence for expression, regulation, localization, and even *in vitro* activity of *Miscanthus* laccases strongly links these enzymes to lignin biosynthesis. Transgenic approaches including complementation of mutants and ectopic overexpression can further provide direct evidence to evaluate the function of these enzymes.

The *Arabidopsis lac4-2/lac17* double mutant exhibits a semi-dwarf phenotype and irregular xylem cells with divergent interfascicular fibers when grown under continuous light (Berthet *et al.*, 2011). In my study, after complementation with *MsLAC1* or *MsLAC2* under the control of the native *AtLAC17* promoter, the double mutant restored the normal size under continuous light, and the shape of xylem cells became regular. However, the phenotype of the *lac4-2 lac17* double mutant under constant light was not complemented by *MsLAC3* and *MsLAC5*, resulting in unchanged suppression of growth (Fig 3.6-2, Fig 3.6-3). Screened by Mäule staining, the complementation of *lac4-2 lac17* double mutant phenotype by all the *Arabidopsis* laccases except AtLAC16 was evaluated (Chaibang, 2014). To be more specific, AtLAC4, 10, 11 and 17 transgenes could fully complement the xylem and fibers, AtLAC3 and 15 as well as two fungal laccases showed no influence to the phenotype of double mutant, while other tested laccases could only partially substitute the function of AtLAC4 and 17. To understand the mechanisms underlying the observed phenotypes, more detailed metabolic analysis is required.

The stems of *lac4-2 lac17* double mutants exhibit lower lignin content concurrent with more phenolic compounds, further, the altered lignin contains higher amounts of G subunits (Berthet *et al.*, 2011). The *Arabidopsis* single mutants of either *lac4* or *lac17* exhibit decreased lignin content, and additionally, an increased S/G ratio was detected in the *lac17* mutant (Zhao *et al.*, 2013). Surprisingly, by expressing four *Miscanthus* laccases under the control of AtLAC17 promoter, lignin content in all the

transgenes increased (Fig 3.6-4), but only MsLAC1 and MsLAC2 can restore the deposition of monolignols (Table 3.6-1). Complementation of *lac17* with AtLAC17 totally reconstitutes the lignin profile to wildtype levels (Berthet *et al.*, 2011). However, expression of *SofLAC* under the control of the *AtLAC17* promoter in *lac17* mutant restores the lignin content but not the lignin composition (Cesarino *et al.*, 2013). Unfortunately, no metabolic data regarding to the non-complementation lines was presented in previous studies about mutant complementation (Chaibang, 2014). Laccase isoforms in *Miscanthus* seem to share the common ability for lignin polymerization when the laccases were expressed under the native AtLAC17 promoter. More relevant detail about the role of laccases in lignification will be discussed in the following section. To connect the metabolic data with the phenotype of *lac4-2/lac17* double mutant, possible reasons for complementation of the phenotype are discussed below.

4.7.1 The light stress-induced semi-dwarf phenotype.

Experiments have shown that the phenylpropanoid pathway is closely related to light stress and the transcript abundance of many phenylpropanoid genes are induced by light stress. In *Arabidopsis*, the expression of monolignol biosynthesis genes involving C4H1, COMT, CCoAOMT1, CCR1 and CAD6 exhibited circadian oscillations (Rogers *et al.*, 2005). After 48 h of continuous light illumination, most of monolignol biosynthesis genes were significantly upregulated in flax (Le Roy *et al.*, 2017). Consequently, within 2 weeks of light exposure, the concentration of monolignol and flavonoid glycosides in *Arabidopsis* roots increased 100-400 folds (Hemm *et al.*, 2004). Furthermore, the accumulation of monolignols always leads to higher lignin content in the plants. The lignin content in soybean hypocotyls grown 3 days with constant light increased 3 folds compared with that of dark treatment (Su *et al.*, 2005). The lignin formation reduced the extensibility of cell wall, and inhibited the growth of plant (Müse *et al.*, 1997).

It was hypothesized that the reduced lignin amount in the *lac4-2 lac17* double mutant

might lead to the repressed elongation of inflorescence stem. Indeed, the severe reduction of lignin content caused by mutation of monolignol biosynthesis genes (Voelker *et al.*, 2010; Thévenin *et al.*, 2011) or *lac4-2 lac11 lac17* triple mutant could result in dwarfism in the mutants. However, the *lac4-2 lac17* plants grown without light stress exhibited a similar size to that of WT plants (Berthet *et al.*, 2011). Nevertheless, mutations of *BdLAC5* and *BdLAC6* in *Brachypodium* displayed reduced lignin content, but did not show significant alteration of growth and development (Wang, Y *et al.*, 2015). In this case, the semidwarf phenotype is more likely to be related to the stress-inducing conditions.

The mutation of lignin biosynthesis genes always lead to redirection of metabolic flux in phenylpropanoid pathway. The soluble phenolic pools in the *AtLAC17*-deficient mutants were affected by redirecting the flux for sinapoyl malate biosynthesis (Berthet *et al.*, 2011). Silencing of HCT in *Aeabidopsis* also leads to repression of lignin biosynthesis and redirection of the metabolic flux into flavonoids (Besseau *et al.*, 2007). Consequently, the accumulation of flavonoids negatively affected the plant growth, which can be restored by chalcone synthesis (CHS) repression (Besseau *et al.*, 2007). The concentration of monolignol and flavonoid glycosides increased dramatically with light exposure (Hemm *et al.*, 2004), resulted in inhibition of auxin transport and repressed growth of plant. Since the function of some laccases in flavonoid polymerization is confirmed (Pourcel *et al.*, 2005), it is possible that the deficiency of *AtLAC4* and *AtLAC17* will also lead to flavonoid accumulation. High level of sinapoyl malate was accumulated in *lac4-2 lac17* mutant (Berthet *et al.*, 2011), but whether accumulation of those phenol compounds will also lead to suppression of plant growth is still unclear. According to the proposed schematic pathway (Fig 4.7-1), the accumulation of both flavonoids and monolignols under continuous light condition may be the true reason for the repression of plant growth, owing to the lack of laccases for polymerization and oxidation.

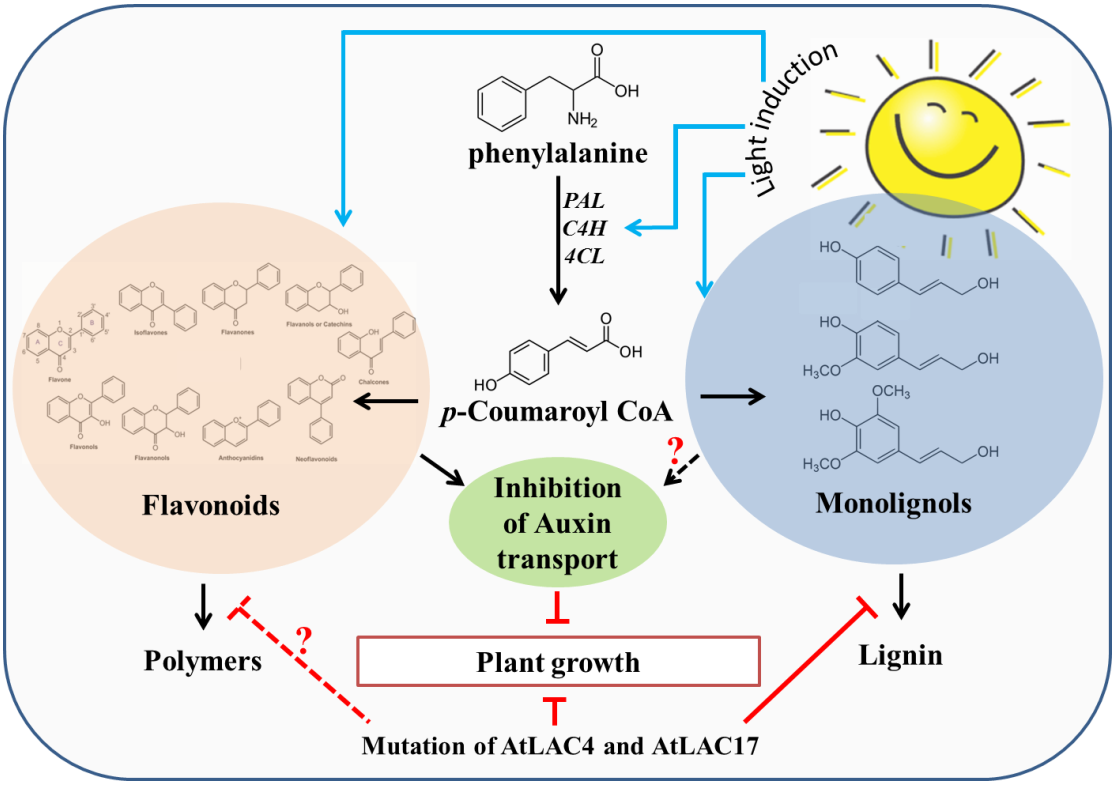

Fig 4.7-1 Schematic illustration of possible mechanisms for the semi-dwarf phenotype of lac4-2 lac7 double mutant grown under continuous light. Blue arrows indicate induction of gene expression and increased synthesis of chemicals. Red lines stands for the function of inhibition or downregulation.

