



The identification of laccases involved in lignin formation in *Brachypodium distachyon* culm and the regulation of laccases in *Arabidopsis* stems

Yin Wang

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UNIVERSITÉ PARIS-SUD

ÉCOLE DOCTORALE 145 :
SCIENCES DU VÉGÉTAL

Institut Jean-Pierre Bourgin (IJPB), INRA Versailles

THÈSE SUR TRAVAUX

par

Yin WANG

The identification of laccases involved in lignin formation in
Brachypodium distachyon culm and the regulation of laccases
in Arabidopsis stems

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Chapter 1 Introduction

1.1 Plant cell wall structure and composition

The plant cell wall matrix is dynamic. Its structure and composition are diverse among plant species as well as cell types. Among adjacent cells, plant cell walls often play important roles in intercellular communication and also interactions with external signals (such as biotic and abiotic stress) (Ellis et al., 2010).

Plants form two types of cell wall. The primary walls of growing cells are relatively thin and flexible, allowing the cell expansion. They mainly contain cellulose, hemicelluloses and pectins. Pectins are branched polysaccharides containing lots of negatively charged galacturonic acid residues and they are thought to be deposited early after cell division to form a middle lamella, while other components are deposited later (Keegstra, 2010). Cellulose is synthesized at the plasma membrane, but the insoluble cellulose microfibrils are deposited directly into the extracellular matrix and randomly arranged. The cellulose microfibrils consist of β -1,4-linked glucan chains which are linked each other via hydrogen bonds (Somerville, 2006) and they are also bound tightly with hemicellulose. The secondary cell wall is produced after the primary cell wall is formed and when the cell has ceased expanding (Cosgrove, 2005). Not all cells have lignified secondary walls which are thicker and more rigid than primary walls. Indeed, they provide additional rigidity and strength to plant cells that are engaged in tissues subjected to mechanical stresses for instance. Secondary walls contain highly ordered cellulose fibers and lignin, but generally lack pectin. Lignins are deposited following the secondary thickening of cell walls and are particularly important in cell types responsible for conducting water and providing mechanical strength to the plant (Donaldson et al., 2001; Myburg and Carolina, 2001).

1.2 Differences of plant cell walls between monocots and dicots

There are significant differences in polymer types and their relative abundance between cell walls of dicots and monocots. For example, relatively high amounts of xyloglucan, pectins and 5-10% structural proteins are typically present in the primary walls of dicots, non-commelinoid monocots and gymnosperms (Jamet et al., 2006). The commelinoid monocots like grasses contain arabinoxylans, small quantities of pectin, structural proteins and high amounts of the hydroxycinnamic acids, *p*-coumaric acid (CA) and ferulic acid (FA) in their walls (Vogel and Hill, 2008). In grasses, the CA is either linked to arabinoxylan (Petrik et al., 2014) or to syringyl (*S*) unit within the lignin polymer (Ralph et al., 1994). Indeed, it has been shown that CA can acylate the side-chains of monolignols and then monolignol-CA conjugates become incorporated into lignin via oxidative radical coupling (Ralph et al., 1994). The carboxylic acid group of FA is attached to the side chains of xylan with ester bonds and the hydroxyl group of FA can be covalently linked to lignin monomers (Ralph et al., 1994; Grabber et al., 1996; Ralph et al., 2004; De, 2009). Actually, it was proposed that arabinose-linked FA coupled with monolignol (coniferyl and/or sinapyl alcohol) act as the initiation and nucleation site for the formation of lignin. (Ralph et al., 1994; Jacquet et al., 1995; Grabber et al., 1996; Ralph et al., 2004; De, 2009). Despite more data are necessary, we may hypothesize that the creation of lignin-hydroxycinnamic acid-polysaccharide complex provide cell wall stiffening to resist against mechanical force and pathogen/ insect invasion (Santiago et al., 2007; Santiago et al., 2008; Lanoue et al., 2010).

Some researchers have observed that cell wall-associated FA content is inversely correlated with enzymatic saccharification efficiency (Grabber et al., 1998; Lam et al., 2003; Casler and Jung, 2006; Jung and Casler, 2006). In consequence, the FA ester-ether bridges between lignin and arabinoxylan are

often considered as a significant limiting factor that inhibits cell wall degradability of grasses (Grabber et al., 1998).

1.3 Plant cell wall lignification and monolignol metabolism

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Plants are built of various specialized cell types that differ in their cell wall composition and structure. The cell walls of certain tissues (xylem, sclerenchyma) are characterized by the presence of the heterogeneous lignin polymer that plays an essential role in their physiology. This phenolic polymer is composed of different monomeric units – the monolignols – that are linked together by several covalent bonds. Numerous studies have shown that monolignol biosynthesis and polymerization to form lignin are tightly controlled in different cell types and tissues. However, our understanding of the genetic control of monolignol transport and polymerization remains incomplete, despite some recent promising results. This situation is made more complex since we know that monolignols or related compounds are sometimes produced in non-lignified tissues. In this review, we focus on some key steps of monolignol metabolism including polymerization, transport, and compartmentation. As well as being of fundamental interest, the quantity of lignin and its nature are also known to have a negative effect on the industrial processing of plant lignocellulose biomass. A more complete view of monolignol metabolism and the relationship that exists between lignin and other monolignol-derived compounds thereby appears essential if we wish to improve biomass quality.

Keywords: monolignol, lignin, lignan, metabolism, biomass

The majority of plant biomass consists of different cell wall polymers produced by living plant cells. In most cases, these polymers are energy-rich linked sugars that form the major structural components in plant cell walls, particularly in the thick secondary cell walls that characterize certain tissues. In addition to polysaccharides, another major cell wall polymer – lignin – limits access to cell wall sugars and negatively affects human utilization of biomass (livestock feed, paper manufacturing, and lignocellulosic biofuel production; Chapple et al., 2007). Because of its significant economic impact and central role in higher plant development, lignification is an important topic in plant biochemistry.

Despite a few exceptions (Martone et al., 2009), lignin is a phenolic biopolymer that is only detected in vascular plants. It is generated by radical coupling of hydroxycinnamyl alcohols named monolignols: coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol. When introduced in the lignin polymer the corresponding monolignols are named guaiacyl (G), sinapyl (S), and hydroxyl-coumaroyl (H) units, respectively. The biosynthesis of monolignols is initiated from the general phenylpropanoid pathway (Dixon et al., 2001). Although tyrosine was proposed to be the starting point of phenylpropanoid metabolism in some plants such as grasses (Neish, 1961; Higuchi, 1990), it is generally recognized that monolignols are derived from phenylalanine via a series of enzymatic reactions, catalyzed by the following enzymes: phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate coenzyme A ligase (4CL), ferulate 5-hydroxylase (F5H), *p*-coumarate 3-hydroxylase (C3H),

p-hydroxycinnamoyl-CoA:quinic/shikimate hydroxycinnamoyl transferase (HCT), caffeoyl-CoA *O*-methyltransferase (CCoAOMT), cinnamoyl-CoA reductase (CCR), caffeic acid *O*-methyltransferase (COMT), and cinnamyl alcohol dehydrogenase (CAD; Vanholme et al., 2009).

The composition of lignin varies between plant species and tissues. In general, lignins from gymnosperms and related species are rich in G units and contains low amounts of H units, whereas dicot lignins are mainly composed of G and S units (Weng and Chapple, 2010). It is important to note that differences in monolignol content are controlled by key enzymes that are often limiting (e.g., F5H is limiting for S unit production; Weng and Chapple, 2010). Consequently, certain natural genotypes/varieties may show unusual proportions of monolignols as illustrated by the recent discovery of a *Populus nigra* variety containing a truncated HCT enzyme and producing substantial amounts of H units (Vanholme et al., 2013) that are usually almost completely absent in poplar lignin. Similarly, a loblolly pine naturally affected in cinnamyl alcohol dehydrogenase activity and showing modified lignin structure was also detected 20 years ago in a natural population (MacKay et al., 1995; Ralph et al., 1997).

From a developmental point of view, lignification is generally initiated during the formation of the secondary cell wall although cell-/tissue-specific differences in the developmental pattern can occur. For example, lignification is initiated in the compound primary cell wall of xylem elements (middle lamella/cell corners) at the start of secondary cell wall formation (Donaldson, 2001; Fromm et al., 2003). In contrast, lignin may be deposited at later

stages in other tissues (Boerjan et al., 2003; Baghdady et al., 2006; Harrington et al., 2012). For instance, parenchyma cells present in the secondary xylem of *Arabidopsis* hypocotyls are devoid of lignin during the vegetative stage (Sibout et al., 2008). This period may range over months according to different *Arabidopsis* accessions but ends as soon as the plant starts flowering when parenchyma cells start to reinforce their secondary cell walls by producing lignin. Parenchyma therefore develops into sclerenchyma, a tissue dedicated to mechanical support. Biotic- and/or abiotic-stresses can also induce lignification in the walls of cells that do not normally lignify under non-stress conditions (Srivastava et al., 2007; Kim et al., 2008). Compression wood in coniferous trees also contains significant amounts of *p*-coumaryl alcohol (Sjöström, 1993). The observation that lignin biosynthetic genes are up-regulated (or activated) following mechanical injury suggests that stress lignification is a tightly controlled phenomenon. In contrast, the expression of monolignol biosynthesis genes is not always correlated with the presence of the lignin polymer. Although this is quite surprising at first glance, this situation can be explained by the fact that a wide range of other (non-lignin) products (hydroxycinnamic acids, phenols esters and phenol amides, or lignans; Figure 1) are also produced by the phenylpropanoid pathway.

In summary, lignification is a dynamic, flexible process reinforcing cell walls according to the different needs (water conduction, mechanical support, defense) of the plant during its life. Nevertheless, once produced, lignins (and the associated carbon) remain anchored in the cell wall since plants do not possess the enzymatic machinery necessary to recycle this polymer in contrast to other cell wall polysaccharides such as (1,3;1,4)- β -D-glucans for instance (Fincher, 2009). The irreversibility of this situation also underlines the necessity for tight regulation of the lignification process.

Metabolic engineering can be used not only to reduce or modify natural lignin structure, but can also be employed to produce novel lignin structures in biomass crops with the goal of improving its structure for biomaterial or bioethanol production (Dixon et al., 1996; Vanholme et al., 2012a). Nevertheless, as underlined by a recent systems biology approach that investigated the consequences of lignin perturbations in *Arabidopsis* mutants, it is clear that our knowledge of this complex process is far from complete and that modifications in the lignification process can be accompanied by unexpected changes in gene expression and metabolism (Vanholme et al., 2012b). In this review, we focus on another aspect of lignin biology where our understanding is only partial – the different processes that occur after monolignol biosynthesis, including the acylation and glycosylation of monolignols in the cytoplasm, their transport through the membrane to the apoplast, and their deglycosylation, and polymerization into lignins. We also discuss lignans and related compounds produced by the same biosynthetic pathway. In this review we aim to point out some gaps in our knowledge of lignification that are potentially limiting for biomass use and engineering.

TRANSPORT AND COMPARTMENTATION

Monolignols are synthesized in the cytoplasm and translocated to the cell wall for subsequent polymerization (Alejandro et al.,

2012). For many years, the mechanism of transport remained unknown and different hypotheses were suggested (passive diffusion, exocytosis, active transport, etc.; Liu et al., 2011; Liu, 2012). An elegant biochemical study has recently demonstrated that the glycosylation status determines monolignol transport and subcellular compartmentation (Miao and Liu, 2010). Plasma membrane-derived vesicles prepared from *Arabidopsis* and poplar cells transported coniferyl alcohol (the aglycone form), but not coniferin (the glycosylated form of coniferyl alcohol), in an ATP-dependant manner, whereas tonoplast-derived vesicles transported the glycosylated form but not the aglycone. Taken together these results would suggest that coniferyl alcohol is transported into the cell wall across the plasma membrane in an ATP dependant process by an ABC-transporter and is subsequently polymerized by laccases (LACs) and/or peroxidases (PRXs). In contrast, the glycosylated form (e.g., coniferin) is transported into the vacuole for storage. Genetic confirmation of the involvement of ABC transporters in monolignol transport into the cell wall was recently reported (Alejandro et al., 2012). Co-expression studies in *Arabidopsis* identified an ABCG transporter gene (*AtABCG29*) co-regulated with phenylpropanoid gene expression and that was expressed in lignin-containing organs and tissues. Protein-fusion studies demonstrated that *AtABCG29* was localized in the plasma membrane and transport studies in yeast suggested that the protein was involved in transporting *p*-coumaryl alcohol, but not coniferyl alcohol. A minor activity was also observed towards sinapyl alcohol. *Abcg29* mutant lines showed reduced root growth when grown on medium containing *p*-coumaroyl alcohol, but not coniferyl alcohol or sinapyl alcohol supporting the idea that *AtABCG29* is involved in *p*-coumaryl alcohol transport. Lignin analyses of roots from two mutant lines revealed decreases in the amounts of H, G, and S subunits as compared to WT roots, an unexpected result since *AtABCG29* is highly specific for *p*-coumaryl alcohol and it would be expected that only H units would be reduced. Interestingly, the mutant lines also showed metabolomic modifications in soluble phenolics, flavonoids and glucosinolates together with reductions in associated gene expression. These modifications are reminiscent of changes observed in different lignin mutants (Vanholme et al., 2012b) underlining the fact that phenylpropanoid metabolism can be perturbed not only at the biosynthesis level, but also by modifications of monolignol transport. The results also raise the possibility that the observed cell-specific variation in lignin composition (e.g., vessel element walls are rich in G lignin whereas fiber walls contain S-G lignin) may also be regulated via specific ABC transporters. Plant genomes contain large ABC transporter gene families (e.g., 130 genes in *Arabidopsis*) but ABC transporters for coniferyl alcohol and sinapyl alcohol remain to be identified and characterized. In addition, it is probable that monolignol glucosides from the vacuole participate to tracheary element lignification following cell death and membrane disruption (Pesquet et al., 2013). Additional information on the potential role(s) of ABC transporters in the lignification process is discussed by Sibout and Hofte (2012).

MONOLIGNOL GLYCOSYLATION AND DEGLYCOSYLATION

Glycosylation of monolignols is catalyzed by UDP-glycosyltransferases (UGTs) belonging to the glycosyltransferase (GT) family 1.

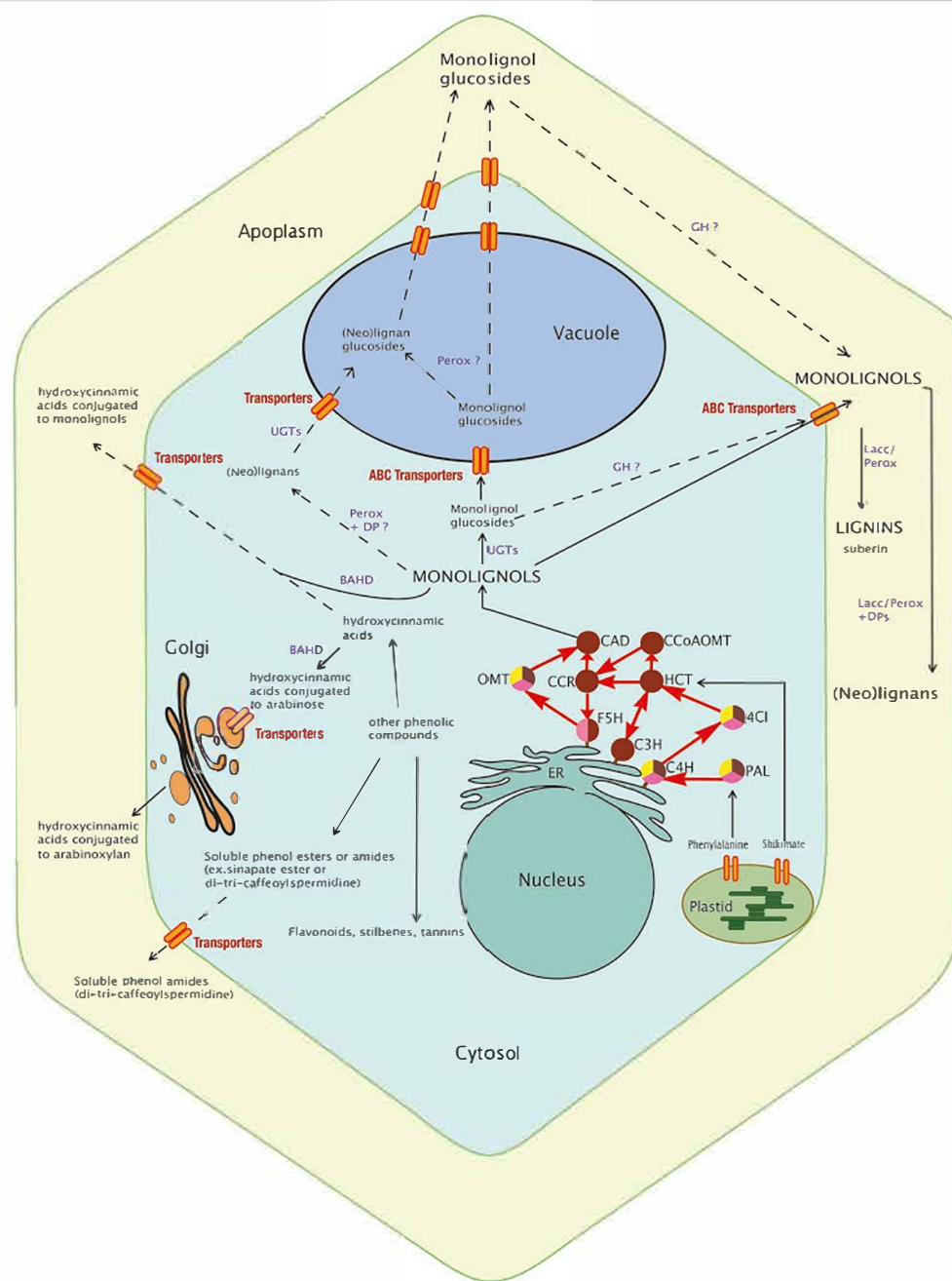


FIGURE 1 | Schematic view of the monolignol biosynthetic pathway. The synthesis of monolignols from phenylalanine and shikimate involves cytosolic (PAL, HCT, 4CL, CCR, CAD, CCoAOMT, and OMT) and ER membrane-anchored (the cytochrome P450 enzymes F5H, C3H, and C4H) enzymes. Monolignols and lignans may be conjugated by UGTs and then transported to the vacuole or directly transported to the cell wall for oxidative cross-linking by apoplastic peroxidases and laccases into lignins. In grasses, some hydroxycinnamic acids are conjugated to arabinose by BAHD enzyme for export via the Golgi apparatus and future incorporation into polysaccharides (pectins or arabinoxylans) whereas some are conjugated to monolignols (mainly sinapyl alcohol) in the cytosol and incorporated into lignin. Other phenolic compounds (soluble phenolic esters and amides, flavonoids) are shown and may indirectly interact with lignification. Their production implies common steps (enzymes) with the monolignol pathway. Most of these transport routes and storage forms still remain to be discovered and the localization of some enzymes (GH,

Perox, Lacc) confirmed. The dashed lines delineate putative pathways and full lines delineate known routes. Enzymes of the monolignol pathway are represented by circles. The brown color indicates involvement of the enzyme in the monolignol pathway; yellow indicates involvement in the flavonoid pathway; and pink indicates involvement in the sinapate ester pathway. PAL, phenylalanine ammonia lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-hydroxycinnamoyl-CoA ligase; HCT, hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyl transferase; C3H, *p*-coumaroyl shikimate 3'-hydroxylase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, hydroxycinnamoyl-CoA reductase; F5H, ferulic acid 5-hydroxylase; COMT, caffeic acid/5-hydroxyferulic acid *O*-methyltransferase; CAD, cinnamyl alcohol dehydrogenase; UGT, UDP-glycosyltransferase; GH, beta-glucosidase; Perox, peroxidase; Lacc, laccase; DPs, dirigent proteins; BAHD, hydroxycinnamic acid transferase; ER, endoplasmic reticulum.