4.7.2 The collapsed xylem and less lignification in fibers.

MsLAC1 and MsLAC2 are capable of restore a normal type of xylem cells, and also restore a normal level of lignification in fibers, when expressed under control of AtLAC17 promoter in *lac4-2 lac17* mutant (Fig 3.6-3). Only middle lamella in fiber cells is still lignified in the double mutant as shown in (Chaibang, 2014). GUS staining of the stem cross sections of *Arabidopsis* line expressing *proAtLAC17::GUS* indicates the tissue-specific localization of *AtLAC17* in the interfascicular fibers but not in vascular bundles (Berthet *et al.*, 2011; Turlapati *et al.*, 2011), which means that *Miscanthus* laccases can be specifically expressed in fibers of the transgenes. In *Arabidopsis*, peroxidases and laccases showed distinct subcellular localization in cell wall. AtPRX64 is only detected in middle lamella and cell corners while AtLAC4 is detected in secondary walls but not middle lamella or cell corners (Yi Chou *et al.*,

2018). It is quite clear that the promoter of AtLAC17 ensured the proper localization of MsLAC1 or MsLAC2, thus the fiber secondary cell wall was lignified and phenotype been restored.

What's more, overexpressing MsLAC1 in the ecotype *Arabidopsis* plant even leads to earlier lignification in interfascicular fibers and stronger lignification in mature plants (Fig 3.7-4). It is clear that genes involved in monolignol biosynthesis pathway will affect the lignin content and composition in *Arabidopsis* (Van Acker *et al.*, 2013). We noticed that there is strong co-expression profile between *MsLAC1*, *MsLAC2* and several genes involved in the phenylpropanoid pathway (Fig 3.2-4). Based on feedback regulation models of biosynthesis and enzyme activity *via* metabolites, which can also affect the expression of the corresponding genes (van der Knaap & Verrijzer, 2016), with the knowledge that disruption of *LAC4* and/or *LAC17* downregulated several monolignol biosynthesis genes (Berthet *et al.*, 2011), the accelerated consumption of monomers after overexpression of *MsLAC1* may lead to increased biosynthesis of monolignol substrates. Preliminary qPCR results suggest that most monolignol biosynthesis genes display a higher expression level in inflorescence stems of *p35S:MsLAC1* plants (Fig 3.7-7) but some key flavonoid biosynthesis genes were downregulated (Fig 3.7-8). The re-direction of phenylpropanoid pathway might results in a higher level of monolignols biosynthesis, in turn promoted the lignin accumulation in cell wall.

Although the collapsed and irregular xylem cells were observed in the double mutant, the lignin content seems not changed (Fig 3.6-3). Down-regulation of laccases in poplar showed no quantitative differences in lignin, but xylem possessed a highly irregular cell contour as compared with controls (Ranocha *et al.*, 2002). Instead of lignin content, the development of normal lignin structure is more likely to cause the collapsed xylem phenotype (Turner & Somerville, 1997). The deficient of *AtLAC4* and *AtLAC17* altered the phenolic metabolism, and even further increased the release of structural sugars (Fig 3.6-5), indicating alterations of xylem and cellulose deposition in cell wall, which decreased the resistant of cell wall to compressive

forces and formed the irregular cells (Turner & Somerville, 1997; Hao & Mohnen, 2014).

4.8 Unraveling the function of laccases in lignification.

The involvement of laccases in lignin polymerization has been studied for the last decade (Sterjiades *et al.*, 1992; Bao *et al.*, 1993). Plant laccases are able to not only oxidize lignin precursors but also polymerize monolignols *in vitro* (Berthet *et al.*, 2011; Zhao *et al.*, 2013). In *Arabidopsis*, disruption of a single laccase leads to a moderate reduction of lignin content, while the *lac4-2 lac17* double mutant contains up to 40% less lignin than the control (Berthet *et al.*, 2011). Moreover, lignin deposition in roots is almost completely abolished in the *lac11 lac4 lac17* triple mutant (Zhao *et al.*, 2013). These findings indicate that the lignification in *Arabidopsis* has to be controlled by several laccases rather than just one. Further, G lignin deposition is affected by AtLAC17, as *lac17*-deficient lines contain less G lignin, which cannot be restored by the sugarcane homolog SofLAC, even though lignin content is recovered completely (Cesarino *et al.*, 2013). Based on the most recent publications, it is assumed that laccases in *Arabidopsis* can influence both lignin composition and content. However, since the laccase gene family is rather large, it still needs to be clarified whether there is redundancy or whether individual laccases have certain functions in specific metabolic pathways.

In our study, laccases from *Miscanthus* show similar functions in lignification but exhibit differences in the distribution of monolignols. MsLAC1, 2, 3 and 5 can be categorized into different subgroups (Fig 3.1-3), indicating that those enzymes may have different functions *in vivo*. Surprisingly, by expressing these laccases under the control of *AtLAC17* promoter, lignin content in the lignin-deficient plants were restored or partially restored (Fig 3.6-4), however, only MsLAC1 and MsLAC2 were able to restore the composition of the *lac4-2 lac17* double mutant completely (Table 3.6-1). Overexpression of the *Miscanthus* laccases in *Arabidopsis* leads to increased lignin levels when plants are grown under long-day conditions (Fig 3.7-5), although

different S/G ratios indicate changed proportions of the involved monolignols (Table 3.7-1). From these results, it becomes apparent that the function of *Miscanthus* laccases in lignification is conserved as long as they are expressed in the correct tissue, although the specific function of each laccase in lignin composition needs to be studied in more detail.

4.8.1 *Miscanthus* laccases redundant in lignin polymerization but lignification likely limited by substrate supply.

During my research, I was able to successfully express and characterize one laccase (MsLAC1), and express all of the other studied laccase isoforms under control of *AtLAC17* promoter. The obtained results confirm that the increased lignin content in the transgenic lines results from complementation (Fig 3.6-4). Similarly to the successful complementation of the *lac4-2 lac17* double mutant using *AtLAC17* (Berthet *et al.*, 2011) and *SofLAC* (Cesarino *et al.*, 2013), a laccase from *B. distachyon*, *BdLac5,* can restore the lignin content in the Bd4442 mutant (Wang, Y *et al.*, 2015), indicating the involvement of those laccases in lignin polymerization. However, all laccases used in the presented studies can be categorized into subgroup 1 (Fig 3.1-3). To our knowledge, my research is the first report showing that laccases from all subfamilies can equally contribute to the complementation of lignin content in the *lac4-2 lac17* double mutant. Moreover, it is the proportion of insoluble lignin that contributes to the increased lignin content (Fig 3.6-4). Since the ASL mainly originates from hemicellulose and syringyl lignin (Yasuda *et al.*, 2001), the unchanged ASL content in all the lines in this study indicates that the S lignin reservoir may not be affected.

Notably, all generated complementation lines (except *pAtLAC17:MsLAC5-2*) show close or less lignin content when compared to WT, indicating a limiting factor in lignin polymerization. Involvement of AtLAC4 and AtLAC17 in lignin polymerization has been confirmed (Berthet *et al.*, 2011), and their overexpression in *Arabidopsis* is sufficient to lignify the primary cell wall in cotyledon epidermal cells

with exogenously supplied monolignols (Schuetz *et al.*, 2014). These findings indicate that total lignin content can be altered as a result of laccase overexpression as long as monolignols are supplied sufficiently. Our results show that only the overexpression of *MsLAC1* increases lignin content under both long day (36.4%) and continues light (10.6%) conditions (Fig 3.7-5). Although it was not reported if lignin content in transgenic yellow poplar overexpressing a laccase gene had been changed (Dean *et al.*, 1998), a 2.1% to 19.6% increase in total lignin content in stems was found in transgenic poplar plants overexpressing a cotton laccase (*GaLAC1*) (Wang, J *et al.*, 2008c). Similarly to our results, overexpression of two *Pyrus bretschneideri* laccase genes (*PbLAC1* and *PbLAC14*) in *Arabidopsis*, only *PbLAC1* significantly increased the lignin content (approximately 17%) (Cheng *et al.*, 2019).

Continued lignification of cell walls requires new hydrogen-abstraction of monomers as well as the growing lignin oligomer following each coupling reaction (Vanholme *et al.*, 2012). Previous research has shown that metabolites can regulate the expression of enzymatic genes, enzyme biosynthesis and activity *via* feedback loops (van der Knaap & Verrijzer, 2016). Therefore, we hypothesized that laccases could induce biosynthesis of monolignol substrates by induction of related genes. Preliminary results suggest higher expression levels of most monolignol biosynthesis genes in the middle part of young inflorescence stems of *MsLAC1* overexpression lines (*Arabidopsis*) (Fig 3.7-7). However, to further elucidate the function of the involved laccases, more data is required and the analysis of phenolic compounds is of primary interest for our further studies.

4.8.2 *Miscanthus* laccases display defined roles in the distribution of monolignols in transgenic *Arabidopsis* stems.

The analysis of the S/G ratio in different transgenic lines highlights the potential of *Miscanthus* laccases to modify lignin composition (Table 3.6-1, Table 3.7-1). It has been suggested that AtLAC17 preferentially metabolizes G-lignin units during lignin polymerization (Cesarino *et al.*, 2013). Accordingly, the *Miscanthus* homolog

MsLAC1 reduces the S/G ratio from 0.78-0.82 in *lac4-2/lac17* mutants to 0.57-0.62 in complementation lines, and even to approx. 0.4 in *MsLAC1*-overexpression lines. One reasonable explanation is that laccase isoforms have distinct specificities amongst the different monomers. Our initial hypothesis that MsLAC1 can selectively oxidize coniferyl alcohol instead of syringyl alcohol was not further supported after the characterization of rMsLAC1 (Section 3.5.5). Nonetheless, the analysis of a *lac4-2/17/fah1-2/C4H-F5H* quadruple hybrid stated that absence of G-subunits does not lead to recovery of the lignin content to wild type levels, indicating that AtLAC4 and AtLAC17 do not specifically metabolize S- or G-subunits (Chaibang, 2014). Still, further studies are needed to understand the underlying mechanism of changes in H/G/S subunit composition.

The incorporation of each monomer and the lignification of cell walls are programmed both temporally and spatially (Verma & Dwivedi, 2014). In particular, the distribution of H, S and G units in different tissues and cell types, and between primary and secondary cell walls is not constant (Meyer *et al.*, 1998; Grabber *et al.*, 2004; Mccann & Carpita, 2008; Grabber *et al.*, 2010). The silencing of *AtLAC4* or *AtLAC17* largely increases the S/G ratio in fibers, while no significant change can be detected in vascular bundles (Berthet *et al.*, 2011). Accordingly, lignin staining of *p35S:MsLAC1* stem cross sections shows increased lignification in the fibers with additional layers of lignified cells (Fig 3.7-4), but the change cannot be detected in other overexpression lines. In *Miscanthus*, *MsLAC1* shows highest expression in stems in different growth phases and in young tissues of mature plants in which fibers are still developing, which is not consistent with other laccases (Fig 3.2-3, Fig 3.2-4). Additionally, *Miscanthus* laccases show pronounced subcellular localization when transiently expressed in tobacco leaves (Fig 3.4-3). The distribution of laccases in different tissues and localization within the cell can largely influence the catalysis, thus contributing to differences in the composition of lignin in different transgenic lines.

4.8.3 Unique functions of *Miscanthus* laccases in lignification.

In the generated *MsLAC1* transgenic plants, yield analysis after thioacidolysis shows that the amounts of H-units and S-units are not significantly affected, whereas G-lignin positively correlates with an increase of *MsLAC1* expression (Table 3.7-1, Table 3.7-2). Considering that *Miscanthus* has similar levels of S- and G-units in cell walls to *Arabidopsis* (Lygin *et al.*, 2011), MsLAC1 may also be the key enzyme responsible for the high proportion of G lignin in *Miscanthus*. Furthermore, lignin in monocots contains higher levels of H units (Vanholme *et al.*, 2010), and expression of *MsLAC5* in the *Arabidopsis lac4-2 lac17* double mutant increases its content 2-7 fold compared either to the mutant or the WT plants (Table 3.6-2), indicating a possible function of this enzyme in the distribution of H units in lignin. However, overexpression of MsLAC5 in WT *Arabidopsis* did not change levels of H lignin significantly (Table 3.7-2), possibly suggesting feedback loop regulation of the metabolites.

Since lignin thioacidolysis is based on the cleavage of β-O-4 bonds that predominate in secondary cell walls (Russell *et al.*, 1996), the increased relative yield observed for the *35S:MsLAC1* lines (Table 3.7-2) suggests that more coniferyl and sinapyl residues were incorporated into lignin, resulting in less condensed lignin. Compared with lignin in dicot plants, grass lignin contains more condensed bonds (i.e., C–C linkages) and has a higher content of phenolic hydroxyl (Verma & Dwivedi, 2014). In contrast, our results indicate that laccases may have less influence on the coupling of monolignols compared to the chemical properties of monolignols, the speed of delivery to the cell wall and the oxidative environment in the wall (Vanholme *et al.*, 2008; Parijs *et al.*, 2010).

4.9 Outlook

In this work, I tried to explore the function of some *Miscanthus* laccases in lignification, which provides promising breeding targets to improve the biomass

quality and quantity of *Miscanthus* for biofuel and biomaterial applications. The catalytic ability of MsLAC1 on oxidizing monolignols is confirmed, and the enzyme also plays important role in lignin biosynthesis *in vivo* when expressed in *Arabidopsis*. However, the direct evidence linking laccases and lignification in *Miscanthus* is still missing. Previous trials for *Miscanthus* transgenic operations were unsuccessful due to problems of plant regeneration from callus.

All the *Miscanthus* laccases were shown to be able to increase the lignin content in *lac4-2 lac17* double mutant, when expressed under control of AtLAC17 promoter. But only MsLAC1 and MsLAC2 can complement the phenotypes, more specifically, restored the irregular xylem cells and promoted the plant growth under continuous light. These results negate the direct connection between lignin content and plant growth, and thus urge further studies to identify the essential causes of semi-dwarf phenotype under constant light.

Furthermore, *Miscanthus* laccase isoforms may play an important role in the distribution of monolignols in lignin. MsLAC1 complemented the S/G ration in *lac4-2 lac17* and further increased the G-lignin amount after ectopic expression in *Col-0*. Expressing MsLAC5 in the double mutant surprisingly increased the H-lignin amount for almost 5 folds, indicating a possible role of this laccases in coupling of H-lignin. Our findings encourage further works to elucidate the regulatory pathway that altered the lignin composition in those transgenic plants. Analysis of those plants in metabolic and transcriptome level may facilitate the understanding the multiple functions of laccase isoforms.

5. Materials and methods

5.1 *In silico* analysis of *Miscanthus* laccases

5.1.1 Analysis of putative laccase sequences in *Miscanthus* transcriptome.

Based on a published *Miscanthus* EST database from a variety of species, including *sinensis*, *sacchariflorus* and *giganteus* (Barling *et al.*, 2013), we performed a local tBLASTn search to find putative laccase sequences. Laccase sequences from *Arabidopsis thaliana* (17 isoforms, http://www.arabidopsis.org) and two C4 plants including *Sorghum bicolor* (25 isoforms, http://www.phytozome.net /sorghum) and *Brachypodium distachyon* (29 isoforms, http://pgsb.helmholtz-muenchen.de/plant/ brachypodium) were used as queries. Contigs were re-blasted to obtain as complete sequences as possible. The integrity of sequences found was further analyzed using the online translate tool (https://web.expasy.org/translate/).

5.1.2 Bioinformatics study of *Miscanthus* laccase sequences.

To categorize Miscanthus laccases, all the contigs with complete coding sequence were aligned with Arabidopsis laccases. To evaluate the relationship between selected *Miscanthus* laccases and lignin-related laccases in other plants, I also did the phylogenetic analysis and multiple alignment with BdLAC5 from *Brachypodium distachyon* (Wang, Y *et al.*, 2015), SofLAC from Sugarcane (Cesarino *et al.*, 2013), PtLAC3 from poplar (Bryan *et al.*, 2016) and AtLAC4/AtLAC17 from *Arabidopsis* (Berthet *et al.*, 2011). All the alignments were performed with MEGA 5, and the Neighbor-joining method was used for phylogenetic analysis, with 1000 replicates bootstrap tests. Figures of phylogenetic trees were then outputted with Evolview (He *et al.*, 2016).

Focusing on five cloned laccase sequences from *Miscanthus*, I then studied the basic properties with various online tools. To be more specific, the N-glycosylation sites

and signal peptides were predicted with Prediction Servers (http://www.cbs.dtu.dk/services/) and expected isoelectric points were calculated on http://web.expasy.org/compute_pi. In order to understand the tertiary structure of those laccases, homology modeling was conducted against the model of one *Rigidoporus lignosus* laccase (1v10.1.A) (Garavaglia *et al.*, 2004b)(Garavaglia *et al.*, 2004b) with SWISSMODEL (https://swissmodel.expasy.org/). Afterwards, the similarities and identities of the cloned laccases and template contigs were analyzed (http://imed.med.ucm.es/Tools/sias.html).

5.1.3 Identification and analysis of *Miscanthus* laccase promoters.

Since the information on the promoter upstream of CDS is lacking in the transcriptome database, we generated a partial genomic DNA database from *M. sinenses var.Sin-13* cooperating with the Deep-Sequencing-Core Facility on Heidelberg Campus. Around 200bp of the 5'-terminal laccase sequences were used as queries to identify promoter sequences from the library via BLAST search. I then did an in-depth computational analysis of the transcription binding sites on those laccase promoters using MatInspector software (www.genomatix.de), with the maximum matrix/core similarity (1.0).

5.2 Plant materials

5.2.1 *Miscanthus*.

Seeds of *Miscanthus sinensis* (identification number: Sin-13) collected in Honshu, Japan (Clifton-Brown & Lewandowski, 2002) were used for all the experiments. *Miscanthus* seeds were soaked in 75% ethanol (v/v) with 1 min shaking for surface sterilization, in 1.5ml Eppendorf tube. Then Buffer C (25% sodium hypochlorite, 0.1% Tween-20, v/v) was used to replace the ethanol, and seeds were shaken for 30 min before washing with 1ml sterilized H_2O for three times. No more than 30 seeds should be used in one batch. Then the seed were sprinkled onto the soil and grown in

glasshouse at 25 ^{o}C under 8/16-hour light period until harvest. Different tissues including root, stem and leaf from the whole plant were collected at different growth times, more specifically, 10 days, 1 month, 2 month and 3 month after germination. Of mature plants (~6 month old), 7 internodes and 5 leaf gradients were collected as shown in Fig. 3.2-2. All samples were kept in 2 ml Eppendorf, immediately frozen in liquid nitrogen, and directly used for protein/mRNA extraction.

5.2.2 *Arabidopsis.*

Arabidposis thaliana ecotypes Col-0 and the lac4-2/lac17 double mutant (kindly offered by Dr. Richard Sibout, IJPB) were used in my project. No more than 20 mg seeds were sterilized in 1.5 ml Eppendorf tube with 1 ml Na/EtOH solution (10% Sodium hypochlorite in 70% ethanol, v/v), 2 min with shaking. Seeds were then washed with 100% ethanol twice and dried overnight under a sterile bench. The dried seeds were then placed onto germination plates (1/2 MS agar media supplemented with 20g/L sucrose) and kept in dark for vernalization at 4 ^{o}C. 1L MS agar media (Murashige & Skoog) contains 2.2 g Murashige & Skoog with vitamins (Duchefa), 10g Sucrose (Roth) and 8g Agar (Duchefa). The pH need to be adjusted to 5.7 with KOH. After seeding, plants were grown under long-day condition (16/8-hour light period, 21^{o}C-25^{o}C) in the glass house, or continuous light condition in a growth chamber (21^{o}C, 60% relative humidity). *Arabidopsis* plants including wildtype and mutant were stably transformed by the floral dip method as described (Clough & Bent, 1998). The dipping operation was repeated after one week to raise the efficiency. Homozygous T3 plants were selected by segregation ratio after BASTA spray.