These enzymes play an important role in stabilization, enhancement of water solubility and deactivation/detoxification of a wide range of natural products including hormones and secondary metabolites (Lim and Bowles, 2004). A recent phylogenetic reconstruction (Caputi et al., 2012) of family 1 UGTs reveals a dramatic evolutionary increase in the number of family 1 GTs from one in the model green alga *Chlamydomonas* to 243 in poplar reflecting the increased complexity of plant life on Earth. Less than 20% plant UGTs have been functionally characterized (Yonekura-Sakakibara et al., 2012). A major problem is that plant GTs may be redundant. Functional analyses in *Arabidopsis* (Lanot et al., 2006) of the UGT72E clade suggested that the UGT72E2 gene product was responsible for monolignol glycosylation. Chemical analyses of soluble phenolics in light-grown roots from the triple mutant showed a significant decrease in the quantity of coniferyl alcohol- and sinapyl alcohol-glucosides as compared to WT plants. Over-expression of the UGT72E2 gene resulted in a 10-fold increase in coniferin levels and a lower increase in sinapyl alcohol glucoside in roots suggesting that this is the principal gene involved in monolignol glycosylation in *Arabidopsis*. Interestingly, rosette leaves accumulated sinapyl glucoside to higher levels than coniferyl alcohol glucoside hinting at organ-specific variations in glycosylation mechanisms. However, no impact on lignin content/composition was observed. Evidence suggesting a link between lignification and monolignol glycosylation was recently demonstrated (Wang et al., 2012) when the over-expression in tobacco plants of a poplar UGT (*PtGT1*) homolog to the *Arabidopsis* UGT72E1-3 genes was shown to be associated with an increased lignification as well as early flowering. Nevertheless, the observation that recombinant *PtGT1* showed no activity toward monolignols and the fact that transformed plants did not contain higher amounts of monolignol glycosides led the authors to suggest that the observed impact of *PtGT1* over-expression in tobacco was indirect. Further research is clearly needed to identify not only the UGTs specifically involved in monolignol glycosylation, but also to demonstrate a functional link between modifications in UGT gene expression and lignification. The potential importance of monolignol glycobiochemistry in regulating lignin biosynthesis is also complicated by the fact that, in addition to glucose, plant UGTs can also use different sugars (glucose, rhamnose, galactose, glucuronic acid, etc.) to glycosylate monolignols (Caputi et al., 2012).

If monolignol glucosides give rise to monolignols that are then incorporated into the lignin polymer, then enzymes (beta-glucosidases) should be co-localized with coniferin and syringin. Beta-glucosidases capable of hydrolyzing monolignol glucosides were first identified in cell wall fractions of *Picea abies* hypocotyls and roots (Marcinowski and Grisebach, 1978). Subsequently, a beta-glucosidase showing high affinity for coniferin was co-localized with PRX activity to cell walls of young differentiating xylem in *Pinus contorta* (Dharmawardhana et al., 1995, 1999). In Angiosperms, beta-glucosidase activity was first located in the cell walls of *Cicer arietinum* L. (Hosel et al., 1978; Burmeister and Hosel, 1981). The co-localization of beta-glucosidase activity and monolignol glucosides in differentiating xylem of conifers and some angiosperms led to the hypothesis that monolignol glucosides could represent lignin precursors. However, subsequent work

in *Ginkgo biloba* (Tsuji et al., 2005) showing that a beta-glucosidase demonstrated a 10 times greater affinity toward coniferaldehyde as compared to coniferin threw doubt on this initial idea. Subsequent work with radioactive precursors indicated that coniferyl alcohol derived from coniferin was only weakly incorporated into the lignin polymer and that greater amounts were converted into coniferaldehyde glucoside followed by subsequent deglycosylation and CAD-mediated conversion of the coniferaldehyde into coniferyl alcohol. These results suggested that coniferin represented the storage form of coniferyl alcohol and was not the direct precursor of coniferyl alcohol for lignin. In *Arabidopsis*, three orthologs (BGLU45/At1g61810; BGLU46/At1g61820; BGLU47/At4g21760) of the *Pinus contorta* beta-glucosidase gene are present and belong to the group 10 of the *Arabidopsis* GH1 family (Xu et al., 2004). Heterologous expressions of BGLU45 and BGLU46 showed that BGLU45 is highly specific for the three monolignol glucosides, whereas BGLU46 preferentially uses *p*-coumaryl alcohol glucoside as well as showing broad activity toward other phenolic glucosides (Xu et al., 2004; Escamilla-Trevino et al., 2006). A recent functional study of *Arabidopsis* *bglu45-1* and *bglu46-2* T-DNA mutants (Chapelle et al., 2012) showed only slight reductions (8.5 and 6.5%, respectively) in global beta-glucosidase activity and protein amounts as estimated by Western blots following Concanavalin-A column chromatography, presumably because of functional redundancy – at least four other glucosidases are expressed in *Arabidopsis* stems – and antibody cross-reactivity. Lignin analyses showed a slight but significant increase in Klason lignin for two BGLU45 mutants of the WS accession (*bglu45-1* and *bglu45-3*) under long-day conditions only but not in Col0 *bglu45* mutants. No significant modification in lignin content was observed for *bglu46* mutants. Similarly, thioacidolysis revealed no changes in lignin subunit structure. Metabolomics (ultra performance liquid chromatography–mass spectrometry, UPLC–MS) identified around 100 different small glycosylated phenolic compounds, most of which were increased in the mutants. For the 10 most important differentials, five were identified/partially identified and all were derived from G units. Interestingly, an important ecotype effect was observed with, for example, an approximate 20-fold increase of coniferin in WS *bglu45-1* mutants, but only a 3.4-fold increase in Col10 mutants. While coniferyl alcohol, sinapyl alcohol, coniferaldehyde, sinapaldehyde, and syringin levels were not significantly modified, certain (neo)lignans increased [lariciresinol hexoside or isodihydrodehydroconiferyl alcohol (IDDDC) hexoside, dehydrodehydroconiferyl alcohol (DDC) hexoside]. Based on these observations, the authors proposed that monolignol glucosides are the storage form of monolignols and not the direct precursors. Nevertheless, it is interesting to note that BGLU45 and BGLU46 expression are deregulated under various biotic stresses suggesting that the monolignol storage pool might be used under pathogen attack in the fabrication of “defense lignin” and/or phytoalexins. Certainly, such a hypothesis allows a better understanding of the basic “spatial problem” associated with beta-glucosidase enzyme and substrate compartmentation (Figure 1). As indicated above, the *Pinus contorta* beta-glucosidase was localized to differentiating xylem walls and BGLU45/46 proteins are secreted to the cell wall (Chapelle et al., 2012), whereas glycosylated monolignols are stored in the

vacuole. Under such conditions it is perhaps not surprising that beta-glucosidase gene down-regulation has little/no effect on lignin levels under standard conditions and that the critical step occurs at the glycosylation step deciding whether monolignols are targeted to the cell wall without glycosylation for lignification, or glycosylated for storage in the vacuole. In contrast, cell and vacuole disruption following pathogen attack, insect/herbivore feeding and potentially severe abiotic stress (cell freezing, desiccation) or during programmed cell death (xylem vessel) would lead to contact between the monolignol glucosides and beta-glucosidases at the cell wall where PRXs and/or LACs could rapidly polymerize the aglycone forms. The role of beta-glucosidases in producing a wide range of bioactive defense molecules has been largely documented in plants (Morant et al., 2008).

Taken together, these observations indicate that the glycosylation/deglycosylation of monolignols play key roles in determining their availability for subsequent lignin biosynthesis thereby representing potentially interesting engineering targets for plant biomass improvement. In addition, similar mechanisms are also likely to affect the availability of ferulic- and *p*-coumaric acids for lignin–hemicellulose/pectin and polysaccharide–polysaccharide covalent cross-links in the cell wall underlining the necessity for further research into the glycosylation/deglycosylation of monolignols and phenolic molecules.

DIMERIZATION OF MONOLIGNOLS AND SYNTHESIS OF (NEO)LIGNANS

Monolignols can also give rise to more than 3,000 different lignans and associated structures such as neo-, sesqui-, and flavanolignans (Schmidt et al., 2010). Lignans are optically active phenylpropanoid dimers that result from the stereo-selective dirigent protein-mediated coupling of the 8 and 8' C-atoms of two hydroxycinnamyl alcohol (monolignol) moieties (Davin et al., 1997). (Note: two different nomenclatures are used to describe the propane carbon atoms involved in monolignol linkages in the lignin polymer and in oligolignols. In oligolignols, the numbers 7, 8, and 9 are used whereas in the lignin polymer α , β , and γ are used to describe the same carbon atoms. A β – β lignin linkage therefore corresponds to an 8–8' *oligolignol* linkage). Lignan formation has been widely studied since these molecules are natural anti-oxidants, possess anti-microbial activity and have numerous beneficial effects on human (Ford et al., 2001). Lignans are known to play a role in the durability, longevity, and resistance of the heartwood of many tree species against wood-rotting fungi and are believed to function as phytoalexins in other (non-woody) plant species (Pohjamo et al., 2003). The formation and accumulation of lignans in flax (*Linum usitatissimum* L.) seeds has been particularly studied since they are one of the richest natural grain sources of certain lignans. The genus *Linum* contains approximately 200 species worldwide that can be divided into two main groups depending upon the major lignan types accumulated – either cyclolignans belonging to the aryltetralin series (AT lignans) or cyclolignans of the arylidihydronaphthalene (ADN)/arylnaphthalene (AN) group (Schmidt et al., 2010). Some plants in both groups can also accumulate dibenzylbutyrolactone (DBBL) lignans such as matairesinol and furofuran (FF) lignans such as pinoresinol. Both FF and DBBL lignans

are precursors of the AT/ADN/AN lignans. In the seeds of flax, dimerization of two E-coniferyl alcohol molecules leads to the formation of 8S,8'S-(–)-pinoresinol, which is sequentially converted into 8S,8'S-(–)-lariciresinol and 8S,8'S-(++)-secoisolariciresinol via the action of the enzyme pinoresinol lariciresinol reductase (PLR). (++)-Secoisolariciresinol is then glycosylated to secoisolariciresinol diglucoside (SDG) that accumulates to high levels in flax seeds (linseed; Ford et al., 2001). The complexity of lignan stereochemistry was underlined in a recent study (Hemmati et al., 2010) that isolated two PLRs (PLR-Lu1, PLR-Lu2) showing opposite enantiospecificity. PLR-Lu1 utilizes 8S,8'S-pinoresinol, whereas PLR-Lu2 uses 8R,8'R-pinoresinol. Interestingly, gene expression analyses have shown that the *LuPLR1* gene is expressed in different flax seed tissues, whereas the *LuPLR2* gene is expressed in flax stem and leaf tissues (Hano et al., 2006; Hemmati et al., 2010; Venglat et al., 2011). In agreement with this organ-specific gene expression, 8S,8'S-SDG accumulates in flax seeds whereas aerial green parts accumulate 8R,8'R-SDG. Further analyses (Schmidt et al., 2012) of lignans in 16 different *Linum* species suggested that lignan stereochemistry also depended upon the species analyzed and whereas R,R-SDG was mainly accumulated in the seeds from species containing relatively high amounts of ADN/AN cyclolignans, S,S-SDG was accumulated in seeds from species containing only trace amounts of cyclolignans.

A further insight into monolignol/lignan biology in flax was provided by a combined transcriptomics and metabolomics study (Huis et al., 2012) of flax inner- (xylem rich) and outer- (bast fiber rich) stem tissues (Figure 2). More than eighty different lignans and neolignans including pinoresinol, lariciresinol, secoisolariciresinol, DDC, and IDDDC, as well as their glycosylated forms were detected in both tissues. In agreement with the presence of lignans and neolignans in the flax stem, transcripts corresponding to PLR, as well as the enzyme phenylcoumaran benzylic ether reductase (PCBER) catalyzing the reduction of DDC into IDDDC and previously identified as the most abundant protein in poplar xylem (Gang et al., 1999; Vander Mijnsbrugge et al., 2000) were also highly abundant. Although PCBER transcripts were more highly abundant in inner stem tissues, IDDDC was not detected in either inner- or outer-stem tissues, despite the presence of DDC in both tissues. In contrast IDDDC hexosides were detected in both stem tissues suggesting that the neolignan is rapidly glycosylated. Overall, the glycosylated forms were more abundant in outer stem tissues in comparison with inner stem tissues. Further work in other species is necessary to know whether such differences in (neo)lignan glycobiology are widespread and also the potential biological role. Interestingly, recent reports showing that lignans are detected in the phloem tissues (Ramos and Kato, 2012) and honey-dew of the stink-bug and that glycosylated flax lignans but not the aglycone forms significantly increased aphid *Myzus persicae* insect mortality (Saguez et al., 2012) raise the possibility that monolignol-derived compounds might be glycosylated and transported in phloem cells as a defense mechanism against insect predators.

The abundance of glycosylated (neo)lignans in flax and other species suggests the existence of different UGTs capable of glycosylating lignans. Lignan UGT activity was first identified in sesame (Noguchi et al., 2008) and more recently in *Forsythia* (Ono

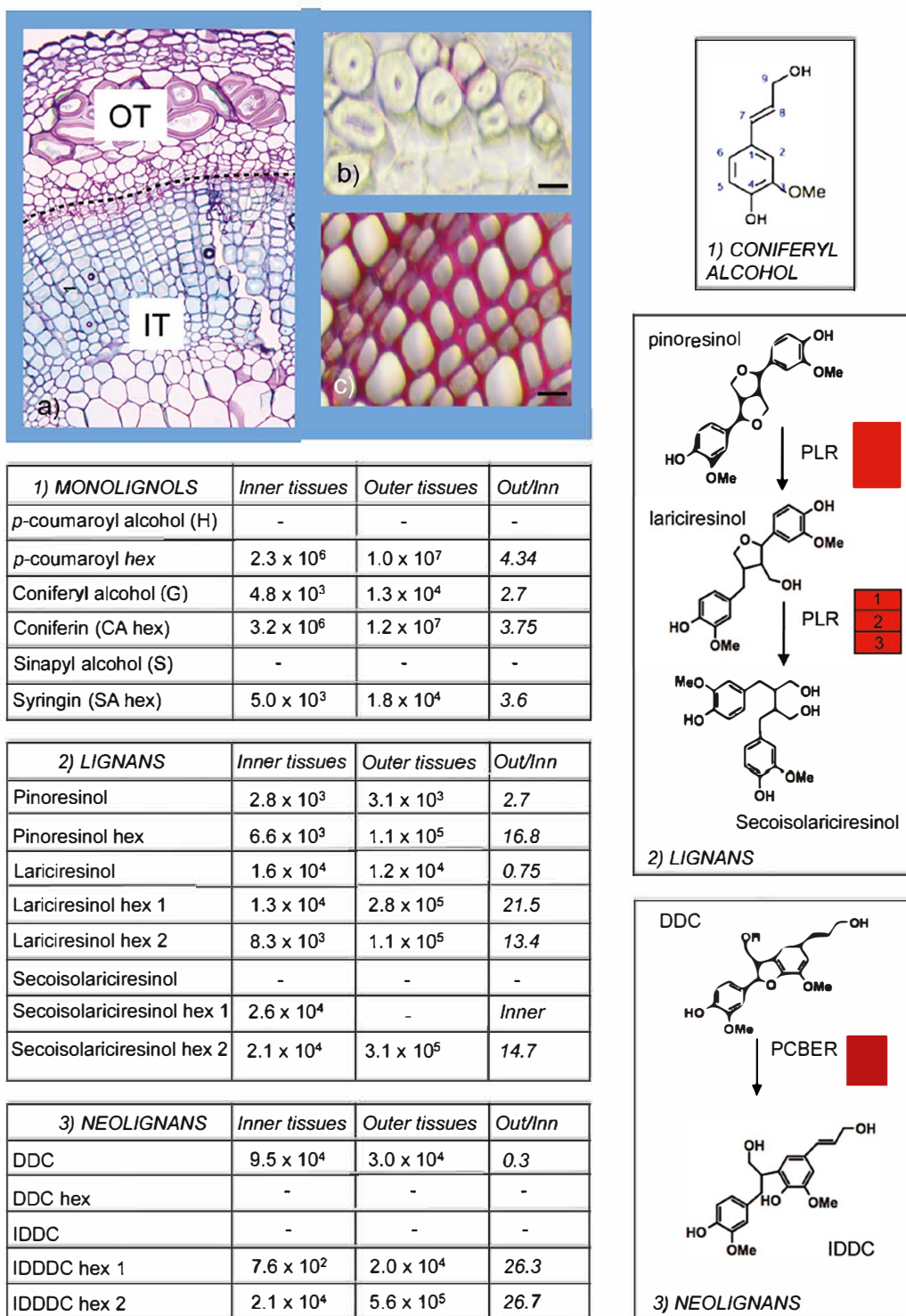


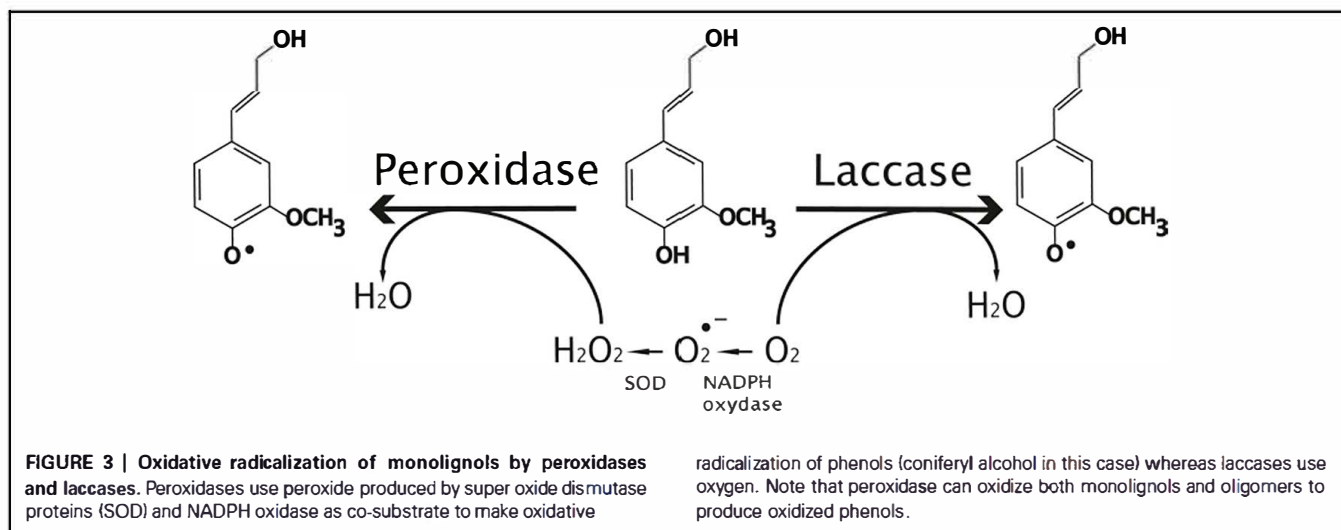
FIGURE 2 | Monolignol metabolism in flax stems. Oligolignols were extracted from inner (IT) and outer (OT) tissues of flax stems (A) and analyzed by ultra-high performance liquid chromatography-Fourier transform-ion cyclotron resonance-mass spectrometry (UHPLC-FT-ICR-MS; Huis et al., 2012). Outer tissues are characterized by the presence of bast fibers with thick cellulose-rich secondary cell walls and extremely low lignin levels as indicated by limited red coloration with phloroglucinol-HCl staining (B). Inner tissues contain xylem tissue with heavily lignified cell walls (C). Aglycone and glycosylated forms of monolignols (1), lignans (2), and neolignans (3) were detected in both inner- and outer-stem samples. Glycosylated forms were generally more abundant in outer stems. DDC, dehydrodiconiferyl alcohol; IDDC, isodihydrodehydrodiconiferyl alcohol; PLR, pinoresinol lariciresinol reductase; PCBER, phenylcoumaran benzylic ether reductase. Red heat blocks indicate corresponding unigene expression in inner- versus outer-stem tissues. This figure illustrates the extremely rich metabolism post-monolignol biosynthesis. (A) Courtesy of Anne-Sophie Biervacq, Université Lille.