5.2.3 *N. benthamiana*.

Nicotiana benthamiana wildtype plants were used for transient expression of laccases. Different *A. tumefaciens* cells were cultured firstly for 2 days in LB medium and then 1 mL suspension was inoculated in 30 mL fresh medium overnight at 28^{o}C, 180 rpm. The cells were harvested by centrifugation (3,000 *g*, 10 min) afterwards. Before

infiltration, Agro Buffer was freshly prepared, containing 10mM $MgCl_2$, 10mM MES, 150μM Acetosyringone (solved in DMF), and adjusted to pH 5.6 with KOH. 4-week-old plants growing in glass house (16/8-hour light period) were transformed with *A. tumefaciens* 'cocktail' re-suspended in Agro buffer. To be more specific, an OD_{600} of 0.1 and 0.4 for P14 cells and Agrobacteria containing fluorescence-tagged constructs, respectively, were mixed in the buffer and incubated for at least 2 h at RT. The transformed plants were grown under the same condition for another 2-3 days before the infiltrated leaves were harvested.

5.3 Microbiological techniques.

5.3.1 *E. Coli, A. tumefaciens* and *P. pastoris* strains.

Organism	Strain	Description of the genotype
E. Coli	XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17(r_K^-, m_K^+) supE44 relA1 lac [F´proAB+ $lacI^qZ\Delta M15$::Tn10(tet^r)]*
	DH5α™	*F⁻φ80lacZΔM15Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (r_k^-, m_k^+) phoA supE44 λ⁻ thi-1 gyrA96 relA1*
	DB3.1	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIq Z&M15 Tn10 (Tetr)]*
	Rosetta-gami	*Δara-leu7697 ΔlacX74 Δ phoAPvu* II *phoR araD139 ahpC galE galK rpsL* F'[lac+(lacIq)pro] *gor522 ::Tn10 trxB* ::kan pRARE.
A. tumefaciens	ASE (pSOUP⁺)	*Chl^r Tet^r Kan^r, pSOUP* ⁺ helper plasmid that confers replicase activity for *pSa* replication origin on *pGreen*-derived plasmids.
	C58C1 (P14)	*Rif^r Tet^r Kan^r, 35S:p14* silencing suppressor from Pothos latent virus (*PoLV*).
P. pastoris	X33	wildtype

Table 5.2-1 *E. Coli, A. tumefaciens* and *P. pastoris* Strains used in this study.

The *E. coli* strains XL1-Blue (Stratagene), DH5α (invitrogen) and DB3.1 were used for cloning and concentration of plasmids depending on the vector and clone procedures. Xl1-Blue was used for Greengate cloning while DB3.1 and DH5α were used for Gateway procedure. To be more specific, DB3.1 was used for ccdB-containing plasmids and destination vectors, while DH5α was used for entry clones.

For the purpose of recombinant protein expression, *E.coli* Rosetta-gami (Novagen) and *P. pastoris* X33 were used. Transient expression of fluorescence-tagged protein in *N. benthamiana* and stable transformation of *A. thaliana* were based on genetically modified *A. tumefaciens* ASE strains. P14 cells were used as silencing suppressor to avoid protein degradation.

5.3.2 Media and antibiotics.

Low salt LB medium was used for cultivation of *E. coli* bacteria, and SOC medium was used for the recovery of *E. coli* and *A. tumefaciens* after transformation. Agrobacteria were grown in YEB medium, and yeasts were incubated in YPD medium. The BMGY and BMMY media were used for expression of recombinant protein in *P. pastoris*. All the media were autoclaved at 120^{o}C for 20 min; antibiotics were added after the medium cooled down to 50^{o}C under the sterile bench (Table 5.3-2).

Low salt LB medium:

0.5% (w/v) yeast extract, 1% (w/v) peptone, 0.5% (w/v) NaCl, pH 7.0 with NaOH.

For agar plates 2% (w/v) Bacto agar was supplemented before autoclaving.

SOC medium:

0.5% (w/v) yeast extract, 2% (w/v) peptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 10 mM glucose, pH 7.0 with NaOH.

YEB medium:

0.1% (w/v) yeast extract, 0.5% (w/v) peptone, 0.5% (w/v) beef extract, 0.5% (w/v) sucrose, 2 mM MgSO4, pH 7.5 with NaOH.

For agar plates 2% (w/v) Bacto agar was supplemented before autoclaving.

YPD medium:

1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose.

For agar plates 2% (w/v) Bacto agar was supplemented before autoclaving.

BMGY and BMMY medium:

1% (w/v) yeast extract, 2% (w/v) peptone, 100mM K_3PO_4 (pH 6.0), 1.34% (w/v)

YNB, 4×10^{-5}% (w/v) biotin, 1% (v/v) glycerol for BMGY or 0.5% (v/v) methanol for BMMY.

MDH and MMH medium:

1.34% (w/v) YNB, 4×10^{-5}% (w/v) biotin, 0.004% histidine, 1.5% (w/v) agar, 2% (w/v) dextrose for MDH and 0.5% (v/v) methanol for MMH.

Antibiotic	**Stock**	**Dissolve in**	**Final conc.**
Ampicillin	100 mg/ml	Sodium salt in water	100 μg/ml
Carbenicillin	50 mg/ml	50% Ethanol	50 μg/ml
Chloramphenicol	34 mg/ml	Ethanol	34 μg/ml
Kanamycin	50 mg/ml	water	50 μg/ml
Rifampicin	25 mg/ml	100% Methanol^{+}	25 μg/ml
Spectinomycin	50 mg/ml	water	50 μg/ml
Tetracyclin	12.5 mg/ml	70% Ethanol	12.5 μg/ml
Zeocin	100 mg/ml	Water	100 μg/ml*

Table 5.2-2 Antibiotics used in media for selection of *E. Coli, A. tumefaciens* and *P. pastoris*. +: with 3-4 drops of 5M NaOH, *: used in P. pastoris media only.

5.3.3 Preparation of competent cells.

For the production of *Escherichia coli* competent cells, a single colony was inoculated first in 25 ml LB medium overnight and then in 1 liter LB medium at 37^{o}C to an OD_{600} of 0.6. The suspension was cooled to 4^{o}C and cells were collected by centrifugation (1,500 *g*, 4^{o}C, 10 min). The pellet was then washed with 100 ml Inoue buffer and centrifuged as above. Finally, 50 μl of the cells re-suspended in 20 ml Inoue buffer were aliquoted into 1.5 ml Eppendorf tubes. The aliquots were frozen in liquid nitrogen immediately and kept in -80 ^{o}C until use.

Inoue Buffer:

55mM MnCl2, 2.5M KCl2, 15mM CaCl2 and 10 mM PIPES (pH 6.7). Freshly prepared, filtrated with 0.45μm filter and cooled to 4^{o}C before use.

For the production of *A. tumefaciens* competent cells, a single colony was inoculated firstly in 25 ml YEB medium overnight and then in 200 ml YEB medium at 30^{o}C to an OD_{600} of 0.7-1.0. The cells were cooled to 4^{o}C, collected by centrifugation (1,500 *g*,

4°C, 10 min) and then washed twice with the resuspension buffer. Aliquots were prepared and stored like *E. coli* competent cells.

Resuspension buffer for *A. tumefaciens*:

10% (v/v) glycerol, 1mM HEPES (pH 7.0). Freshly prepared, filtrated with 0.45µm filter and cooled to 4°C before use.

For the production of *P. pastoris* competent cells, a single colony was inoculated into 5 ml YPD medium in a 50 ml conical flask at 30°C overnight. 0.5 ml of the overnight culture was then pipetted into 500 ml YPD in a 2 liter flask and cultured overnight till an OD_{600} of 1.3-1.5. The suspension was then centrifuged at 1,500 g for 5 min at 4 °C, and washed three times with 500 ml water, 250 ml water and 20 ml 1M sorbitol, respectively. Finally, the pellet was re-suspended in 1 ml 1M ice-cold sorbitol and used on that day without storage. All the buffers were ice-cold and sterilized before use.

5.3.4 Transformation.

The chemically competent *E. coli* cells were thawed on ice and gently mixed with circular plasmid DNA (30-100 ng). The mixture was incubated on ice for 30 min before heat-shocking at 42°C for 90 s. 1ml SOC medium was immediately added and the cells were then cooled down on ice for 15 min. After incubation for 1 h at 37 ℃, 200 rpm, the cells were plated onto LB-agar containing the appropriate antibiotics.

The *A. tumefaciens* ASE competent cells were also transformed via heat shock. The cells were thawed on ice for 45 min. 1 µg plasmid DNA was then well-mixed with the cells, followed with frozen in liquid nitrogen for 5 min. The cells were heat-shocked at 37°C for 5 min in a water bath and then supplemented with 1 ml SOC medium. After incubation for 3 h at 28 ℃, 180 rpm, the cells were plated onto LB-agar containing the appropriate antibiotics.

For the transformation of *P. pastoris* competent cells, 80 µl freshly prepared cells were mixed with 10µg if linearized DNA and transferred to an ice-cold electroporation cuvette. After incubation on ice for 5 min, the cells were pulsed with

the following settings: R=200 Ω, C=25 μF, U=1.8 kV, U=1.6 kV. 1 mL ice cold 1M sorbitol was immediately supplemented and the cuvette content was transferred to a sterile 15ml tube. The tube was incubated at 30 ℃ without shaking for 2 hours and cells were spreaded on YPDS plates with 100μg/ml Zeocin. The plates were incubated at 30 ℃ for 3-10 days until colonies form.

5.3.5 Glycerol stock preparation.

A single colony of bacteria or yeast was inoculated in appropriate medium overnight. 750μl of culture was mixed with 250μl 60% glycerol to a final concentration of 15% glycerol. The mixture was flash frozen in liquid nitrogen and stored at -80 ^{o}C.

5.4 DNA techniques.

5.4.1 Isolation of plasmids DNA from *E. coli*.

Plasmids were extracted from bacterial overnight cultures with GeneJET Plasmid Miniprep Kit (Thermo Scientific, 2-4 ml) or JETSTAR2.0 Midi Kit (Genomed, 50ml) according to the manufacturer's instructions.

5.4.2 Isolation of genomic DNA from plants.

For the extraction of genomic DNA, seedlings of *Miscanthus* or *Arabidopsis* were used. Plant material (80-100 mg) was harvested into a 1.5 ml tube and homogenized with 200 μl extraction buffer. 150 μl of the cleared supernatant was transferred to a fresh tube after centrifugation (15,000 *g*, 15 min), and mixed with 150 μl isopropanol. The mixture was centrifuged as above for 10 min, and supernatant was removed carefully. This step was repeated after supplementation of 1 ml 70% ethanol. The pellet was air-dried for 5 min and dissolved with 40μl 1/2TE buffer and kept in 4^{o}C.

Extraction buffer:

150 mM Tris (pH 8.0), 250 mM NaCl, 25 mM EDTA, 0.5% SDS

1/2TE buffer:

10 mM Tris, 0.2 mM EDTA, pH 8.0

5.4.3 Determination of DNA and RNA concentration.

The concentration of nucleic acid was measured by the NanoDrop2000 Spectrophotometer (Thermo Scientific). To evaluate the purity of sample, the ratio of OD_{260}/OD_{280} was used, which should be theoretically around 1.8 for DNA and 2.0 for RNA.

5.4.4 Amplification of DNA fragments by Polymerase Chain Reaction (PCR).

Based on the purpose of PCR, different polymerases were used, including One*Taq*® DNA Polymerase (New England Biolabs), Phusion or Phire polymerase (Thermo Scientific). The PCR reactions were carried out according to the manufacturer's instructions.

5.4.5 Gel electrophoresis of DNA.

To determine the purity or molecular weight of DNA, DNA samples were mixed with 5×loading buffer and loaded onto 1% agarose gels in 1×TAE buffer. The GeneRuler 1 kb DNA ladder (Thermo Scientific) was loaded as a molecular weight marker. After running (U=100V, constant), DNA was stained with 0.1µg/ml ethidium bromide for 10 min and imaged with the INTAS science imaging instruments (Göttingen, Germany) under UV-light.

5×loading buffer:

50% glycerol, 5x TAE buffer, 1% Orange-G

1×TAE buffer:

40 mM tris-base, 20 mM sodium acetate, 1 mM EDTA, pH 7.2

5.4.6 Purifiaction and gel extraction of PCR restriction digested DNA fragments.

Fur purification of PCR products, GeneJET PCR Purification kit (Thermo Scientific)

was used. After the gel electrophoresis, desired bands were cut with razor blade and DNA was extracted with GeneJET Gel Extraction kit (Thermo Scientific). All the experiments were performed according to the manufacturer's instructions.

5.4.7 DNA sequencing.

According to the sample submission guides (www.eurofinsgenomics.eu), samples for sequencing were prepared and reactions were conducted by Eurofins Genomics (München, Germany).

5.4.8 Cloning strategies.

5.4.8.1 Cloning using restriction digestion.

To clone laccase sequences into pPICZαA (Fig 5.4.1), up to 1 µg of plasmid DNA and PCR products of *Miscanthus* laccase were digested using FastDigest restriction enzymes (Thermo Scientific), and then purified. Ligation of the digested DNA fragments was conducted with T4-DNA Ligase (NEB). All the experiments were performed according to the manufacturer's instructions.

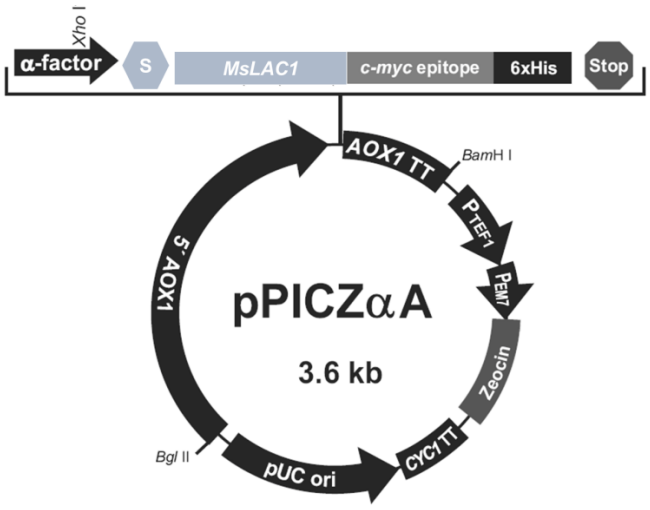

Fig 5.4-1 The map of the recombinant plasmid pPICZ*α*A_MsLAC1_S+ used for expression of MsLAC1 gene in *Pichia pastoris* X-33. The vector contains the sequence coding for the native signal peptide of MsLAC1, which is shown as 'S'.

5.4.8.2 Gateway cloning.

For the aims of expressing the *Miscanthus* laccases in *E. coli* and dual luciferase assay, Gateway vectors were used. DNA fragments were amplified by gene-specific primers with Getaway compatible attB overhangs. pDONR201 was used as entry vector for all the clones by BP reaction. The entry clone was then used for the construction of destination clones with different vectors according to the purpose by LR reaction.

BP reaction:

1) Mix 75 ng of pDONR201 vector and purified PCR products with 1 μl BP ClonaseII enzyme mix in a 5 μl reaction.
2) Incubate the reaction at 25 ℃ for 1 h.
3) Add 0.5 μl Proteinase K to each sample to terminate the reaction at 37 ℃ for 10 min (optional).
4) 2 μl of the mix is used for transformation.

LR reaction:

1) Mix 75 ng of entry clone and destination vector with 1 μl LR ClonaseII Enzyme mix in a 5 μl reaction.
2) Incubate the reaction at 25 ℃ for 1 h.
3) Add 0.5 μl Proteinase K to each sample to terminate the reaction at 37 ℃ for 10 min (optional).
4) 2 μl of the mix is used for transformation.

5.4.8.3 Greengate cloning.

For plant transgenic operations, different promoters, resistances and fluorescence or purification tags need to be fused with *Miscanthus* laccases. Thus, the Greengate cloning method was applied (Lampropoulos *et al.*, 2013). DNA fragments were amplified by gene-specific primers with module overhangs (Fig 5.4-2). The purified fragments were then constructed into entry modules and sequentially selected entry modules were constructed into destination module.

Entry module creation:

1) DNA fragments and the empty vector (Table 5.4-1) were digested with Eco31I FD (Thermo Scientific) following the manufacturer's instructions.
2) Purify the digestion products.
3) Ligation with T4-DNA Ligase (NEB) following the manufacturer's instructions.
4) 3 µl of the mix is used for transformation.

Destination module creation:

1) Mix 1.5 µl of each of the six entry modules (A-F) with 1 µl of destination vector (pGGZ003) in PCR tube.
2) Add 2 µl FastDigest buffer, 1.5 µl 10 mM ATP, 1 µl T4 DNA ligase (NEB) and 1 µl Eco31I FD (Thermo Scientific).
3) Put the reaction mix into a PCR thermocycler with following cycles:

37°C for 2 minutes
16°C for 2 minutes } 50 cycles
50°C for 5 minutes
80°C for 5 minutes

4) 5 µl of the mix is used for transformation.

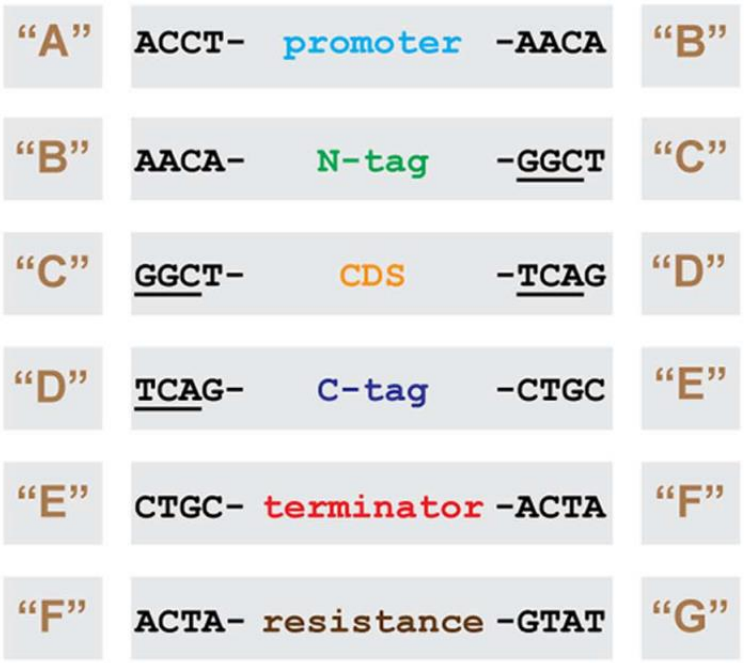

Fig 5.4-2 Module overhangs used for Greengate cloning. The figure is deduced from Figure 2 in (Lampropoulos *et al.*, 2013).

5.4.9 Vectors.

All the vectors used in my project are listed below (Table 5.4-1). A brief description of the application is included.