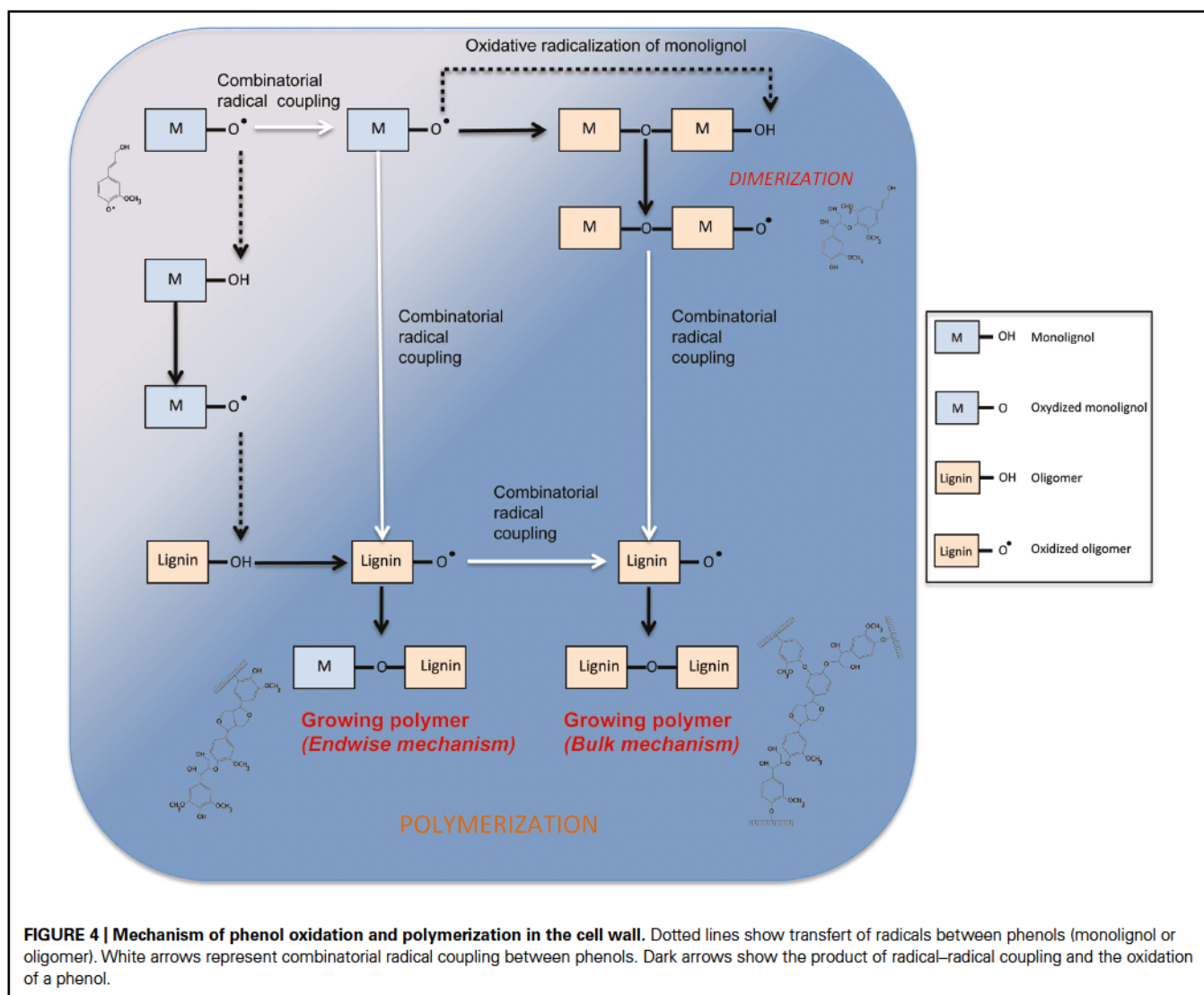
et al., 2010). Subsequent screening of a *Forsythia* cDNA library and biochemical characterization of recombinant UGT71A17 and UGT71A18 proteins showed that both proteins were capable of glycosylating (+)-pinoresinol albeit UGT71A17 activity was much weaker than UGT71A18 activity. Further characterization of the latter showed that it was active against different FF and DBBL lignans. Expression analyses indicated that both genes were expressed in leaves, floral buds, and petals, but that only *UGT71A18* was expressed in cell suspension cultures. It would obviously be interesting to see whether *UGT* genes are also expressed in other organs/tissues. Monolignol dimerization is presumably catalyzed by class III PRXs that are localized in the cell wall and/or vacuole suggesting that lignans should also be localized in these compartments. Although one study (Attoumbre et al., 2010) localized lignans to the cell wall in flax seeds, a positive signal was also observed in cytoplasmic inclusions raising the possibility that lignans might also be present in this compartment. Plant UGTs, unlike their mammalian counterparts, do not possess peptide signals and are therefore probably localized in the cytoplasm. In this case, where does (neo)lignan glycosylation take place – do plants possess plasma membrane/tonoplast ABC transporters capable of transporting neolignans into/out of cells/tonoplasts? In conclusion, although we now have a relatively good understanding of monolignol biosynthesis (even if our knowledge of transcriptional and post-transcriptional regulation processes is far from complete), it is clear that we have much to learn about the mechanisms regulating the availability of synthesized monolignols in the cell wall for subsequent lignification. A better understanding of these mechanisms should lead to the elaboration of new targets for engineering more efficient plant biomass.

POLYMERIZATION

Beyond dimerization formation of lignins requires a polymerization step. The complex structure of the polymers built up of different subunits (H, G, and S), the presence of different types of linkages between subunits and the absence of repeated motifs suggest a highly unpredictable polymerization mechanism probably affected by its microenvironment (pH, polysaccharides, etc.;

Wingender et al., 1999). In the developing cell wall, lignin polymerization occurs by radical coupling reactions (Freudenberg, 1959; Figures 3 and 4). Type III PRXs and LACs are probably both involved in monolignol oxidation and participate to lignification (Figure 3). PRXs involved in lignification are exported to the apoplast in lignifying tissues where monolignol oxidation takes place (Koutaniemi et al., 2005; Vanholme et al., 2010). The oxidation reaction uses H_2O_2 as co-substrate which is probably provided by combined action of NADPH oxidase or germin-like proteins, even though firm genetic evidence for the involvement of these enzymes is currently lacking (Davidson et al., 2009). PRX exist as large multigene families (73 and 138 in *A. thaliana* and *Oryza sativa*, respectively; Welinder et al., 2002) making the clarification of the biological role of each member a considerable challenge. Clear characterization is also complicated by the fact that PRXs generally show low substrate specificities (Hiraga et al., 2001). Nevertheless recent studies suggesting that some PRX isoforms may be more specialized in the polymerization of guaiacyl or/and syringyl units could help to explain how plants are able to control lignin monomeric structure (Barcelo et al., 2007; Martinez-Cortes et al., 2012). Both the isoelectric point of the proteins and specific motifs in the PRX primary structure could support protein–substrate interaction with one monomer type over another and therefore influence lignin composition. Two examples of genetic manipulation illustrate this hypothesis. Antisense suppression of one PRX gene led to a global reduction of both G and S units in tobacco (Blee et al., 2003) whereas analyses of PRX down-regulated poplar (Li et al., 2003) revealed that lignin contained less G units but that S content was unchanged. More recently, Herrero et al. (2013a) used *Zinnia elegans* PRX protein sequence data to identify *Arabidopsis* orthologs for functional characterization. Histochemical analyses of the *atprx72* mutant suggest that it has reduced overall lignin levels and a low syringyl unit content as compared to WT plants (Herrero et al., 2013b). However, the mutant shows a strong developmental phenotype and it would be necessary to confirm this result on a second mutant allele. In parallel, Shigeto et al. (2013) used similar strategies to functionally characterize other PRX gene candidates in





Arabidopsis. Chemical analyses indicated a significant decrease in the total lignin content and modified lignin structure in *atprx2* and *atprx25* mutants and altered lignin structure in *atprx71* mutants.

The mechanism of oxidative radicalization of phenols followed by combinatorial radical coupling is well described (Vanholme et al., 2010; van Parijs et al., 2010). Nevertheless, the kinetics of monomer incorporation into the lignin polymer by PRX remains poorly understood. In a recent article, Demont-Caulet et al. (2010) propose a general scheme for S–G copolymerization coordinated by a PRX (PRX34) purified from *A. thaliana*. The purified enzyme shows a high substrate specificity and seems unable to directly oxidize S monomers in the absence of G units. The authors studied kinetics of the monomer conversion and subsequent formation of di-, tri-, and tetramers by using monomer feeding experiments. Their results suggest that endwise and bulk polymerization mechanisms are likely to co-exist within the same system, the bulk mechanisms occurring after total consumption of the monomers. During endwise polymerization, monomers are added to the polymer one-by-one whereas during bulk polymerization different

oligomers are linked together at the same time (Figure 4). Interestingly, Demont-Caulet et al. (2010) show that while monomer availability may influence the relative preponderance of the two mechanisms, it is the nature of the monomer that exerts the most influence. Indeed, under *in vitro* oxidation conditions, the presence of S monomer converted the PRX34-mediated polymerization of G monomers from a bulk to an endwise mechanism in agreement with data from Argyropoulos et al. (2002). These observations underline the central role of PRXs in controlling lignin biosynthesis. In a recent article, the group of Niko Geldner proposed a very nice model where PRXs are the last pieces of a puzzle that orchestrate lignification in Casparian strips in *Arabidopsis* roots (Lee et al., 2013). The authors show that the PRX enzymes colocalize with the very specific Casparian strip domain protein (CASP) and that their activity is indirectly under the control of an NADPH oxidase named respiratory burst oxidase homolog F protein (RBOHF) that provides H_2O_2 to PRXs via the action of an unknown superoxide dismutase (SOD). These fascinating results show for the first time that a *trans*-membrane protein

(CASP) is able to control peroxidase activities thereby representing a potential mechanism for localized spatial control of lignification.

Therefore, PRXs represent interesting targets for modifying not only lignin content but also lignin structure, even if high redundancy due to the existence of large gene families complicates genetic approaches. It is also important to emphasize that PRXs exported to the extracellular matrix of some tissues may not only be involved in lignification. For instance, they may participate to suberin polymerization, cell membrane protection or else, catalyze di-ferulate bonds between polysaccharides particularly in grasses where arabinoxylans are highly feruloylated (Fry, 2004; Arrieta-Baez and Stark, 2006). Tannins that contribute to the heartwood color in some trees (e.g., walnut), as well as lignans (see above) may also be produced by PRXs.

Laccases are a large group of copper-containing glycoproteins in plants, bacteria, and fungi. In contrast to PRXs, LACs use O_2 instead of H_2O_2 to oxidize the monolignols (Figure 3). Although there is not enough evidence to confirm that plant LACs are localized in the cell wall, the presence of signal peptides in the N-terminal protein region suggests a subcellular localization (Cesarino et al., 2013) whereas some PRXs could be detected in other compartments. A number of *in vitro* experiments have identified different putative LAC substrates including flavonoids, 4-Hydroxyindole, coniferyl alcohol, *p*-coumaryl alcohol, and sinapyl alcohol (Mayer and Staples, 2002). While certain studies confirm that tree LACs can polymerize monolignols *in vitro* (Sterjiades et al., 1992; Ranocha et al., 2002), the broad substrate specificity of plant LAC needs to be confirmed *in vivo*. LACs, like PRXs, belong to (smaller) multigenic families in plants and different members may have redundant or specific biological functions. This is based upon two contrasting observations showing that in single antisense poplar plants with reduced LAC transcripts, neither lignin content nor composition is significantly altered (Ranocha et al., 2002). In contrast, the transgenic poplars exhibited an increase in total soluble phenolic content and perturbation of xylem fiber integrity (Ranocha et al., 2002). In *Arabidopsis*, 17 LACs have been reported (McCaig et al., 2005) but until 2011, only indirect evidence linked these enzymes to lignification in *Arabidopsis*. *AtLAC2* mutants showed a reduced root elongation under PEG treatment suggesting a possible impact on vasculature (Cai et al., 2006) and *AtLAC4* mutants under continuous light conditions were associated with a weak irregular xylem phenotype (Brown et al., 2005). Similarly, the *Arabidopsis AtLAC15* mutant was identified during a transparent testa screen since it possesses seeds with a pale brown seed coat due to altered flavonoid condensation (Pourcel et al., 2005). Mutants also showed a 59% increase of soluble proanthocyanidins when compared to wild-type and the extractable lignin content in *tt10* seeds was reduced by nearly 30% (Egertsdotter et al., 2004). This observation would suggest that both monolignols and proanthocyanidins could serve as substrates for *AtLAC15* in *Arabidopsis* seed coats. Transcriptomics (Ehlting et al., 2005) indicate that four LACs (*AtLAC4*, *AtLAC11*, *AtLAC12*, *AtLAC17*) show moderate or high expression levels in *Arabidopsis* developing stems. These four genes are up-regulated during the transition from young to intermediate stage stems and were therefore considered as good targets for modifying lignin.

Direct evidence showing that specific LACs are involved in monolignol polymerization *in planta* was obtained by the characterization of *Arabidopsis lac4lac17* double mutants (Berthet et al., 2011). The stems of double mutants possess collapsed vessels and show reduced G lignin deposition in fibers. Mutants also show a 20–40% decrease in total lignin content and a 130% increase in saccharification yields when compared to the wild-type control. Lignin composition (higher S/G ratio) is also modified and is mainly due to the decrease of G lignin in fibers. This is not surprising since LAC and lignin genes are tightly co-expressed (Berthet et al., 2011) and promoter-GUS analyses showed that *LAC4* is expressed in vascular bundles and interfascicular fibers of stems while *LAC17* is specifically expressed in fibers (Berthet et al., 2011). Phylogenetic analyses of *Arabidopsis* LACs showed that *LAC4* and *LAC17* are the best homologs (McCaig et al., 2005) suggesting redundant or at least similar activity for both proteins in lignification. Recently, Cesarino et al. (2013) identified a sugarcane LAC gene, *SofLAC*, putatively involved in lignification. Its transcripts are localized in the inner- and outer-lignified zones of stem sclerenchymatic bundle sheaths. *SofLAC* was expressed preferentially in young internodes and the corresponding transcript level decreased along with stem maturity. The *Arabidopsis lac17* mutant was partly complemented by expressing *SofLAC* under the control of the *AtLAC17* promoter. *SofLAC* expression restored the lignin content but not the lignin composition in complemented *lac17* mutant lines. These results suggest that specific LACs may also play a role in lignification in monocots.

Studies on mutants affected in different specific steps of monolignol biosynthesis indicate that plants are able to incorporate substantial amounts of components (e.g., aldehydes, 5-hydroxyconiferyl alcohols) that are poorly incorporated in “normal” conditions. Currently, it is not known whether incorporation is mediated by PRXs or LACs, or by both proteins. Some attempts have also been made to engineer plants so as to incorporate unusual compounds (e.g., rosmarinic acid or hydroxybenzaldehyde and hydroxybenzoate derivatives) into their lignins (Eudes et al., 2012; Tobimatsu et al., 2012). The incorporation of such specific compounds has been proposed as a strategy to reduce the degree of lignin polymerization. The incorporation of novel compounds together with the existence of large LAC and PRX gene families showing broad substrate specificities raises exciting new possibilities for the production of modified lignins and improved biomass.

ACYLATION

Plant lignins often contain various acids (acetates, *p*-hydroxybenzoate, and *p*-coumarate) in addition to conventional monolignols. It is usually accepted that some of them (ferulate, *p*-coumarate) are by-products or intermediates of the monolignol pathway since there is no evidence that an independent pathway exists. Several observations suggest that the degree of acylation could be reasonably expected to impact on biomass quality. First, amounts of acylated monomers show high natural variations between different species. Kenaf and Agave possess up to 60 and 80% of acylated lignins, respectively when compared to maize and wheat that show 18 and 3% of acylated lignins, respectively

(reviewed by Withers et al., 2012). Second, it is possible that acylation may affect lignin structure during polymerization as well as subsequent degradation by chemical or biochemical processes such as saccharification and digestibility. In grasses, *p*-coumarates are linked to syringyl units and plants with low coumarate levels also generally show modifications in their S, or S and G content (Harrington et al., 2012). Importantly, coumaroylation of cell wall polysaccharides is most likely (or at least partly) catalyzed in the cytosol prior to export and polymerization in the cell wall strongly suggesting the existence of specific monolignol transferases and transporters (Withers et al., 2012; Molinari et al., 2013; Figure 1).

Until recently, the genetics underlying coumarylation of both lignin and arabinoxylans remained unknown, mainly because of the lack of suitable plants (specifically engineered to modify acylation) for functional characterization. It is generally accepted

that the genes associated with such reactions would encode BAHD acyl-CoA transferase proteins. Recent work showing that transgenics have higher or lower feruloylation levels has suggested that some members of the BAHD acyl-CoA transferase protein family may be responsible for catalyzing the addition of ester-linked coumarate to cell wall arabinoxylans (Fernando et al., 2009; Withers et al., 2012; Bartley et al., 2013; Molinari et al., 2013). A member of the BAHD acyl transferase family was recently identified in the rice genome (Withers et al., 2012). Analyses of the recombinant protein produced in *E. coli* showed that it had a specific coumaroyl transferase activity. This enzyme OsPMT (*O. sativa p*-coumaric acid monolignol transferase) catalyzes the acylation of a monolignol with *p*-coumaric acid via *p*-coumaroyl CoA and the corresponding transcript is co-expressed with the monolignol biosynthetic 4CL gene. Based on catalytic efficiency

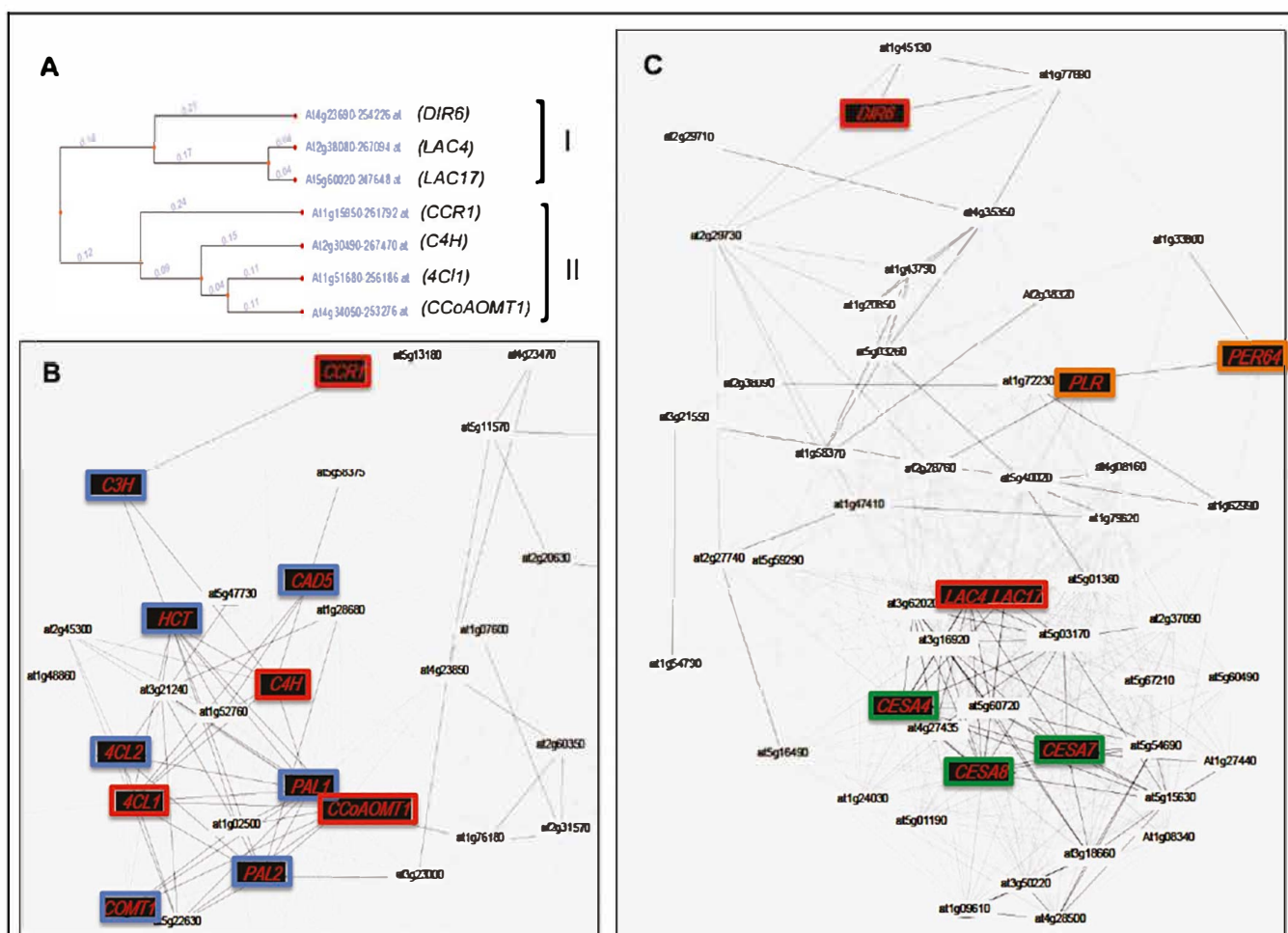


FIGURE 5 | Co-expression network of genes involved in lignin and lignan biosynthesis in *Arabidopsis*. (A) Dendrogram representing the co-expression of genes involved in the biosynthesis and polymerization of monolignols. Two separate clusters (II and I, respectively) are obtained using the tool "expression tree" from Genecart (<http://genecart.mpg.de>). (B) Co-expressed gene network of genes present in cluster II. (C) Co-expressed gene network of genes present in cluster I. Nodes and edges represent genes and significant positive co-expression relationships between genes. Red nodes represent genes used as baits to produce gene co-expression

networks. Blue nodes depict genes known to be involved in monolignol biosynthesis. Green nodes represent specific genes involved uniquely in cellulose synthesis of secondary cell walls. Orange nodes represent genes putatively involved in polymerization and lignan formation. DIR6, dirigent protein 6; LAC4, laccase 4; LAC17, laccase 17; CCR1, hydroxycinnamoyl-CoA reductase 1; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-hydroxycinnamoyl-CoA ligase 1; CCoAOMT1, caffeoyl-CoA O-methyltransferase; PLR, pinorensinol-lariciresinol reductase; PER64, peroxidase 64; CES4, 7, 8, cellulose synthase 4, 7, and 8. Other abbreviations are given in Figure 1.

and reaction rate, the authors show that OsPMT would produce more *p*-coumaryl *p*-coumarates in saturating conditions but would favor the synthesis of sinapyl *p*-coumarate over coniferyl *p*-coumarate *in planta*. Interestingly this gene is grass specific and its enzymatic hallmark therefore perfectly correlates with the high level of coumaroylated sinapyl alcohols in these types of plant. In addition, the BAHD protein accepts coumarate, but not ferulate as a substrate. The production of rice plants with disrupted OsPMT expression may be difficult because of gene redundancy since there are at least two paralogs in this species. Nevertheless, it is interesting to note that there are less paralogs in *Brachypodium* (Withers et al., 2012) and the recent article published (Molinari et al., 2013) could help to identify suitable candidate genes for further study. The authors established a correlation between candidate *p*-coumaric acid monolignol transferase transcript levels and amounts of bound ferulate in different organs at different developmental stages. They propose different candidate genes similar to OsPMT genes to be responsible for the lignin bound *p*-coumaric acid.

CO-EXPRESSION DATA MAY HELP TO SCREEN FOR PROTAGONIST INVOLVED IN THE LAST STEPS OF LIGNIN BIOSYNTHESIS

The last 20 years have seen the elucidation of the complete monolignol biosynthetic pathway and a number of limiting enzymes have been identified. Importantly, the expression of monolignol biosynthesis genes is not always correlated with the presence of the lignin polymer. This observation can be attributed to the fact that the monomers can also be used to generate a wide range of monolignol-derived compounds located in intra- or extracellular compartments and underlines the complexity of plant monolignol metabolism and regulation (Figure 1). One illustration of this phenomenon can be seen with the use of co-expression tools. Recent years have seen the emergence of large transcriptomic data sets and co-expression tools have been developed by different research groups (Ruprecht and Persson, 2012). These tools allow the identification of genes putatively involved in the same biological process as, in general, the expression of related genes should be transcriptionally coordinated (Usadel et al., 2009). Two recent articles have used this approach to analyze lignin biosynthesis in *Arabidopsis* and *Brachypodium* (Ruprecht and Persson, 2012). When monolignol biosynthesis genes (*4CL*, *C4H*, *CCoAOMT*) and monolignol polymerization/isomerization genes (*Dirigent protein*

6, *LAC4*, *LAC17*) are used as baits for the co-expression analysis with genecat (<http://genecat.mpg.de/>), two distinct gene networks clearly appear (Figure 5). LAC genes are clearly co-expressed with the cellulose synthase genes *CESA4*, *7*, and *8*, that are specific to secondary-, but not primary-cell wall formation. This co-expression is even stronger than that observed with *4CL*, *C4H*, and *CCoAOMT* that are all highly co-expressed with other monolignol biosynthetic genes such as *PAL*, *COMT*, *CAD*, *CCR*, *C3H*, and *HCT* (Figure 5). Since the monolignol biosynthetic pathway shares steps with the general phenylpropanoid pathway involved in the production of various non-lignin compounds (Goujon et al., 2003; Bassard et al., 2010), the co-expression data could suggest that *LAC4* and *LAC17* might represent a more efficient bait than monolignol biosynthesis genes when trying to identify other “lignin-related” genes including those encoding transporters and other polymerization enzymes.

CONCLUSION

It is clear that the metabolism and polymerization of both monolignols and other more unusual monomers following their biosynthesis are more complex than previously thought. A number of key potential regulation points are involved in these processes allowing plants to rapidly respond to different developmental and environmental cues and that could also constitute interesting novel engineering targets for optimizing lignin production and composition. This will be achieved by answering a long list of crucial questions. For instance, where are glucosidases located – in the cell wall, in the vacuole or in the cytosol? In which conditions (development, stress, etc.) are they active? How are glycosylated monolignols released from the vacuole? What are their roles? Where does the dimerization occur for lignan formation? Are cytosolic PRXs involved? How do lignin polymers grow in the cell wall? What are the specific roles of LACs versus PRXs? Are CASP-like proteins also present in other lignified tissues (xylem and sclerenchyma for instance)? The regulation of lignification has long been studied and modified by scientists at the monolignol biosynthetic level only, often with the sole goal of reducing lignin contents. It is now becoming clear that many other important control points exist that will allow the development of novel strategies for improving plant biomass for both energy and material production. We can reasonably expect that some of these key questions will be answered during the next decade.

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1.4 Lignification: different mechanisms for a versatile polymer

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Abstract

Lignins are cell wall phenolic polymers resulting from monolignol radical coupling. They have characteristically high diversity in their structures which is a direct consequence of the versatile character of the lignification mechanisms discussed in this review. We will relate the latest discoveries regarding the main participants involved in lignin deposition in various tissues. Lignification is often described as a cell autonomous event occurring progressively in all cell wall layers during lignifying cell life and stopping with the cell death. However, recent data combined to old data from studies of tree lignification and zinnia cultures challenged these entrenched views and showed that the lignification process is cell-type dependent and can involve neighboring cells. Therefore, we consider recent data on cell-autonomous and non-cell autonomous lignification processes. We conclude that the role of lignins still need to be assessed during plant development and that control of polymerization/lignin deposition remains elusive and need to be investigated.

polysaccharide network in the cell wall, providing rigidity, impermeability and structural reinforcement.

It is often claimed that lignins are deposited in cells that undergo early cell death. This assumption stems from first, the high lignin content in xylem that is made of dead conductive vessels (wood) and second, the late induction of lignification during cell wall formation. Nevertheless, lignification is a tightly controlled mechanism and it can occur early in tissue development and in cells that do not systemically undergo programmed cell death (PCD). Moreover, some studies show cooperation between different types of cells for lignification suggesting that this process can continue after the lignified cell death. Here, we present and discuss recently proposed lignification models and the diversity in lignin structures and compositions.

Lignin is a versatile polymer

Lignin polymers are characterized by beta-O-4 ether bonds and by resistant carbon-carbon and biphenyl-ether linkages between H, G and S units [2-4]. However, it is important to note that lignin content as well as monomer composition and inter-unit linkage distribution are extremely variable between species and tissues. A well-documented example is the absence of S units in most gymnosperm lignins that contain mainly G units with minor amounts of H units, whereas most angiosperms contain S lignin-units in addition to G units. For a long time the nature of lignin polymers was considered as a marker of evolution [5]. This assumption now needs to be reassessed. For instance, *Selaginella moellendorffii*, a lycophyte that represents an ancient lineage of vascular plants produces S lignin units. Indeed, a *Selaginella F5H-like* gene that evolved independently of angiosperm *F5H* (the limiting enzyme for S unit production) was discovered [6]. Interestingly, this phenylpropanoid dual meta-hydroxylase bypasses several steps of the canonical lignin biosynthetic pathway in which most angiosperms require two phenylpropanoid meta-hydroxylases *C3H* and *F5H* [7,6]. Beyond 'classical' H, G and S lignin units, monolignol polymers are often associated to other phenolic compounds in the cell wall. Grasses possess high amount of hydroxycinnamic acids, mainly *p*-coumaric and ferulic acid [8]. *p*-Coumaric acid is mainly esterified to the phenylpropane side chain of S lignin units [9]. *p*-Coumarate monolignol transferases (PMTs) that acylate sinapyl alcohol were recently discovered in rice, brachypodium and maize [10,11,12]. Unexpectedly the brachypodium *Bdpm1* mutant, which possesses less than 0.5% of

Introduction

The cell wall of terrestrial plants comprises middle lamella (ML) and primary wall (PCW). In specific tissues, like wood, a secondary cell wall (SCW) is deposited. All wall layers may accumulate phenolic polymers called lignins that are mainly made of cytosol-synthesized hydroxycinnamoyl alcohols. *p*-Coumaryl, coniferyl and sinapyl alcohols are the most abundant monolignols and give rise to *p*-hydroxyphenyl (H), guaiacyl (G) and sinapyl alcohols (S) lignin units when polymerized after transport into the apoplast. Lignins which represent 20–30% of dry matter [1] act as glue which embeds the

p-coumaric acid in its lignins, does not show any obvious phenotype regarding size, growth, lignin content or saccharification yield [11^{**}]. Therefore, the role of PMTs and *p*-coumarylated monomers in the development of lignified tissues in grasses remains elusive. Another amazing example to illustrate how lignin polymers may vary between plants and tissues is the discovery of a homopolymer formed by endwise beta-*O*-4-coupling of caffeyl alcohol monomers onto the growing polymer resulting in benzodioxane units in some taxa of cactaceae as well as in vanilla seeds [13]. Interestingly, hydroxyguaiacyl polymers that were only previously observed in COMT deficient plants [14–17] were also discovered in a cactaceae subgroup [18,19^{**}]. Although the molecular bases of these unusual biosynthetic pathways are not known their discovery suggests that polymerization and export to the cell wall are both extremely flexible. All this data provides evidence that the lignin pathway is plastic and open to manipulation, allowing the production of desired polymers for industrial application (reviewed in [20]), an opportunity that is less flexible with cellulose for instance. A very nice example is the production of lignin zippers in poplar by overexpression of the *Angelica sinensis* feruloyl-coenzyme A (CoA) monolignol transferase (FMT) to facilitate lignin degradation [21^{*}]. In these lines monolignol ferulate conjugates incorporate into the lignin backbone thereby introducing readily cleavable ester bonds. Therefore the saccharification yield is increased after pre-treatments.

Cell autonomous lignification

In the extraxylary fibers and sclereids that compose the sclerenchyma, SCW lignification occurs while lignifying cells are still alive and stops only after programmed cell death. In this scenario, each lignifying cell controls the whole process of its own lignification and therefore undergoes Cell-Autonomous Lignification (CAL, Figure 1, [22^{**}]).

In xylem fibers and presumably in interfascicular sclerenchyma fibers originating from cells with primarily parenchyma functions, the lignification starts in the Compound Middle Lamella (CML) which comprise the middle lamella and the primary cell wall. This phenomenon starts when cellulose is progressively deposited in the first layer of SCW (S1) and continue during S1 and S2 thickening [23]. As CML lignification is slowing down/stopping and the polysaccharide matrix of the S2 layer is almost complete, SCW lignification starts in S1 and proceeds continuously in S2 and S3 following cellulose and hemicellulose deposition. This process results in a higher lignin accumulation in the CML as compared with the SCW [23]. G and H are the first units incorporated into cell walls prior to S units [24,25], which might explain why ML and PCW are enriched in condensed units. A pivotal gene that ensures the transition between lignification of CML and SCW in Arabidopsis might be

MYB103, a transcription factor that controls *F5H* expression, the limiting enzyme for sinapyl alcohol biosynthesis [26]. Nevertheless, more data would be necessary to confirm this hypothesis. On the contrary, in alfalfa sclerenchyma fibers that differentiate from the procambium and are associated with vascular bundles, the onset of lignification would occur in the SCW [25] resulting in a late lignin deposition and the presence of S units in the ML and PCW. In Arabidopsis, the S units are absent (or present in very low amounts) from the CML of interfascicular and xylem fibers which is consistent with the event chronology of the lignification in these tissues.

Non-cell autonomous lignification

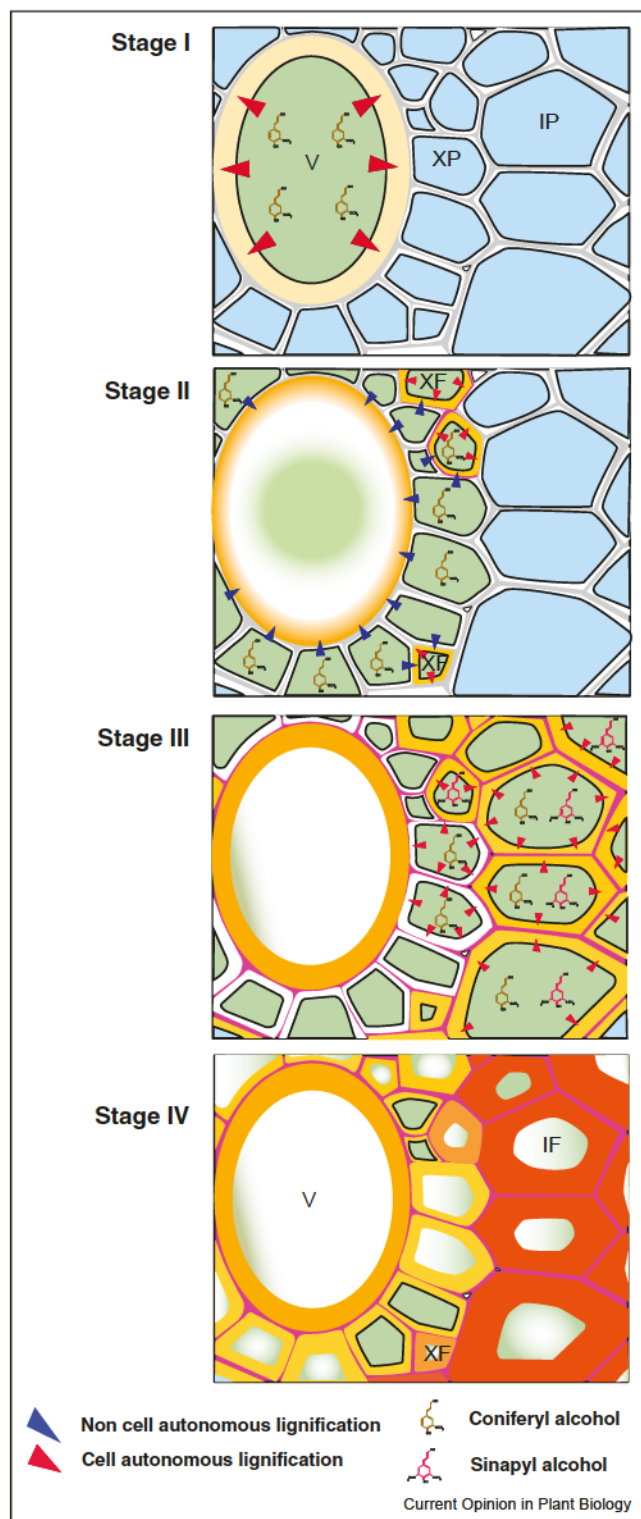
In contrast to sclerenchyma fiber cells, which stay alive throughout the lignification process, vessel formation involves rapid programmed cell death. In consequence, vessels require the help of neighboring cells to achieve full lignification after their programmed cell death. This is called the ‘good neighbors’ scenario or the non-cell autonomous lignification [22^{**},27,28] (NCAL, Figure 1).