Vector	Resistance	Application
pPICZαA	Zeocin	Protein expression in *P. pastoris*
pDONR201	Kanamycin	Gateway entry vector
pETG20A	Ampicillin	Gateway destination vector, protein expression in *E. coli*
pETG30A	Ampicillin	Gateway destination vector, protein expression in *E. coli*
pART7	Ampicillin	Gateway destination vector, in planta protein expression
pLUC	Ampicillin	Gateway destination vector, promoter driven firefly luciferase expression
pRluc	Ampicillin	35S promoter driven Renilla luciferase expression
pGGA004	Ampicillin	Greengate A module, 35S promoter
pGGAlac17	Ampicillin	Greengate A module, 2 kb promoter of AtLAC17
pGGB003	Ampicillin	Greengate B module, B-Dummy
pGGC087	Ampicillin	Greengate C module, C-Dummy
pGGC001	Ampicillin	Greengate C module, GFP
pGGCml1	Ampicillin	Greengate C module, *Miscanthus* laccase 1
pGGCml2	Ampicillin	Greengate C module, *Miscanthus* laccase 2
pGGCml3	Ampicillin	Greengate C module, *Miscanthus* laccase 3
pGGCml4	Ampicillin	Greengate C module, *Miscanthus* laccase 4
pGGCml5	Ampicillin	Greengate C module, *Miscanthus* laccase 5
pGGD002	Ampicillin	Greengate D module, D-dummy
pGGD039	Ampicillin	Greengate D module, Strep II tag
pGGD001	Ampicillin	Greengate D module, GFP tag
pGGD010	Ampicillin	Greengate D module, mCherry tag
pGGE009	Ampicillin	Greengate E module, UBQ10 terminator
pGGF001	Ampicillin	Greengate F module, pMAS::Basta^r::tMAS
pGGZ003	Spectinomycin	Greengate destination vector

Table 5.4-1 Information of vectors used in this study.

5.4.10 Primer list.

Oligonucleotides were designed with Primer Premier 5, and ordered at Eurofin MWG Operon (Ebersberg, Germany). The Oligonucleotides were dissolved to 100 pmol/µl (100 µM) in water and stored at -20^{o}C. Primers were then diluted 10 times to 10 µM

for standard PCR reaction, sequencing and qPCR.

Gene	Description	Sequence (5'-3')
MsLAC1	EcoRI, with SP	ccgGAATTCATGGCGTCCTCGTCTGC
	EcoRI, without SP	ccgGAATTCACGAGGAAGTACCAGTTCGATG
	XbaI, without SC	tgcTCTAGATAGCACTGAGGCAGATCCAAT
MsLAC2	EcoRI, with SP	ccgGAATTCCAGCTAGCTGGAGCAATG
	EcoRI, without SP	ccgGAATTCGCGCCTGTGCGTGACGAA
	XbaI, without SC	tgcTCTAGACACCTGGGGATATCGGAC
MsLAC3	EcoRI, with SP	ccgGAATTCATGGCTCGGCTCCCATGTAC
	EcoRI, without SP	ccgGAATTCGCCATTGTTGAGCACACCTT
	XbaI, without SC	tgcTCTAGATAGCACCGGGGGTAGTCT
MsLAC4	EcoRI, with SP	ccgGAATTCATGGCGAGGTCAGGTCTC
	EcoRI, without SP	ccgGAATTCGAGGAGCGCTTCTACGAGT
	XbaI, without SC	tgcTCTAGATACAGCACGCAGATGGG
MsLAC5	EcoRI, with SP	ccgGAATTCATGTCAAGCTCAAGCAGCAG
	EcoRI, without SP	ccgGAATTCAAGGAGCAGTACCATGAGTTCG
	XbaI, without SC	tgcTCTAGACAGCAGACCGGCAGGT

Table 5.4-2 Primers used for protein expression in *P. pastoris*. SP, the predicted signal peptide in laccase sequences; SC, stop codon.

Gene	Description	Sequence (5'-3')
MsLAC1	GW_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACGAGGAAGTACCAGTTCGATG
	GW_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTCTAGCACTGAGGCAGATCCAA
MsLAC2	GW_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACGACTGTGCGTGACGAA
	GW_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCACCTGGGGATATCGGAC
MsLAC3	GW_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCATTGTTGAGCACACCTT
	GW_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTCTAGCACCGGGGGTAGTCTG
MsLAC4	GW_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAGGAGCGCTTCTACGAGTTC
	GW_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTCTACAGCACGCAGATGGG
MsLAC5	GW_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAAGGAGCAGTACCATGAGTTCG
	GW_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAGCAGACCGGCAGGT

Table 5.4-3 Getaway primers for protein expression in *E. coli*.

Gene	Description	Sequence (5'-3')
MsLAC1	GW_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAGGATAAGGTAAGGGCAAATAT

promoter	GW_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTAATTGGTAGCACGGGGGTA
MsLAC2	GW_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCCATGAAGTTGTTTTTCTTCC
Promoter	GW_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTTGCTCCAGCTAGCTAGC
MsLAC3	GW_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTCTCACCACTAATAGCTCTCAA
Promoter	GW_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTGCGAGCGAACTCAATTC
MsLAC4	GW_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTCACGCAAGAGCTTACGTGAGC
Promoter	GW_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTGATCGCAGCCACCGCT
MsLAC5	GW_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCACATTTCTGGCTCCATTATGG
Promoter	GW_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTGCCAAGGCTGAAGCAGGCT

Table 5.4-4 Getaway primers for cloning of *Miscanthus* laccase promoters.

Gene	Description	Sequence (5'-3')
AtLAC17 promoter	Module A_F	AACAGGTCTCAacctGACTACACTTTACTACGTAGTAAT
	Module A_R	AACAGGTCTCTtgttGTGTCCAAGCTCACTTCA
MsLAC1	Module C_F	AACAGGTCTCAggctCAATGTCAAGCTCAAGCAGCAG
	Module C_R	AACAGGTCTCTctgaGCAGACCGGCAGGTCAG
MsLAC2	Module C_F	AACAGGTCTCAggctCAATGGCGTCCTCGTCTGC
	Module C_R	AACAGGTCTCTctgaGCACTGAGGCAGATCCAAT
MsLAC3	Module C_F	AACAGGTCTCAggctCACAGCTAGCTGGAGCAATG
	Module C_R	AACAGGTCTCTctgaCCTGGGGATATCGGACGG
MsLAC4	Module C_F	AACAGGTCTCAggctCAATGGCTCGGCTCCCATGTAC
	Module C_R	AACAGGTCTCTctgaGCACCGGGGGTAGTCTG
MsLAC5	Module C_F	AACAGGTCTCAggctCAATGGCGAGGTCAGGTCTC
	Module C_R	AACAGGTCTCTctgaCAGCACGCAGATGGGCAA

Table 5.4-5 Greengate primers for construction of A and C modules.

Vector	Description	Sequence (5'-3')
pDONR201	SeqL_A	TCGCGTTAACGCTAGCATGGATCTC
	SeqL_B	GTAACATCAGAGATTTTGAGACAC
pART7	35S_F	CTATCCTTCGCAAGACCCTT

	OCS R	GGCGGTAAGGGAGCTA
pLUC	LUC_F	CTAACATACGCTCTCCATCA
	LUC_R	GGATAGAATGGCGCCGG
pPICZαA	pPICZαA_F	TCTCTCGAGAAAAGAGAGGCTG
	PICHIA_REV2	AGGAACAGTCATGTCTAAGGCTAC
pETG20/30A	TrxA_Seq	AAGGTCAGTTGAAAGAGTTCC
	T7term	CTAGTTATTGCTCAGCGGT
pGGA/C000	F	ACCTCTCGGGCTTCTGG
	R	CGATTTTTGTGATGCTCGTC
pGGZ001	A00111_F	GTATTCAGTCGACTGGTACCAAC
	A01924_R	TTGGTACCAGTCGACTGAATAC
pGGB003	B-dummy_F	GTGGATCCTAGATAACCTTTAC
	B-dummy_R	ACAGGGAATGAAGGTAAAGG

Table 5.4-6 Primers for colony PCR and sequencing.

	Primer	**Sequence**	**Product**
Laccase genes	qMsLac1_F	GCCGAGACGAGGAAGTACCAGT	184
	qMsLac1_R	CGTGCCAGTGGATGCTGATGTT	
	qMsLac2_F	CTACGCCTACGACTTCCGCATC	179
	qMsLac2_R	CGTTGAACCACTCACCGAGCAT	
	qMsLac3_F	GGTACAACGAGACGGTGGAGGT	166
	qMsLac3_R	TGCTGCGGGTTGACGAGGTT	
	qMsLac4_F	GGTGTTGCTGCTGGCTTGCT	87
	qMsLac4_R	TGCCCGTTCACCGTGACGAT	
	qMsLac5_F	ACCACCACCACGGCCATCTT	188
	qMsLac5_R	CCGACGGTGAAGAAGAGGCTCT	
Transcription factors	SND1_F	GACATCCAAGAGAAGTGCCG	84
	SND1_R	CGTCGGGTACTTCTTGTCCT	
	NST1_F	GACATCCAAGAGAAGTGCCG	84
	NST1_R	CGTCGGGTACTTCTTGTCCT	
	VND7_F	TCCCGCCTTCGTTATACTCC	104
	VND7_R	GACGTCGGAACAGTAGGCTA	
	MYB63_F	ACATAGCAAGCTTCAGCCCA	105
	MYB63_R	CCACCAGGAGTTCAGGTTCC	
	MYB85_F	GCAGCCCTACGGAATCGA	93
	MYB85_R	CCAGCGGGTCTTGGTCAT	
	MYB43_F	AAGGCAGCTTCCTCACAGTC	132
	MYB43_R	GCTGGACTGCTCCGATGAAT	
Monolignol biosynthesis	HCT_F	GGAGCACTGGATAGGATGGA	161
	HCT_R	AAGTCGGCATCATGGATAGG	
	CCoAOMT_F	ACGCCGACAAGGACAACTAC	155
	CCoAOMT_R	GTCACGGTAGAAGCGGATGT	
	CCR_F	AAGGAAGCAGCCGTACAAGA	102

	CCR_R	TGGAAGCATATGACGGTGTC	
	CAD_F	ACCACCCGCTAGAACCCTAC	185
	CAD_R	CTTGTCGACGCAGAACTGG	
	C3H_F	AACGTGATGGTGAACGTGTG	108
	C3H_R	GCCCTTGATGTCGATGTTCT	
Cellulose	CesA7_F	CCTCTACCCGTTCCTCAAGG	101
biosynthesis	CesA7_R	AGACGAGCGAGAAGATGGAG	
Report genes	PP2A_F	GCTAGCTCCTGTCATGGGTC	89
	PP2A_R	TCATGTTCGGAACCCTGTCC	
	UBC_F	CTGAACCAGACAGCCCACTT	63
	UBC_R	CTCTGATATCACCCGACCGC	

Table 5.4-7 Specific primers for expression of lignin-related genes in *Miscanthus* using RT-qPCR.