During the early stage of vessel formation and prior to programmed cell death, SCW polysaccharides are deposited by the tracheary element (TE) themselves thanks to the expression of cellulose and xylan biosynthetic genes. Preliminary vessel lignification depends on monolignols produced by the vessel cell itself and then, following vessel PCD, continued lignification of the TE wall requires monolignols produced by neighboring parenchyma cells.

Xylem parenchyma cells (the good neighbors) are non-lignified metabolically active cells that express genes associated with lignin monomer biosynthesis such as the cinnamyl alcohol dehydrogenase (CAD) and cinnamoyl CoA reductase (CCR) genes [29–31]. It was hypothesized that these cells participate in the TE lignification *in vitro* process by producing and exporting monolignols [28,32] and are also the source of H₂O₂ necessary for the monolignol polymerization in the secondary cell walls of lignifying xylem vessels [33].

According to results from [22^{**}], the unique monolignol biosynthetic activity of parenchyma cells is necessary for vessels to display normal levels of lignification. In Arabidopsis TE, monolignols are then oxidized by LACCASE 4, LACCASE 11 and LACCASE 17 enzymes prior to free radical polymerization to form lignin [34,35^{*}]. Like metaxylem TE, protoxylem cells also undergo a non-cell autonomous lignification [22^{**}]. However, by contrast with metaxylem TE, lignification occurs exclusively in the SCW and the PCW remains un-lignified. Indeed, very recently, Schuetz *et al.* [36] showed that the laccases necessary for this process would be precisely delivered to the SCW while proteins synthesizing or transporting

Figure 1



Schematic of two lignification models during primary growth of dicots (*Arabidopsis thaliana*): non-cell autonomous lignification (NCAL) and cell autonomous lignification (CAL). Lignification is divided into 4 stages. **Stage I:** Vessel element are lignifying. They produce monolignols (mainly coniferyl and hydroxycinnamyl alcohol) prior to programmed cell death (PCD). Interfascicular parenchyma cells are not

monolignols do not show specific pattern in the cell or the plasmalemma during protoxylem TE differentiation.

Since lignification of xylary fibers benefits from the activity of neighboring parenchyma cells [22^{••}], the lignin composition of xylary fiber cell walls may depend on the cell's immediate surroundings in the vascular bundles. In wood, fibers next to vessels are enriched in G units whereas fibers next to other fibers are enriched in S units [37]. Since it is likely that vessels from wood also undergo CAL, this suggests the hypothesis that parenchyma cells are only able to synthesize coniferyl alcohol and that the biosynthesis of sinapyl alcohol could be specific to the NCAL process. This would also explain why vessels do not possess S units in their cell wall.

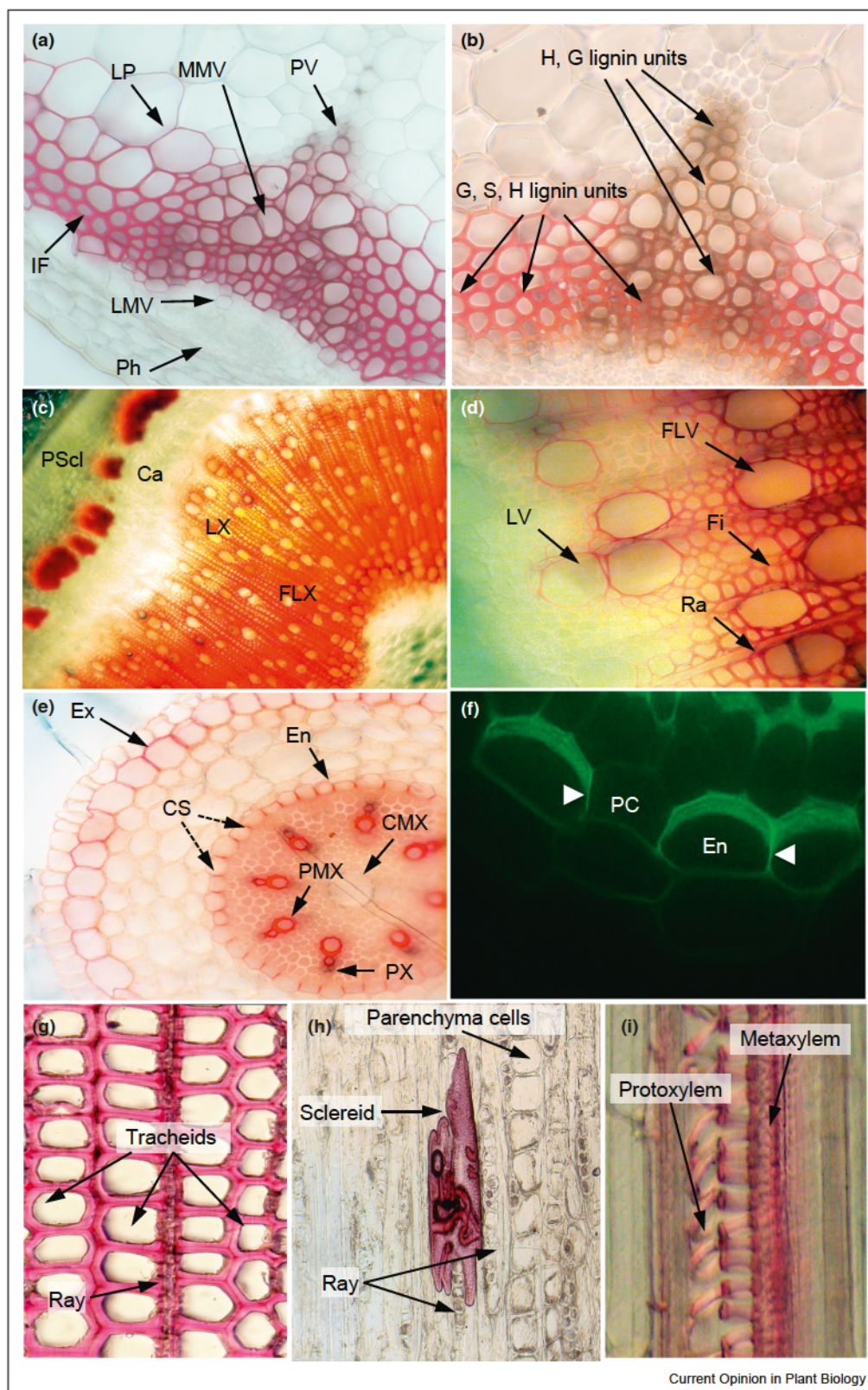
It would be interesting to check whether the good neighbor model is a widespread system or not. Cells that may lignify can have different shapes and biological roles (Figure 2). For instance, conifers only develop tracheids in their secondary xylem and in consequence do not benefit from non-lignified axial parenchyma that could supply them with lignin precursors. This role might be assumed by ray parenchyma cells where, for instance the CAD promoter is active [30,38].

The transcriptional regulation of lignin biosynthesis during cell autonomous lignification and non-cell autonomous lignification.

In *Arabidopsis*, lignification during SCW formation is regulated by NAC transcription factors: SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEINS (SND1, also named NST3)/NAC SECONDARY WALL THICKENING PROMOTING FACTORS (NST1 and NST2) and VASCULAR RELATED NAC DOMAIN TFs (VND1-7). Their biological role during development is well documented [39–44]. Interestingly, expressions of SND1/NST3 and VND6/7 are highly specific to interfascicular fibers and vessels, respectively [43,44]. We can therefore try to decipher their respective roles in the context of CAL and NCAL because these cell types differ in their mechanisms of lignification [22^{••}]. The SNDs and VNDs cited above are master transcriptional switches activating the developmental program of secondary wall biosynthesis through the regulation of a

lignified. **Stage II:** Vascular bundles initiate PCD. Non-lignifying xylary parenchyma cells produce monolignols and transport them to the vessel cell wall in order to finalize lignification (NCAL). Xylary fibers produce lignin in their cell wall by CAL and benefit from NCAL. Interfascicular parenchyma cells are not lignified. **Stage III:** CAL starts in interfascicular parenchyma that develops into interfascicular fibers. G and H units are deposited in the middle lamella and primary cell wall (colored in pink when lignified) and secondary cell wall prior to S unit deposition in the secondary cell wall. **Stage IV:** most cells are lignified and ultimately die. Cell walls of interfascicular fibers and a few xylary fibers are enriched in S units. V: vessel; XP: xylary parenchyma; IP: Interfascicular parenchyma; XF: xylary fiber; IF: interfascicular fiber.

Figure 2



Examples of lignified cells or tissues. (A) and (B) cross sections of *Arabidopsis thaliana* stem (magnification 50×). LP: lignifying parenchyma; IF: interfascicular fibers; LMV: lignifying metaxylem vessel; MMV: mature metaxylem vessel; Ph: phloem; PX: protoxylem. (C) and (D) cross sections

cascade of downstream transcription factors, such as SND2, SND3, MYBs (MYB46, MYB83, MYB103, MYB85, MYB52, MYB54, MYB69, MYB42, MYB43, MYB20) and KNAT7 [39,45]. Some MYBs are able to control expression of other MYBs making the lignin regulation network complex but well-orchestrated. For instance, MYB46 and MYB83 are involved in the transcription of MYB58, MYB63, MYB85 and MYB103 that have been reported to clearly play direct or indirect roles in regulating lignin biosynthesis genes [39,46,47]. MYB58, MYB85 and MYB103 are expressed in xylem or protoxylem area of elongating internodes as well as in fibers and xylem of non-elongating internodes [39] [46] while MYB63 is mainly expressed in fibers but not in protoxylem of elongating and non-elongating internodes [46]. MYB61, another MYB involved in regulation of lignification, is expressed in protoxylem but not in fibers of elongating internodes [48]. In the NCAL scenario, good neighbor cells do not lignify at an early stage but are able to synthesize and transport monolignols to the lignifying cell walls. This hypothesis suggests that master genes necessary for the activation of monolignol biosynthesis genes are expressed in parenchyma cells. In contrast, master genes activating oxidase coding genes would not be expressed in order to avoid lignin polymerization in parenchyma cell walls as this would prevent monolignol transport to the lignifying TE [22^{••}]. MYB58 and MYB63 are able to activate both monolignol synthesis and polymerization genes such as LACCASE4 (but not LACCASE17) which was recently shown to be involved in monolignol polymerization [46]. If, as suggested above, cell wall lignin structure is related to (N)CAL then variations in lignin composition might represent a good marker for distinguishing CAL from NCAL and providing information on the involvement of different master genes. Indeed, in MYB103 knock-out mutants [26], the transcript abundance of *F5H* was strongly decreased, and syringyl unit biosynthesis is severely affected. Interestingly, metabolomes of *myb103* mutant and *F5H* null mutant were very similar and there were only very minor changes in the overall lignin content in the inflorescence stem of *myb103* when compared with wild type despite its high expression in lignified tissues. These results might place MYB103 as an important protagonist in CAL of *Arabidopsis* because interfascicular fibers produce mainly S-enriched lignin in their cell wall. In summary, expression of master genes in lignifying cells or adjacent cells may give interesting clues about lignification mechanisms. For instance, the precise expression of SHATTERPROOF MADS-box1/SHATTERPROOF

MADS-box 2 and MYB26 in the lignifying cells of the endothecium or adjacent to the dehiscence zone in siliques, respectively [49,50] suggests that they undergo CAL. However, the effectiveness of activators of lignification might be counterbalanced and restricted in some cells (xylary fibers, parenchyma cells) by other transcription partners or inhibitors such as bHLHs that interact with MYBs, or VASCULAR-RELATED NAC-DOMAIN-interacting 1 and 2 that interact with VND7 [22^{••},44,51]. Finally, monolignols are present in some non-lignified tissues [29,30,52,53] as these molecules can also have different (non-lignin) metabolic fates: they are detected in suberin, lignans, ectopic wax [53,54]. Certainly, this would explain ectopic expression patterns often observed for genes encoding monolignol biosynthesis enzyme [52]. Other transcription factors (WRKY, APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) have also been identified as important regulator of lignification, and their role in CAL and NCAL needs to be clarified [55–57].

Lignin polymerization/deposition where, when and how?

Lignification is a non-reversible mechanism, therefore monolignol biosynthesis and lignin deposition/polymerization should be tightly controlled. Polymerization and lignin deposition are often described as simultaneous phenomena since ‘genuine’ polymeric lignin cannot exist until monolignols are transported into the apoplasm. However, the recent discovery of cytosolic PMTs [11^{••},21[•]] that link *p*-coumaryl alcohol to sinapyl alcohol prior to export to the apoplasm and integration into the lignin polymer raise the question of when polymerization starts and how many dimers, trimers or higher oligomers the cell is capable of exporting for lignification. Until recently it was thought that only monolignols are actively transported through the plasma membrane by ABC transporters [58,59]. Despite debate, it remains to be clarified as to whether lignans (stereoactive monolignol dimers) may participate in lignification or not and, if so, to what extent? Interestingly, in *Arabidopsis*, the loss of function of a pinorensinol reductase *PrR1* results in decreased levels of both lignans and lignins [60]. Amazingly, a dirigent domain containing protein (ESB1) plays an essential role in building the extracellular lignin-based Casparian strip (CS) of *A. thaliana* roots [61[•]]. CSs are lignified and suberized fine structures located between adjacent endodermis cells and constitute an apoplastic barrier to force nutrients and water through the symplasmic pathway.

of *Populus tremuloides* secondary xylem (magnification 20× and 50× respectively). PScI: phloem sclereids; CA: cambium area; LX: lignifying xylem; FLX: mature lignified xylem; LV: lignifying vessel; FLV: mature lignified vessel; Fi: fiber; Ra: ray. (E) and (F) cross section of *Brachypodium distachyon* roots (25× and 100×). Ex: exodermis; En: endodermis; PMX: peripheral metaxylem; CMX: central metaxylem; PX: protoxylem; En: endodermis cell; PC: passage cell in endodermis. Arrows show lignin fluorescence of casparian strip. (G) cross section of *Picea abies* secondary xylem (80×). (H) tangential section of *Picea abies* secondary phloem (80×). (I) radial section of *Brachypodium distachyon* culm (100×). In A, C, D, E, G, H, I, sections were stained with phloroglucinol-HCl that colors lignin aldehydes in red. In B, section was colored with Maile staining method that colors G unit-enriched tissues in orange/brown and S unit-enriched tissues in red. In F, root section was visualized under UV illumination.

Nevertheless, an active and direct role in lignification remains to be shown for ESB1.

The control of lignin deposition in the cell wall is poorly known. In the grass cell wall, ferulic acid, which accounts for 2–10% of total monolignols present in cell wall, can be both ester-linked to arabinoxylans and ether-linked to lignins [8]. Therefore, these molecules may act as initiation or nucleation sites of lignification in grasses [62–64]. The formation of ferulate cross-linked structures between lignins and arabinoxylans would be a key step in switching between the elongating primary cell wall (which contains few ferulates) and SCW deposition [65–67]. The nature of initiation sites is intriguing for most dicots that do not possess many hydroxycinnamic acids. The study from [36], suggests that monolignols may diffuse freely throughout the apoplast and become polymerized only in the secondary cell wall of Arabidopsis protoxylem TE. Some proteins capable of orientating lignin deposition in the cell wall despite being located in the plasma membrane were recently discovered. The most detailed data was obtained by studying the lignification of the CS in Arabidopsis. The correct deposition of lignins in the CS is controlled by Casparian strip membrane domain proteins (CASPs). Niko Geldner and colleagues [68**] discuss a model in which CASP scaffolds located in specific regions of the plasma membrane are involved in correctly organizing the polymerization of monolignols. This model implies specific root peroxidases and ROS to oxidize monolignols prior to polymerization. An NADPH oxidase (Respiratory burst oxidase homolog F protein) is probably involved in the production of Reactive Oxygen Species (ROS) since a mutant (*sgn4/rbohF*) shows a strong delay in the formation of the CS. NADPH oxidases produce $O_2^{\bullet}$ from O_2 and in a perfect scenario, a superoxide dismutase protein (SOD) would be necessary to reduce $O_2^{\bullet}$ into H_2O_2 , the substrate of peroxidases. As yet, the candidate SOD has not been identified. Upon reduction of $O_2^{\bullet}$ into H_2O_2 , SOD also produces O_2 , the substrate of laccase. Although more data are necessary to prove the role of laccases in the CASP complex, it is tempting to imagine that NADPH oxydase, SOD, peroxidases and laccases all function together in a ‘lignification complex’.

Conclusion

The recent discovery of natural ‘non-classical’ lignin polymers like catechyl polymers illustrates the high plasticity of lignification even though it still remains to be seen whether these molecules have a specific role during plant/organ development. Lignins accompany cell maturation and although their deposition is usually synonymous of PCD, these polymers are also present in living cells with specific functions such as the basal endosperm transfer cells of maize kernel [69] or endoderm cells [53]. These observations suggest another function for these polymers other than providing rigidity

to the wood for instance. The most recent data show that the lignification is a complex trait that cannot be reduced simply to monolignol biosynthesis. For example, flax fibers which are poorly-lignified cellulose-enriched cells produce oligolignols that apparently do not polymerize into the cell wall [70]. This phenomenon can be compared to the good neighbor lignification model in which non-lignified parenchyma cells synthesize monolignols for polymerization in cell walls of dead or dying neighbor cells [22**,28,29,30,32,71]. These phenomena highlight how polymerization/deposition is an important checkpoint in lignification. It remains to understand whether the gatekeepers of the last act in the lignification scenario are oxidase proteins (laccase/peroxidase), ROS or other unknown molecules present in the polysaccharide matrix that determine lignin deposition.

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1.5 Altered lignin biosynthesis to improve biofuel production, forage quality and paper manufacture

In recent years, researchers are desiring to find renewable alternatives for gasoline and diesel fuels. Biofuels derived from plant biomass conversion are considered as a great solution. However the global food shortage indicates biofuels production should not compete with food consuming. First generation biofuels are made from the sugars, starches and vegetable oils found in economic crops, which can be easily extracted using conventional technology. In comparison, second generation biofuels are made from lignocellulosic biomass, provided by forest, agricultural residues, waste or even now dedicated plants such as grasses (De, 2009). However, renewable and sustainable liquid transportation biofuels based on lignocelluloses conversion face several obstacles that must be overcome in order for them to become commercially viable and cost-competitive.

The presence of lignin is the most significant contributors to biomass recalcitrance and consequently increases the costs associated with conversion. Lignins are a barrier to the release of cellulose fibers by chemical pulping and the enzymatic hydrolysis of cell wall polysaccharides and then production of fermentable sugar – a process called saccharification. Indeed, lignin negatively affects the saccharification process by prohibiting cellulose fiber swelling, thus reducing the surface area exposed to the enzyme (Mooney et al., 1998), by immobilizing cellulase (and associated enzymes) and by preventing them to reach their polysaccharide substrates (Chang and Holtzaple, 2000; Palonen, 2004). Reducing lignin content or manipulating its composition would reduce the high processing costs associated with saccharification, so it is of great interest in the utilization of biofuel production, forage quality and paper manufacture (Grabber et al., 1998; Ralph et al., 2004; Vanholme et al., 2010). In most lignin mutants, the saccharification yield was improved. The saccharification models and

Pearson correlation matrix revealed that the lignin content was the main factor determining the saccharification yield (Van Acker et al., 2013a). Strikingly, cellulose content did not significantly affect saccharification yield (Van Acker et al., 2013a).

Although genetic engineering of lignin, using mutants and transgenics, promises to increase the accessibility of cell wall polymers to hydrolytic enzymes during the biofuel production process, the potential problems associated with this approach must be addressed before large-scale application. For example, most efforts to reduce lignin content during plant development resulted in severe biomass yield reduction, and also it should be assumed that plants with decreased lignin content may become more susceptible to pathogens or may be attacked by insects against which they are usually resistant or tolerant (Franke et al., 2002b; Voelker et al., 2010).

1.6 Roles of Lignins in plant growth and development

Considering the hydrophobic and strengthening nature of lignins, these polymers enable xylem vessels to transport water and nutrients from the roots to photosynthetic organs while withstanding the negative pressure caused by transpiration (Eastham and Gregory, 2000). Perturbation of lignin biosynthesis during development cause growth defects, a phenomenon which often correlate with the loss of vessel integrity that adversely affects water and nutrient transport in plants. In *Arabidopsis*, mutations or perturbations of genes early in the pathway lead to drastic lignin reduction and a severe inhibition of plant growth. For instance, knocking out four phenylalanine ammonia-lyase genes (*PAL*) results in stunted and sterile plants with severely decreases in lignin content (Rohde et al., 2004; Huang et al., 2010). Plants affected in *p*-coumaroyl shikimate 3'-hydroxylase, (*C3H*), cinnamate 4-hydroxylase (*C4H*) and hydroxycinnamoyl-

CoA shikimate: hydroxycinnamoyl transferase (HCT) respectively exhibit severe dwarfism and sterility (Franke et al., 2002a; Hoffmann et al., 2004; Abdulrazzak et al., 2006; Besseau et al., 2007; Li et al., 2010). Developmental defects were also noticed in *c4h-2*, *ccr1(-3*, and *-6)* mutants which are affected in coumaric 4-hydroxylase and coumaroyl CoA reductase. In these plants, the height of their inflorescence stems at mature stage was reduced by 29%, 83%, and 34% as compared to the wild-type, respectively (Vanholme et al., 2012). Notably, the *ccr1-3* mutant had a bushy appearance with a small primary inflorescence (that died early) and many secondary inflorescences. A strong reduction in biomass (measured by the weight of the primary senesced inflorescence stem) was observed for *c4h-2*, *ccr1-3*, *ccr1-6* and *ccoaoomt1-3* (Van Acker et al., 2013a). For most authors, the stunted growth and collapsed vessels of *ccr1* mutants, is attributable to the lower lignin contents and also to the incorporation of ferulic acid into lignin polymer and other metabolic changes (Dauwe et al., 2007; Leplé et al., 2007; Mir Derikvand et al., 2008; Ralph et al., 2008).

The association between lignin modification and plant growth reduction has also been reported in several other species including poplar, tobacco, and alfalfa (Kim et al., 2014). Although this association widely occur, it is still not clear how the perturbation of lignin biosynthesis influences plant growth and development because phenylpropanoid metabolism produce many compounds like flavonoids and hydroxycinnamate esters which play important roles in many aspects of plant growth and development (Kim et al., 2014).

1.7 *Brachypodium distachyon* is a good model for monocots

Brachypodium distachyon (Brachypodium) is a temperate grass. Its close relationship to the major cereal grain species like wheat (*Triticum aestivum*), rice (*Oryza sativa*), oat (*Avena sativa*) and maize (*Zea mays*) makes it an ideal

model organism for understanding the genetic and molecular biology of cereals and most grasses dedicated to human food consumption, animal forage and also biofuel production. Moreover, it has many qualities that make it an excellent model organism for functional genomic research (Draper et al., 2001). These attributes include a high-quality genome sequence, small genome (~280Mbp), diploid accessions (often sequenced), self-fertility, a short lifecycle, a small physical stature, simple growth requirements and an efficient transformation system (Vogel and Hill, 2008b). The promise of *Brachypodium* led the U.S. Department Of Energy (DOE) to call for the development of *Brachypodium* as a model system to help accelerate the development of superior grasses to serve as feedstocks for the emerging biofuel industry (DOE 2006).

Resources currently available include: whole genome sequence (Vogel et al., 2006b), freely accessible inbred lines (Vogel et al., 2006a), cDNA libraries and EST sequences (Vogel et al., 2006b), BAC libraries and BAC end sequences (Hasterok et al., 2006; Huo et al., 2011, Huo et al., 2009). Moreover, physical and linkage maps are available for some populations (Garvin et al., 2008). Three web portals (<http://www.brachypodium.org>, <http://pgsb.helmholtzmuenchen.de/plant/brachypodium/> and <http://phytozome.jgi.doe.gov>) for *Brachypodium* contain genome browsers and links to community resources. In addition, two project-specific websites (<http://brachypodium.pw.usda.gov/> and <http://www-urgv.versailles.inra.fr/tilling/brachypodium.htm>) provide access to T-DNA and Tilling resources, respectively. All of these information will help *Brachypodium* reaching its potential as an important model system for developing new energy and food crops.

Cell wall biosynthesis has been studied mostly in the model dicot plant *Arabidopsis*. However, results on this species cannot always be extrapolated to (commelinid) grasses given the different stem anatomy and cell wall composition (Carpi ta and Gibeaut, 1993). By contrast, the identification of interested genes in *Brachypodium* could work as a handle for orthologous gene discovery in other

important crops thanks to the syntenicity between grasses (Bennetzen and Freeling, 1993; Devos and Gale, 2000). Comparison of the Brachypodium, rice and sorghum genomes shows a precise history of genome evolution across a broad diversity of the grasses, and establishes a template for analysis of the large genomes of economically important pooid grasses such as wheat.

1.8 Brachypodium is a good model to study lignification in grasses

The knowledge regarding lignin biosynthesis in Brachypodium is still limited. However, few articles were recently published. Our lab recently identified two missense mutants of *BdCAD1* (*Bd4179* and *Bd7591*). The mature stems of these mutants accumulated aldehydes and displayed a reddish coloration of lignified tissues. Lignin content was decreased by more than 20 % compared with the control (Bouvier D'Yvoire et al., 2013). Several non-synonymous mutations in *BdCOMT6* were also identified by TILLING and few mutants showed lower lignin content, typical decrease of syringyl units and accumulation of 5-hydroxy-coniferyl alcohol (Dalmais et al., 2013a). Transgenic plants overexpressing artificial microRNA designed to silence *BdCOMT4* (actually *BdCOMT6* according to our own lab classification) were produced by the group of Samuel Hazen lab. The plants exhibited a decrease of lignin content and especially syringyl units in stem (Trabucco et al., 2013). More recently, a transferase involved specifically in monolignol acylation, *BdPMT1* was identified by our team in collaboration with John Sedbrook group. *Bdpmt1* missense mutant and RNAi plants had a very low (less than 0.5 %) amount of CA linked with sinapyl alcohol, but the amounts of CA acylating arabinosyl units on arabinoxylans were not changed. CA acylation of lignin from *BdPMT1*-overexpressing plants was found to be more than three-fold higher than that of wild-type, but again the level on arabinosyl units remained unchanged (Petrik et al., 2014).

1.9 BRACHYTIL: an important tool for Brachypodium functional genomics

To extend the panel of resources for functional genomics in *Brachypodium*, we implemented a mutagenized population and a TILLING (Targeting Induced Local Lesion IN Genome) platform (named BRACHYTIL) was established by our colleagues at URGV. The TILLING method has been established and developed for many plant species including wheat, sorghum, barley, rice and oat (Gilchrist et al., 2006; Gilchrist and Haughn, 2010). TILLING is a reverse genetics technique based on the use of an endonuclease that recognize mismatches in DNA double fragments. This allows the identification of mutations in any target gene present in pool of mutagenized DNA through heteroduplex analysis (Talamè et al., 2008). Chemically-induced mutants are complementary to insertion mutants in several aspects: (i) mutation rates are higher and hence screens can be done on smaller mutant populations, (ii) single base pair changes, as opposed to insertion mutants, more likely yield allelic series of partial loss-of-function, conditional or gain-of-function mutants and (iii) somaclonal variation is avoided in the absence of an *in vitro* culture step that is required for T-DNA-induced mutagenesis (Henikoff et al., 2004; Wang et al., 2012). In the TILLING method, a pool of seeds is mutagenized, the resulting M1 plants are self-fertilized and the M2 generation of individuals is used to prepare DNA samples for mutational screening, while seeds of the M3 families can be stored and distributed. The DNA samples are pooled and subjected to gene-specific PCR. The PCR amplicons of the target gene are carried on with chromophore labelled primers. The PCR products are subjected to digestion with endonuclease enzymes (ENDO 1 or CEL1) that specifically recognize at the site of a mismatch induced by chemical mutagens such as ethyl methanesulfonate (EMS) or Sodium azide

(NaN₃) mutagenesis. The sizes of the cleavage fragments resolved on polyacrylamide gels reveal the approximate position of the mutation within the amplicon (Dalmais et al., 2013b).

A large collection (5530 M2 families) of chemically-induced mutants was generated in Versailles and a subset of the phenotypes can be consulted at <http://urgv.evry.inra.fr/UTILLdb>. We calculated an average mutation rate of one in around 400 kb or 700 mutations per genome for a genome size of 272 Mb (Dalmais et al., 2013b). This frequency of induced mutations is similar to that found in EMS-mutagenized rice (1/294 kb) (Till et al., 2007) or in barley treated with NaN₃ (1/374 kb) (Talamè et al., 2008) or EMS (1/500 kb) (Gottwald et al., 2009).

In this study we intend to provide evidences that laccases are also responsible for lignification in grasses, and to identify specific genes of the laccase family in *Brachypodium*. If laccases were proved to be responsible for lignification, they should be good targets for cell wall genetic modification. Furthermore, we will explore the transcriptional regulatory mechanisms of the laccases which are involved in lignin biosynthesis.

Chapter 2 The identification of laccases involved in lignin formation in *Brachypodium distachyon* culm

2.1 Plant laccases were identified as multicopper oxidases putatively involved in lignification

Laccase is a multicopper oxidase that couples the four-electron reduction of oxygen with the oxidation of a broad range of organic substrates, including phenols, polyphenols and anilines (Mayer and Staples, 2002; Kunamneni et al., 2008). These proteins could remove protons from the phenolic hydroxyl groups with oxygen as the electron acceptor, and then the radicals generate by this reaction can be spontaneously rearranged (Kunamneni et al., 2008). Laccase from fungi are relatively well characterized and a laccase from *Trametes versicolor* was crystallized (Piontek et al., 2002). Fungi laccases participate to the degradation of lignin polymers. They are also used in many industrial processes (Mayer and Staples, 2002).

Plant laccases were found to be widely distributed among plant species, since their discovery in the Japanese tree *Rhus vernicifera* a long time ago (Kunamneni et al., 2008). They are supposed to be involved in the wound healing and monolignol polymerization in plants (Mayer and Staples, 2002; Wang et al., 2013). It demonstrated that the addition of O_2 to spruce cambium extracts stimulated the formation of Dehydrogenation Polymers (DHP) from coniferyl alcohol more effectively than after addition of H_2O_2 (Sterjiades et al., 1992). Later, laccases purified from the differentiating xylem of *Acer pseudoplatanus*, *Pinus taeda*, *Zinnia elegans* and *Liriodendron tulipifera* were also shown to oxidize monolignols (sinapyl, coniferyl, and *p*-coumaryl alcohols) *in vitro* to form DHP (Sterjiades et al., 1992; Bao et al., 1993; Liu et al., 1994; LaFayette et

al., 1999). Bao et al. (1993) and Liu et al. (1994) found a direct correlation between laccase activity and lignification in *P. taeda* and *Zinnia elegans*, respectively. De Marco and Roubelakis-Angelakis (1997) suggested that laccase activity may contribute to cell-wall reconstitution in regenerating protoplasts of higher plants. Few articles relate on the investigation of laccase substrate specificity. For instance, in the presence of O₂, laccases from *Liriodendron tulipifera* are capable of polymerizing sinapyl alcohol and coniferyl alcohol rapidly within a few minutes, but it was demonstrated that *p*-coumaryl alcohol is oxidized much more slowly, taking several hours completion (Dean et al., 1998).

2.2 LACCASE5 Is Required for Lignification of the *Brachypodium distachyon* Culm

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Abstract

The oxidation of monolignols is a required step for lignin polymerization and deposition in cell walls. In dicots, both peroxidases and laccases are known to participate in this process. Here, we provide evidence that laccases are also involved in the lignification of *Brachypodium distachyon*, a model plant for temperate grasses. Transcript quantification data as well as in situ and immunolocalization experiments demonstrated that at least two laccases (LACCASE5 and LACCASE6) are present in lignifying tissues. A mutant with a misspliced LACCASE5 messenger RNA was identified in a targeting-induced local lesion in genome mutant collection. This mutant shows 10% decreased Klason lignin content and modification of the syringyl-to-guaiacyl units ratio. The amount of ferulic acid units ester linked to the mutant cell walls is increased by 40% when compared with control plants, while the amount of ferulic acid units ether linked to lignins is decreased. In addition, the mutant shows a higher saccharification efficiency. These results provide clear evidence that laccases are required for *B. distachyon* lignification and are promising targets to alleviate the recalcitrance of grass lignocelluloses.

Lignins are cell wall phenolic heteropolymers, primarily made from *p*-coumaryl, coniferyl, and sinapyl alcohols, referred to as monolignols. These monolignols are synthesized in the cytoplasm from the phenylpropanoid pathway and then transported to the cell walls, where they are oxidatively polymerized into *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) lignin units. This oxidative polymerization step is driven by hydrogen peroxide-dependent peroxidases and/or oxygen-dependent laccases (Vanholme et al., 2010). While the involvement of peroxidases in lignification has been indicated for many years, the occurrence of lignin-specific laccases has been unambiguously established more recently in *Arabidopsis* (*Arabidopsis thaliana*), the genetic model plant. While the transfer DNA mutants of *AtLAC4* (for LACCASE4) and *AtLAC17* showed moderate alterations in lignification, the double mutant *lac4 lac17* displayed more severe effects, together with poorly lignified interfascicular fibers

and collapsed vessels in stems, when grown in continuous light (Berthet et al., 2011). More recently, *AtLAC4* and *AtLAC17* were shown to direct lignification in secondary cell walls and during protoxylem tracheary element development (Schuetz et al., 2014).

In this study, we aim to establish whether laccases are also involved in the lignification of grass cell walls. Indeed, to our knowledge, no mutants or transgenics affected in laccase activity with a convincing impact on lignification have been reported in grasses, despite a report of a laccase cloned from sugarcane (*Saccharum officinarum*), *SofLAC*, shown to partially complement *AtLAC17* (Cesarino et al., 2013). To this aim, we used the *Brachypodium distachyon* mutant collection and the targeting-induced local lesion in genome platform established previously (Dalmais et al., 2013) to isolate laccase mutants. By doing so, we demonstrated that the *BdLAC5* gene is involved in the lignification of *B. distachyon* culms and that laccases might be good candidates to target for cell wall engineering.