	Primer	**Sequence**	**Product**
Laccase	qMsLac4_F	GGTAGATATCCAGGTCCCACA	184
genes	qMsLac4_R	GTTATGTAAGCAGGCCCATCA	
	qMsLac17_F	TTCTCTTGTGTTCTTCTTCTT	179
	qMsLac17_R	GAACTTCTTTGTGAGGTTTAG	
Transcriptio	qAtMYB63_F	GCGTGGCAACTTCACTTCAG	80
n factors	qAtMYB63_R	CGATTTTCGACCACTTGTTCCC	
	qAtMYB46_F	TTCGCTTTCATTCCATCCTCG	53
	qAtMYB46_R	CAATCGTGCTGCAATCTGAGA	
	qAtMYB85_F	GGCCTTCTCTCGCATGATGA	73
	qAtMYB85_R	TCTTAGACCACTTATTGCCGAGA	
	qAtMYB58_F	CGTGGCAATTTCAGTGCAGAGG	189
	qAtMYB58_R	TGAACCCGCTTCATCGGCAT	
Monolignol	qAtPAL1_F	AGCGCAACGTACCCGTTGAT	175
biosynthesis	qAtPAL1_R	CGTAGGCTGCTCTTGCTGCT	
	qAtC4H_F	AACTGGCTTCAAGTCGGAGA	91
	qAtC4H_R	GACCCATACGGAGGAGGAAG	
	qAtC3H1_F	AGGAGCGGTTGCGTTCAACA	87
	qAtC3H1_R	AGCCCTTGCTCGTCCACAAC	
	qAtCCR1_F	CGCGTGGTCATCACCTCCTC	64
	qAtCCR1_R	CAGCCTCAGGGTCACGGTTC	
	qAtF5H1_F	CCATAGGACGCGACCCAACC	82
	qAtF5H1_R	AAATCCGGTACGCCCGGTTC	
	qAtCOMT_F	GAAGCTGCCCTCTTCGCCAT	189
	qAtCOMT_R	ACGGAGGATACGGTCGAGCA	
	qAtHCT_F	ACACGAGACCAGCTTGTTGCT	118
	qAtHCT_R	CTCGCGCCTTTCCCACTGAT	
	qAtCAD6_F	GGCTGCCAGAGACCCATCTG	176
	qAtCAD6_R	CCACTTCATGCCCAGGAACCA	

	qAt4CL_F	CTAATGCCAAACTCGGTCAGG	104
	qAt4CL_R	AGCTCCTGACTTAACCGGAAA	
	qAtCCoAOMT1_F	CTCACAAGATCGACTTCAGGG	137
	qAtCCoAOMT1_R	ACGCTTGTGGTAGTTGATGTAG	
Flavonoid biosynthesis	qAtCHS_F	CTTGCCTTCTATCTGCCTACCTAC	132
	qAtCHS_R	TCCAGCACATATCACATATCACATC	
	qAtCHI_F	GGAGGCGGTTCTGGAATCTATC	108
	qAtCHI_R	TTCGTCCTTGTTCTTCATCATTAGC	
	qAtFLS1_F	CAAGGATTACAGTTACCGCAAGC	83
	qAtFLS1_R	CCACAACCACAAATTATTCTTCTCG	
Cellulose biosynthesis	CesA7_F	ATGCCACCGATAAGCACATT	108
	CesA7_R	TTCCTCAATGCTAACTCCGC	
Report genes	EF1a_F	GGGTGTGAGGACGAAAGGTA	120
	EF1a_R	TTCTTAACAGCGAGCACACG	
	PDF2_F	TAACGTGGCCAAAATGATGC	61
	PDF2_R	GTTCTCCACAACCGCTTGGT	
	Clath_F	TCGATTGCTTGGTTTGGAAGAT	61
	Clath_R	GCACTTAGCGTGGACTCTGTTTGC	

Table 5.4-8 Specific primers for expression of different genes in *Arabidopsis* using RT-qPCR.

5.5 RNA techniques.

5.5.1 RNA extraction and reverse transcription.

30/50 mg *Miscanthus*/*Arabidopsis* sample was used for RNA extraction with RNeasy Mini Kit (Qiagen). 1 µg of total RNA was then reverse transcribed by AMV reverse transcriptase (Roboklon). All experiments were conducted following the manufacturer's instructions.

5.5.2 qRT PCR.

To determine the transcript levels of genes, quantitative RT-PCR was performed on a

Rotor-Gene Q (Qiagen) with a 15µl reaction mixture. 5 µl 1:16 diluted cDNA was mixed with 10µl qPCR mixture containing 0.5 µl of each primer (10 µM stock), 1.5 µl 10× qPCR buffer (Sigma-Aldrich), 0.3 µl 10 mM dNTPs, 0.3µl JumpStart Taq DNA polymerase and 0.3 µl 1:400 diluted SYBR Green I (Sigma-Aldrich), and 6.6µl H_2O.

The thermal cycling conditions used were 95℃ for 6 min followed by 40 cycles of 95℃ for 15 s, 60℃ for 30 s, and 72℃ for 20 s, followed by a melting cycle with 1℃ increments for 5 s each from 56℃ to 96℃. The analysis of melting curves, measurement of primer pair efficiencies, and determination of cycle threshold values, including the calculation of the mean normalized expression of the genes, were conducted using the Rotor-Gene Q Series Software Q 2.0.2 (Qiagen) and the Q-Gene software. The mRNA levels of the studied genes were normalized with the comparative CT method, using the expression of one or two reference genes (Table 5.4-7, 5.4-8) as internal standard.

5.6 Protein techniques.

5.6.1 Extraction of protein from plant materials.

With same plant material used for RNA extraction, 200 mg homogenized samples were used for protein extraction with extraction buffer. The supernatants after centrifugation (15,000 g, 30 min at 4^{o}C) were directly used for measurement of protein concentration and gel analysis. To extract both cell wall protein (CWP) and intra-cellular protein (ICP) from different internodes and gradient of leaf from *Miscanthus*, a published protocol (Shane & Plaxton, 2014) was used.

For measurement of laccase activity, protein samples were dialyzed against pH 5.0 50 mM HAc/NaAc buffer overnight before the reaction.

Protein extraction buffer:

10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.1mM $MgCl_2$, 1 mM DTT and 1mM PMSF

5.6.2 Gel electrophoresis and immunol blot for protein.

The protein samples were mixed with Roti®-Load 1 (Roth) for SDS-PAGE followed by heating at 100°C for 5 mins, and Roti®-Load 2 (Roth) for Native-PAGE, respectively. Both forms of electrophoresis were performed on a 4.5% stacking gel as well as a 12% separating gel with chambers from Bio-Rad. Loading of samples, running and staining were described previously (He *et al.*, 2015a).

Proteins were electro-blotted onto Immobilon-P PVDF membrane (Sigma-Aldrich) after SDS-PAGE. The expressed proteins were studied by Western blotting with anti-MYC (Thermo Fisher Scientific) or Anti-Strep-tag II antibody (abcam) and detected using SuperSignal West Dura Extended Duration Substrate for HRP (Thermo Fisher Scientific) after incubation with anti-mouse IgG secondary antibody (Bio-Rad).

5.6.3 Measurement of protein concentration.

The concentration of protein was measured with Bradford reagent (Sigma-Aldrich). A calibration curve based on bovine serum albumin (0.1–1 mg/mL) was used for calculation.

5.7 Lignin staining and microscopy techniques.

5.7.1 Lignin staining.

To prepare the cross section of inflorescence stems, samples were embedded in 6% agarose and cut by hand using a razor blade (Mitra & Loqué, 2014). Lignin was stained with HCl-phloroglucinol staining solution (2% phloroglucinol in absolute ethanol, mix with same volume of HCl before use). Lignified tissues were stained red and signals were captured with a Leica DM IRB inverted microscope.

5.7.2 Cell wall labeling using NBD-CA.

After 2 days culture of infiltrated tobacco plants, leaf discs were cut around the

injection site on leaves. The stem cross section of *A. thaliana Col-0* was used as positive control. Plant samples were incubated with NBD-CA and washed as described by (Schuetz *et al.*, 2014). Afterwards, plant tissues were mounted in water for detection of fluorescence signals under confocal.

5.7.3 Microscope setting.

Confocal images were taken using the Perkin-Elmer UltraView VoX spinning disk confocal mounted on a Leica DM16000 inverted microscope with a Hamamatsu 9100-02 CCD camera. The GFP filter (excitation 488nm, emission 525nm) was used to image GFP-tagged constructs and NBD-CA. The RFP filter (excitation 561nm, emission 595-625nm) was used to image mCherry-tagged constructs. Samples were mounted in water and imaged using a Leica oil immersion 20× or63× objective. All images were processed using Volocity image analysis software (Improvision).