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RESULTS AND DISCUSSION

BdLAC5 and BdLAC6 Proteins Are Closely Related to Lignin-Specific AtLAC17 and AtLAC4

In order to identify the laccases of *B. distachyon* in silico, we BLASTed the amino acid sequences of *Arabidopsis* *AtLAC4* or *AtLAC17* (<http://mips.helmholtz-muenchen.de/plant/brachypodium/>) against proteins from the *B. distachyon* genome. This search allowed the identification

of 29 nonredundant laccase genes named *BdLAC1* to *BdLAC29*, according to their chromosome localization (Supplemental Table S1). A phylogenetic tree was constructed with the deduced 29 *B. distachyon* laccase proteins together with the 17 Arabidopsis laccases (Fig. 1). In addition, we incorporated in this tree some other laccases reported to catalyze the oxidative polymerization of various phenolics, such as SofLAC from sugarcane (Cesarino et al., 2013), ZmLAC3 from maize (*Zea mays*; Caparrós-Ruiz et al., 2006), PtLAC3 from *Populus trichocarpa* (Ranocha et al., 2002), GaLAC1 from *Gossypium arboreum* (Wang et al., 2008), and *Brassica napus* Transparent Testa10 (BnTT10; Zhang et al., 2013; Supplemental Table S2). The resulting phylogeny comprised four large clades (Fig. 1). Clades I and II gathered

14 and 3 *B. distachyon* laccases, respectively, and some Arabidopsis proteins of unknown functions. Clade III comprised three *B. distachyon* laccases with AtLAC15 and BnTT10 laccase proteins involved in proanthocyanidin polymerization (Pourcel et al., 2005; Zhang et al., 2013). This result suggests that other members of clade III might also catalyze the oxidation of flavonoids. The other members clustered in clade IV with the Arabidopsis lignin-specific laccases AtLAC17 and AtLAC4 (Fig. 1). More importantly, only one protein, *BdLAC6* (Bradi1g74320), tightly gathered in a subcluster with AtLAC4 (66%/83% identity/similarity), AtLAC11 (Zhao et al., 2013), and PtLAC3 (Ranocha et al., 2002), also was reported to be involved in lignification. *BdLAC5* (Bradi1g66720), *BdLAC8* (Bradi2g23370), and *BdLAC12*

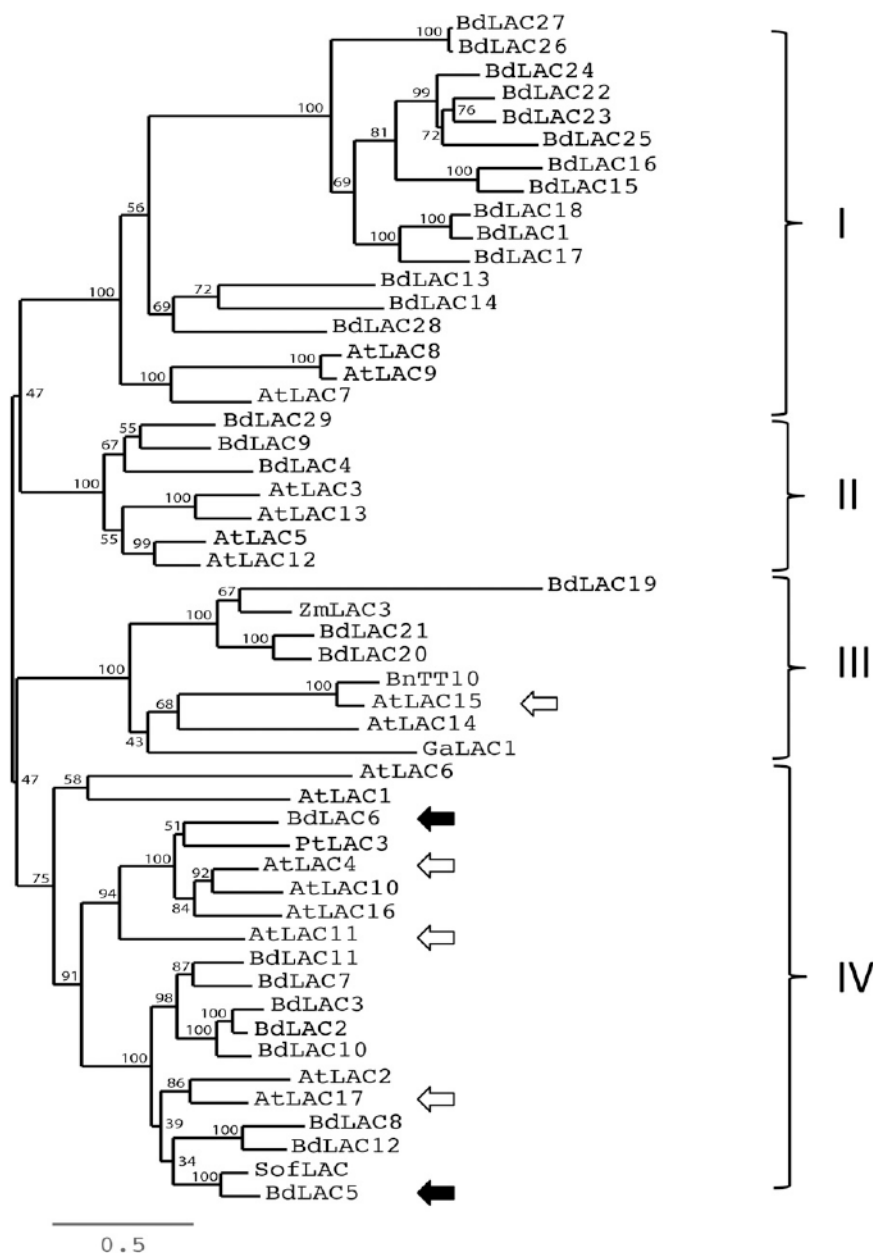


Figure 1. Phylogenetic analysis of laccase proteins. Twenty-nine laccases from *B. distachyon* and 17 laccases from Arabidopsis were used to build the tree. Other proteins putatively involved in the polymerization of phenolics were also included: SofLAC from sugarcane, ZmLAC3 from maize, PtLAC3 from *P. trichocarpa*, GaLAC1 from *G. arboreum*, and BnTT10-1 from *B. napus*. Branch length is proportional to the number of substitutions per site and represents evolutionary distance as indicated by the scale bar. Names and protein sequences are available in Supplemental Tables S1 and S2. White arrows show Arabidopsis laccases for which mutants have already been published. Black arrows show *BdLAC5* and *BdLAC6*.

(Bradi2g54740) gathered together in a subcluster including AtLAC17. BdLAC5, which shares 64% identity and 77% similarity with AtLAC17, is also the closest ortholog of SofLAC, a protein able to partially restore the lignin level of the lignin-deficient *Atlac17* mutant (Cesarino et al., 2013). Based on this analysis, the *BdLAC5* and *BdLAC6* genes were chosen as the best lignin-specific candidates for further investigation.

***BdLAC5* and *BdLAC6* Are Mainly Expressed in Lignified Tissues**

The expression and coexpression of the selected *BdLAC5* and *BdLAC6* genes were studied in different organs and at different development stages using the PlaNet tools (<http://aranet.mpimp-golm.mpg.de/>; Supplemental Fig. S1). Both genes were highly expressed in lignified organs (internode, node, and peduncle) and poorly expressed in organs with low lignin levels (e.g. leaf) or in nonlignified tissue (endosperm). This coexpression study (Supplemental Table S3) revealed that both laccases are coexpressed with genes involved in monolignol biosynthesis, such as *O*-methyltransferase (Bradi3g16530; Bragg et al., 2012; Dalmais et al., 2013), cinnamoyl-CoA reductase (Bradi3g36887), or Phe ammonia lyase (Bradi3g49250 and Bradi3g49260). They are also coexpressed with three cellulose synthase genes specific to the secondary cell wall, CESA4 (Bradi3g28350), CESA7 (Bradi4g30540), and CESA8 (Bradi2g49912; Handakumbura et al., 2013).

The expression levels of *BdLAC5* and *BdLAC6* were analyzed by quantitative reverse transcription real-time

(RT)-PCR on RNA isolated from different organs at three developmental stages (see “Materials and Methods”), the tillering stage (TS), early flowering stage (EFS), and maturity stage (MS). *BdLAC5* and *BdLAC6* transcripts were detected in all tested organs and stages, but with considerable variation (Fig. 2). *BdLAC5* is expressed mainly in the stem, internode or peduncle, and floral rachis, whereas *BdLAC6* is expressed mainly in the root or mesocotyl at earlier stages and also in the internode at MS. Both *BdLAC5* and *BdLAC6* transcript levels were found to be very low in poorly lignified organs, such as young leaves and young spikelets.

In parallel with this transcript study, the kinetic monitoring of *B. distachyon* lignification was performed on stem and leaf samples at the same stages in addition to the senescence stage (SS). Lignin content and the structure of extractive-free material were estimated using thioglycolic acid (TGA) and thioacidolysis methods (Fig. 3, A and B). In agreement with Terashima et al. (1993), the stem lignin level as well as the percentage of S lignin units was found to increase with maturity, whereas H thioacidolysis monomers were recovered in higher frequency at early developmental stages (Fig. 3, A and B). Histochemical Wiesner and Maüle stainings of *B. distachyon* stems (Fig. 3C) were consistent with these chemical analyses. At TS, using Wiesner staining, lignins could be detected mainly in vessels (metaxylem and protoxylem), mestome, and intrafascicular fibers of vascular bundles. It is only at the EFS and later stages that interfascicular fibers were substantially lignified, with enrichment of S units as revealed by its bright red color with the Maüle reagent. By contrast, and whatever the stage, xylem elements remained orange with Maüle

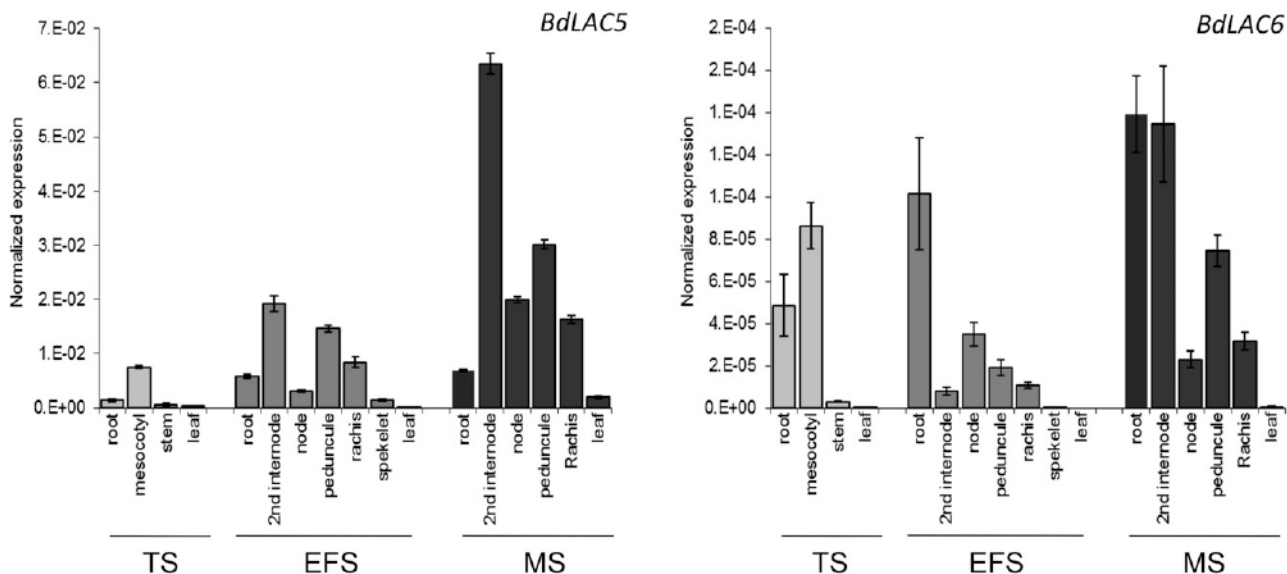


Figure 2. Expression patterns of *BdLAC5* and *BdLAC6* mRNA at different stages of development in wild-type plants (accession Bd21-3). *BdLAC5* and *BdLAC6* transcript levels were quantified with quantitative RT-PCR. The relative transcript levels of *BdLAC5* and *BdLAC6* were normalized with the housekeeping gene *BdUBI4*. The data represent means $\pm$ SD from biological replicates ($n = 3$).

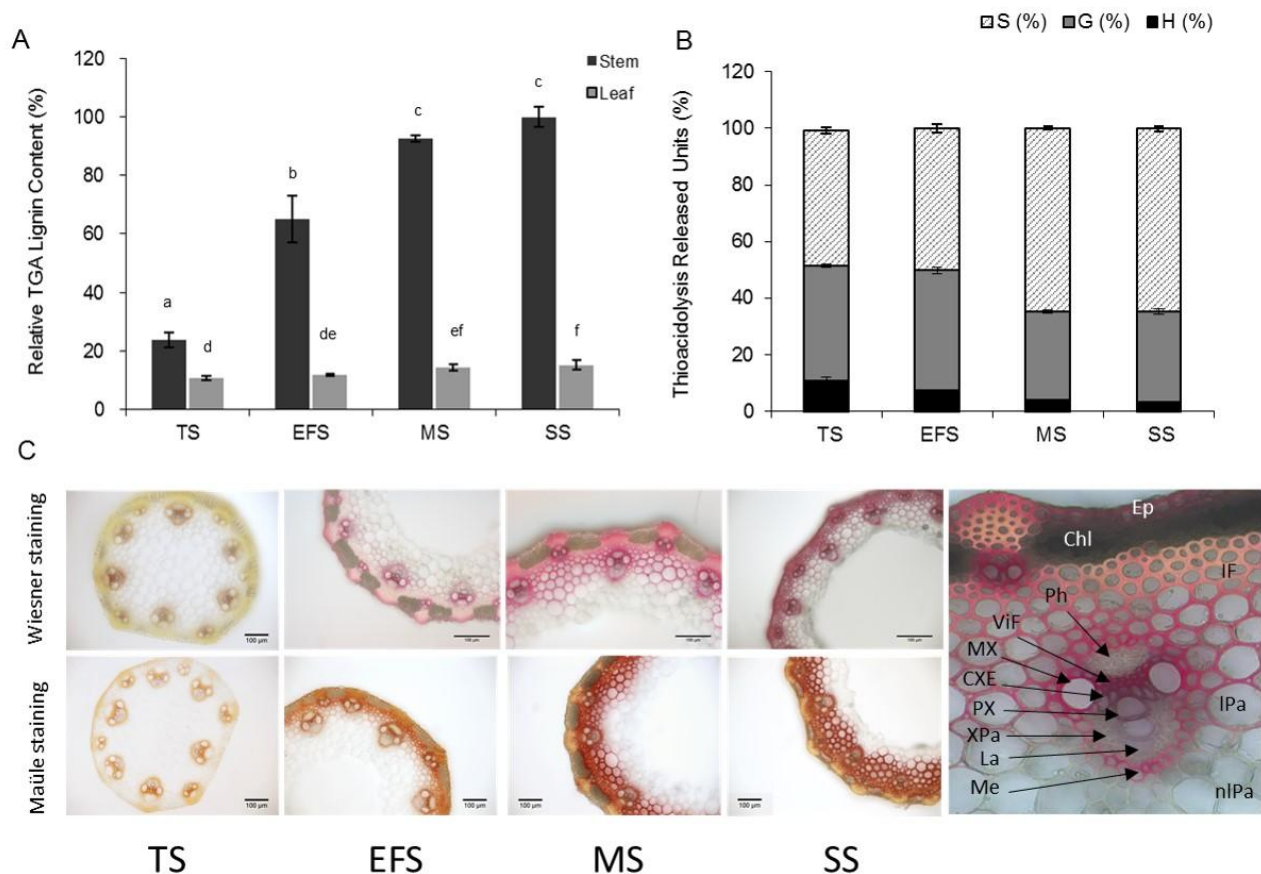


Figure 3. Lignin content and histochemical detection of lignified tissues in wild-type plants at different stages. **A**, Relative TGA lignin content in stems and leaves. Data are shown as percentages of the highest value (stem at SS). Lowercase letters represent significant differences. **B**, Thioacidolysis released monolignols from stems at different stages. Data are shown as percentages of the relative thioacidolysis yield. For **A** and **B**, all data represent means $\pm$ SD from biological replicates ($n = 3$). **C**, Histochemical staining of lignified tissues in internodes at different developmental stages. **D**, Wiesner staining at MS and labeling of tissues in *B. distachyon* stem. Chl, Chlorenchyma; CXE, connecting xylem elements; Ep, epidermis; IF, interfascicular fibers; IPa, lignified parenchyma; Me, mestome; MX, metaxylem; nIPa, nonlignified parenchyma; Ph, phloem; PX, protoxylem; VF, vascular fibers; Xpa, xylem parenchyma.

staining, which suggests the occurrence of G-rich lignins. In summary, the lignification of vascular bundles occurs early, with deposition of G-rich lignins, while interfascicular fibers lignify gradually, with a distribution of G-lignin and S-lignin units.

To more accurately localize *BdLAC5* and *BdLAC6* transcripts at the tissue level, in situ hybridization experiments were performed with appropriate RNA probes applied to internode sections collected at EFS. At this stage, the sclerenchyma (interfascicular fiber) tissue, a few parenchyma cells, and vascular bundles are lignified (Fig. 3C). The intense signal of the *BdLAC5* anti-sense probe was detected in most of these tissues (Fig. 4A) when compared with the negative control performed with the sense probe (Fig. 4C). The metaxylem and protoxylem areas were poorly labeled, a phenomenon that could be accounted for by the fact that these cells are fully functional and cytosol free at EFS (Bollhöner et al., 2012; Schuetz et al., 2013). The most intense signal of *BdLAC5* transcripts was observed in most sclerenchyma cells and some lignifying neighboring parenchyma cells of

the interfascicular area, whereas the nonlignified phloem was not labeled (Fig. 4A). When the hybridization test was carried out on whole leaves (Fig. 4F), only foliar veins were labeled, a result that further confirms the location of *BdLAC5* transcripts in lignifying areas. Most signals of *BdLAC6* transcripts were observed to parallel those of *BdLAC5*, but with a lower intensity (Fig. 4B). In conclusion, the maximum levels of *BdLAC5* and *BdLAC6* transcripts were found in cells and stages undergoing the most active lignification, with *BdLAC5* being expressed to a much higher extent than *BdLAC6* in stems.