5.8 Laccase activity assay.

Laccase activity was determined with ABTS [2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] (Fisher Scientific) as the substrate by measuring the increase of absorbance at wavelength of 420nm (Ng *et al.*, 2013). Sample was diluted in pH=5.0 50 mM HAc/NaAc buffer to 180 μL and mixed with 20 μL ABTS. 180 μL buffer without enzyme was mixed with ABTS in the same way and used as control. After incubation in 30 oC for 3 mins, absorbance at 420 nm was measured and the increased absorbance compared with control (ΔA) was used for the calculation of enzyme activity:

$$\text{Laccase activity } (\text{U} \cdot \text{L}^{-1}) = \frac{V_t \times N \times \Delta\text{A} \times 10^6}{V_e \times \varepsilon \times \Delta\text{T}}$$

Where, V_t, total volume; N, dilution fold; V_e, sample volume; ε, Molar extinction coefficient for ABTS ($3.6 \times 10^4 M^{-1}cm^{-1}$); ΔT, time of incubation.

One unit of enzyme activity was defined as the amount of enzyme that oxidizes 1 μmol of ABTS per min at 30^{o}C.

5.9 Study of recombinant MsLAC1.

5.9.1 Cultivation of yeast.

After transformation, positive colonies were selected on YPD plates containing 50μg/mL Zeocin^{TM} followed by colony PCR using α-factor and 3´AOX1 sequencing primers (Table 5.4-6). Colonies were screened on MMH and MDH plates supplemented with Zeocin for fast methanol utilization (Mut+) phenotypes (Bronikowski *et al.*, 2017). With the selected strains, YPD medium was used for general cultivation. For expression of protein, strains were cultured in 30 ml BMGY medium overnight and then 1 ml suspension was inoculated into 200 ml BMMY medium for 10 days.

5.9.2 Production of recombinant protein.

To check the expression and secretion of recombinant protein, the verified Mut+ colonies were cultured in 5mL BMGY medium (50mL tubes, 30℃, 180rpm) and harvested by centrifugation (5000 rpm, 5 min) when the cultures reached an OD_{600} of 3.0. The pellets were subsequently washed, resuspended in BMMY medium (supplemented with 0.3mM $CuSO_4$) and diluted to a final OD_{600} of 1.0 in 30mL BMMY media (300mL flasks, 30℃, 180rpm). Methanol (1%, v/v) was added daily to maintain the induction. Aliquots were collected daily by centrifugation (5000rpm, 5min) after induction. The supernatants were dialyzed against 20mM sodium acetate buffer (pH5.0) overnight at 4℃ and kept for further analysis at -80℃. Concentration of protein as well as activity of laccase was measured for each sample. The *pPICZαAfeh* strain expressing recombinant fructan exohydrolase (FEH) protein previously created in our lab was cultivated in parallel and used as a control.

5.9.3 Purification of rMsLAC1.

The recombinant protein expressed with pPICZαA was tagged with 6×His tag, and thus rMsLAC1 was purified using His-Tag Purification Columns (Sigma-aldrich)

following the manufacturer's instructions. Purified protein was dialyzed against pH5.0 50mM HAc/NaAc buffer overnight, and stored in 4^{o}C with 10% glycerol.

5.9.4 Characterization of rMsLAC1.

The optimal pH and temperature for the oxidation of ABTS was determined by measuring the activity at different pH (1-9) and temperature (10-50^{o}C). For pH stability assay, rMsLAC1 was incubated in 50mM of HCl/KCl buffer (pH 1-3), HAc/NaAc buffer (pH 3-6) and Tris-HCl buffer (pH 6-9) for 2 hours. The laccase activity was measured at 0, 5, 10, 20, 40, 60, 90 and 120 min after incubation. For the calculation, highest activity in all the conditions was set as 100%.

Chloride salts of Cs^{+}、K^{+}、Na^{+}、Mg^{2+}、Ca^{2+}、Cu^{2+}、Mn^{2+} and Fe^{3+}were selected to determine the effect of metal ions on the laccase activity in HAc/NaAc buffer (50 mM, pH 5.0) containing different concentrations of metal ions (1 and 10 mM). The residual activity was calculated based on the control without adding any metal compound.

The effect of organic solvents on the laccase activity was performed at 30℃ in the presence of 10% and 20% (v/v) of several organic solvents, including methanol, ethanol, isopropyl alcohol, acetonitrile and acetone. The residual activity was calculated based on the control without adding any organic solvent.

The effect of various inhibitors including sodium azide (0.1, 1 and 10 mM), EDTA, SDS and DTT (1 and 10 mM) on the laccase activity was investigated by determining the residual activity of the mixture in HAc/NaAc buffer (50 mM, pH 5.0). The residual activity was calculated based on the control without adding any inhibitor.

All of the measurements were performed in triplicate.

5.9.5 Oxidation of monolignol by rMsLAC1.

To evaluate the ability of rMSLAC1 to oxidize monolignols, cinnamyl alcohol, *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Sigma-aldrich) were used as substrates. Measured with with ABTS, 0.02U of enzyme were mixed with 0.5mM of each substrat. Reactions were performed in 1.5mL reaction vials in 50mM

NaAc-HAc buffer (pH5.0) at 30℃. The absorption of each reaction mixture was scanned between 250nm to 650nm. For the oxidation of sinapyl alcohol the reaction was scanned at 5, 10, 20, 30 and 60min after enzyme addition, while for other monolignols, the mixtures were scanned at 0.5, 1, 2, 6, and 12h. Water replacing the recombinant enzyme was used as control for each monolignol and measured in parallel. Highest absorption was detected at around 280 nm for all the substrates, and the oxidation rate was calculated against the water control.

5.10 Dual luciferase assay.

To determine the activation of promoters by lignin-related transcription factors (TFs), dual luciferase assay was applied in grapevine (*Vitis vinifera*) suspension cells as previously described protocol (Höll *et al.*, 2013; Wei *et al.*, 2017). Transcription factors (*MsSND1*, *MsVND7*, *MsSCM2-4* and *MsMYB52*) were cloned in previous work (Golfier *et al.*, 2017) by my colleague. Vectors and primers for construction of reporter plasmids for DLA are concluded in Table 5.4-1 and 5.4-4.

The value of ratio between firefly and Renilla luciferase for each transfection experiment was then normalized against the control plasmid pRluc, to represent the relative activation fold. All measurements were repeated three times and all the experiments were carried out independently at least 2 times.

5.11 Lignin and sugar analysis.

Mature stems of *Arabidopsis* were dried, and siliques and leaves were removed. Stems with a length of approx. 12cm were ground and the resulting powder washed with acetone in a Soxhlet column to remove of all soluble components. The content of acid soluble lignin (ASL) and insoluble lignin (IL) was measured according to (Cullis *et al.*, 2004). Sugar contents were measured using liquid chromatography and lignin composition was estimated by gas chromatography after thioacidolysis as described before (Robinson & Mansfield, 2009). All the measurements were done in triplicates with two independent lines.

6. List of abbreviations

CSE	caffeoyl shikimate esterase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LAC	laccase
TNC	trinuclear cluster
2,6-DMP	2,6-dimethoxyphenol
4CL	4-coumarate: CoA ligase
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ASL	acid soluble lignin
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
C3H	p-coumarate 3-hydroxylase
C4H	cinnamate 4-hydroxylase
CA	coniferyl alcohol
CAD	cinnamyl alcohol dehydrogenase
CaMV	cauliflower mosaic virus
CB	crude broth
CCoAOMT	caffeoyl-CoA O-methyltransferase
CCR	cinnamoyl-CoA reductase
cDNA	complementary DNA
CDS	coding DNA sequence
CesA	cellulose synthase A
CHI	chalcone isomerase
CHS	chalcone synthase
COMT	caffeic acid O-methyltransferase
CWP	cell wall associated protein
DLA	dual luciferase assay
DMF	dimethylformamide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
E. coli	*Escherichia coli*
e.g.	for example (lat. exempli gratia)
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tag
F5H	ferulate 5-hydroxylase
FEH	fructan exohydrolase
FLS	flavonol synthase
FT	flow through
GFP	green fluorescent protein

GR	glucocorticoid receptor
HCT	hydroxycinnamoyl-CoA shikimate/Quinatehydroxycinnamoyltransferase
ICP	intracellular protein
IL	insoluble lignin
IPTG	isopropyl β-D-1-thiogalactopyranoside
kDa	kilodalton
KL	klason lignin
M	molarity
MES	2-(N-morpholino)ethanesulfonic acid
MW	molecular weight
NBD	nitrobenzoxadiazole
NTA	nitrilotriacetic acid
OD	optical density
PAGE	polyacrylamidide gel electrophoresis
PAL	phenylalanine ammonia-lyase
PCR	polymerase chain reaction
PCW	primary cell wall
pI	isoelectric points
PIPES	piperazine-N,N′-bis(2-ethanesulfonic acid)
POD	peroxidase
PVDF	polyvinylidene difluoride
qRT-PCR	quantitative reverse transcription PCR
RFP	red fluorescent protein
RNA	ribonucleic acid
RT	reverse transcription
SCM	secondary cell wall MYB factor
SCW	Secondary cell wall
SD	standard deviation
SDS-	sodium dodecyl sulfate
SMRE	secondary wall MYB responsive elements
SNBE	secondary wall NAC binding elements
SND	secondary cell wall-assosiated NAC domain protein
SOC	super optimal broth with catabolite repression medium
TAL	tyrosine ammonia-lyase
TBST	tris buffered saline with Tween20
TF	transcription factor
VND	vascular related NAC domain protein
WT	wildtype

7. References

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