BdLAC5 and BdLAC6 Proteins Are Localized in the Apoplasm

The *BdLAC5* gene (1,719-bp coding sequence) encodes a predicted 572-amino acid polypeptide (theoretical molecular mass and pI, 62.7 kD and 8.96), while the *BdLAC6* gene (1,689-bp coding sequence) encodes a predicted 561-amino acid polypeptide (theoretical molecular mass and

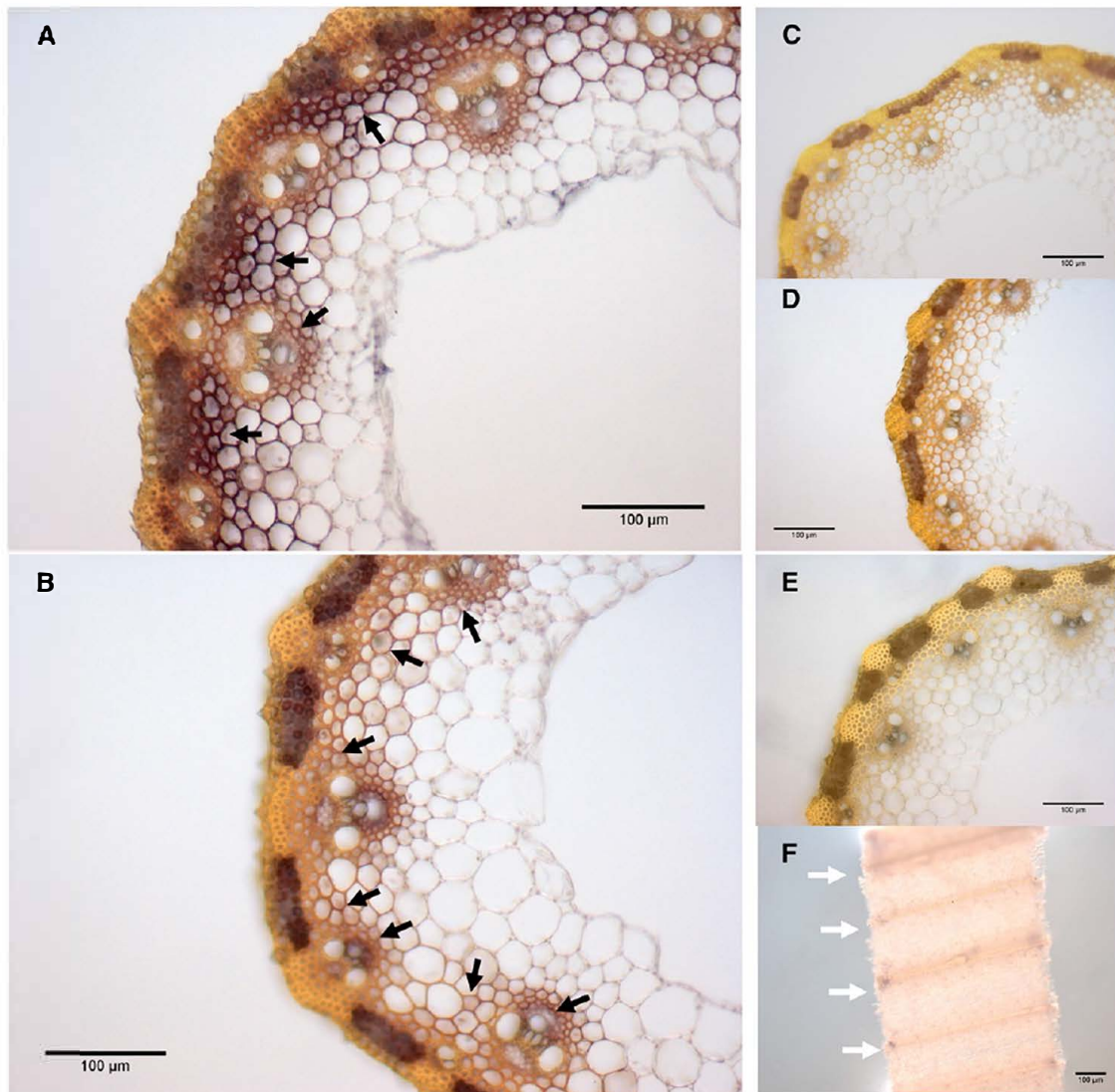


Figure 4. Tissue localization of *BdLAC5* and *BdLAC6* transcripts. Transcripts of *BdLAC5* and *BdLAC6* were detected by in situ hybridization at the heading and flowering stages (EFS). A, Cross sections of the second internode were labeled with *BdLAC5* antisense probes. B, *BdLAC6* antisense probes. C, *BdLAC5* sense probes. D, *BdLAC6* sense probes. E, Cross section of internodes without labeled probes. F, Whole-mount leaves labeled by *BdLAC5* antisense probes. Black arrows show immunolabeling signal. White arrows show veins. Bars = 100 μm.

pI, 62.1 kD and 9.34). According to the prediction software, both *BdLAC5* and *BdLAC6* are hydrophilic proteins that could be glycosylated and exported to the cell wall (Supplemental Fig. S2). Little experimental evidence is published regarding the subcellular location of laccases (Berthet et al., 2011; Cesarino et al., 2013). It is worth noting that Pang et al. (2013) observed the *AtLAC15* protein fused to GFP in the vacuole instead of the cell wall, which would make sense if *AtLAC15* is involved in the polymerization of proanthocyanidins (Pang et al., 2013). More recently, it was shown that *AtLAC17* and *AtLAC4* are located in secondary cell walls throughout protoxylem tracheary element differentiation (Schuetz et al., 2014). We attempted to detect *BdLAC5* and *BdLAC6*

subcellular location using immunolabeling with two specific primary antibodies against these laccases. We focused our attention on the interfascicular fiber cells, because vascular bundles have high levels of autofluorescence. This immunolabeling assay revealed anti-*BdLAC5*-labeled particles in or next to the walls of the interfascicular fibers (Fig. 5). *BdLAC6* showed a similar pattern, but fewer particles were detected, presumably due to the lower expression of *BdLAC6*. However, these data suggest that the two laccases are secreted to the apoplast and accumulate in secondary cell walls of lignified cells, a result in accordance with the location of *AtLAC4* and *AtLAC17* in secondary cell walls of tracheary elements (Schuetz et al., 2014).

Identification of BdLAC5 and BdLAC6 Mutants Affected in the Highly Conserved C-Terminal Domain

A targeting-induced local lesion in genome screen was carried out on the Versailles *B. distachyon* mutant collection in order to identify mutations in *BdLAC5* and *BdLAC6* genes by reverse genetics. This screen led to the identification of several lines with mutations in *BdLAC5* and *BdLAC6* (Dalmais et al., 2013). Among these mutants, one *BdLAC5* mutant, *Bd4442*, and one *BdLAC6* mutant, *Bd5024*, were selected on the rationale that mutations result in the loss of the highly conserved and

essential laccase C-terminal domain. To be active, laccase needs to bind four copper ions (McCaig et al., 2005; Reiss et al., 2013). One copper ion site is in the C-terminal domain of the protein, and it is involved in the oxidation of the reducing substrate and, thus, necessary for oxidase activity (Durão et al., 2006). The mutant *Bd4442* has a mutation in the 5' splice site of the last intron of the *BdLAC5* (*Bradi1g66720*) gene model (Dalmais et al., 2013). In order to verify the effect of this mutation on the transcript, we performed RT-PCR analysis to amplify the *BdLAC5* mRNA in *Bd4442*. When

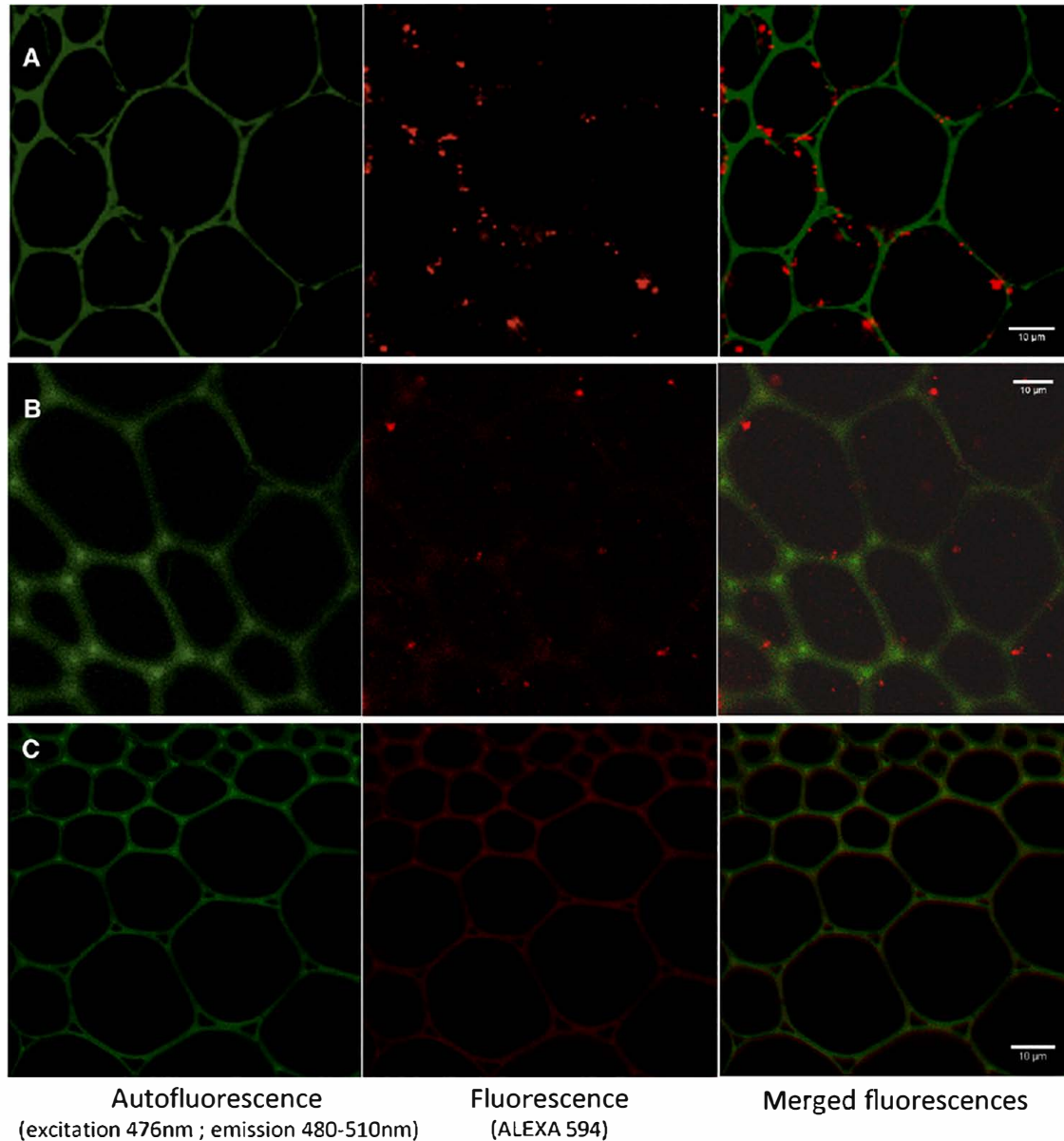


Figure 5. Subcellular localization of BdLAC5 and BdLAC6 in lignifying interfascicular fibers. Immunolocalizations are imaged with confocal microscopy. A, Immunolocalization of Alexa Fluor 594 after the detection of BdLAC5 primary antibody. B, Immunolocalization of Alexa Fluor 594 after the detection of BdLAC6 primary antibody. C, Control immunofluorescence with Alexa Fluor 594 secondary antibody without primary antibody. Green indicates autofluorescence of cell wall (excitation, 476 nm; emission, 480–510 nm), and red indicates fluorescence of Alexa Fluor 594. Bars = 10 µm.

compared with the wild-type sample, the *BdLAC5* mRNA from *Bd4442* displayed an insertion of 13 nucleotides from the last intron, which is predicted to cause a truncation of the C-terminal domain of the *BdLAC5* protein (Supplemental Fig. S3). The mutant *Bd5024* possesses a single G-to-T nucleotide substitution at position 1,270 of the *BdLAC6* complementary DNA (cDNA), which introduces a premature stop codon at Gly-424. Consequently, the two selected lines share truncated laccase proteins, which likely lack the highly conserved C-terminal domain (Fig. 6).

Impact of Mutations in *BdLAC5* and *BdLAC6* on Plant Phenotype and Lignification

Compared with the wild-type sample, the *Bd4442* and *Bd5024* homozygous mutants did not display any drastic

alteration of growth and development in our culture conditions (Fig. 7A). However, closer examination revealed that the stem height and internode diameter of the *Bd4442* line were systematically smaller than those of the wild-type line (Supplemental Table S4). At the tissue level, the area of the vascular bundles from the homozygous *Bd4442* line was also found to be reduced slightly as compared with the wild type.

The lignin level of extractive-free mature stems of *Bd4442* and *Bd5024* mutants was measured by the Klason lignin method (Dence, 1992). Consistent with the histochemical results, the *BdLAC6*-deficient *Bd5024* line did not display any alteration to the lignin content of mature stems. By contrast, disrupting the *BdLAC5* gene in the *Bd4442* mutant induced a modest but significant reduction of the culm lignin level (Table I). This reduction (by about 10% compared with the wild-type level) was systematically observed for independent cocultures of

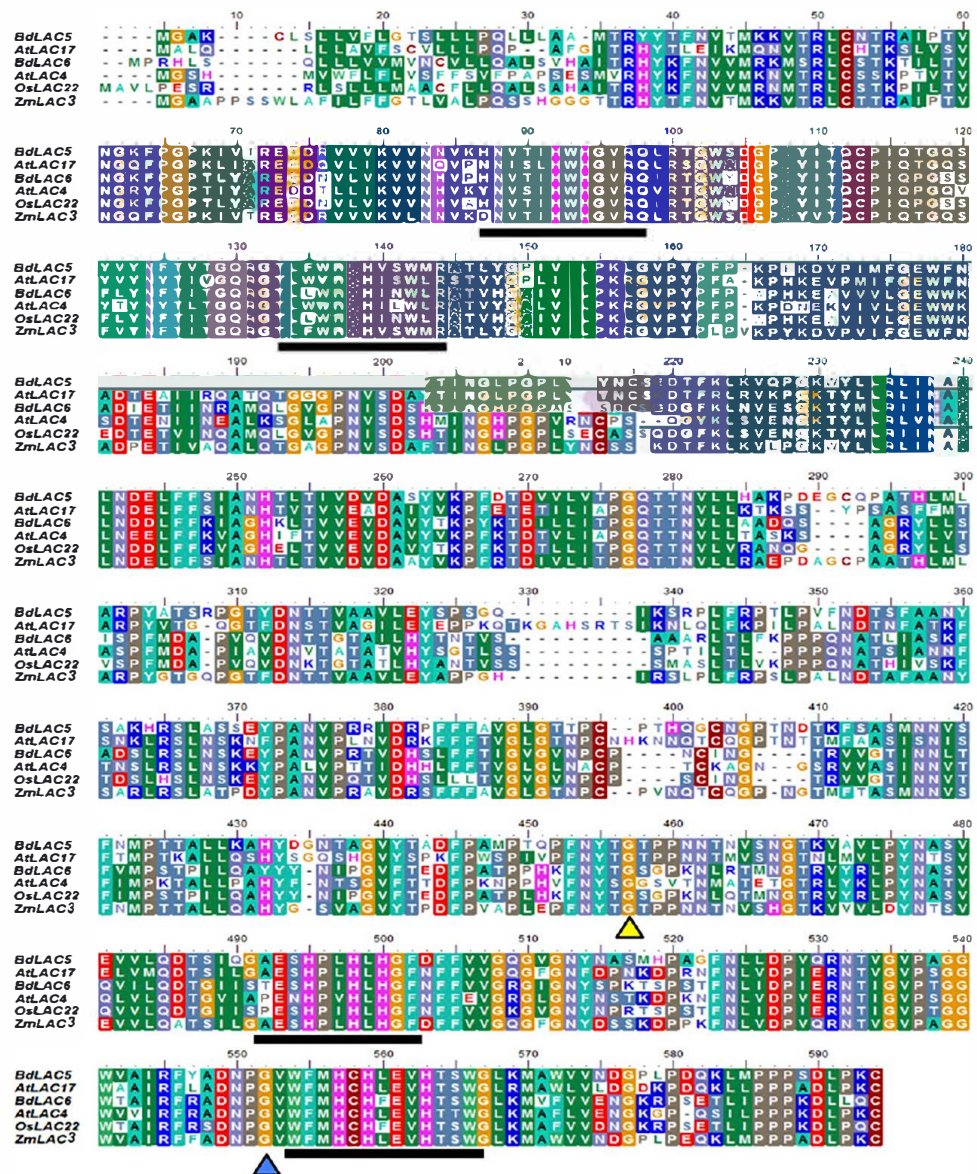


Figure 6. Multiple alignment of deduced amino acid sequences from *B. distachyon* and other plant laccases. Amino acid sequences of both *B. distachyon* laccases, maize (*ZmLAC3*), rice (*Oryza sativa*; *OsLAC22*), and Arabidopsis (*AtLAC4* and *AtLAC17*) laccases were aligned with ClustalW software. The copper-binding domain motifs are underlined. Triangles represent the locations of amino acid changes in *BdLAC5* of *Bd4442* (blue) and the location of the premature stop codon in *Bd5024* (yellow).

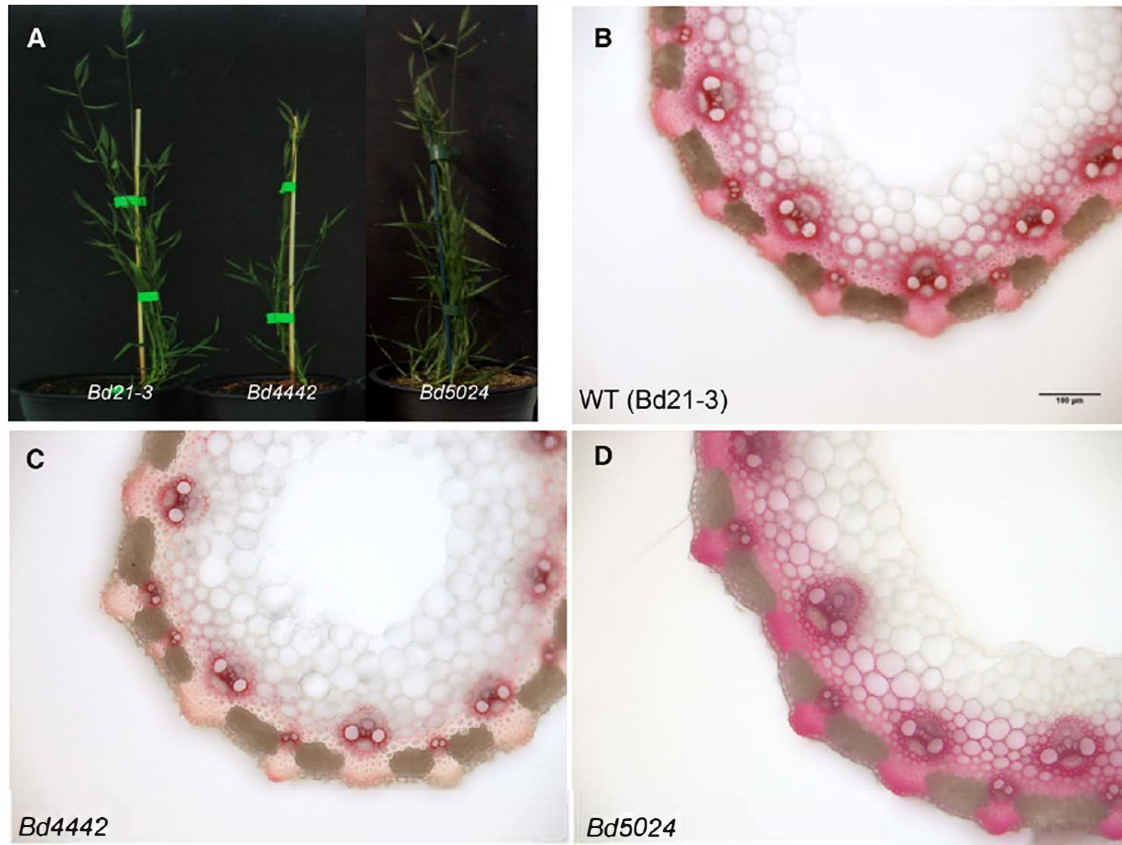


Figure 7. Phenotypes of mutants and wild-type plants. A, Developmental phenotypes of *Bd4442*, *Bd5024*, and the wild type. B to D, Transverse sections of second internodes stained with Wiesner reagent. Bar = 100 μ m. For B to D, photographs were taken at the same magnification.

Bd4442 and wild-type lines carried out over a 2-year period and confirmed by the TGA lignin assays (Supplemental Table S5). The analysis of cell wall

polysaccharides suggested that the decreased lignin content in *Bd4442* stems is balanced by a slightly increased hemicellulose content (Supplemental Fig. S4).

Table 1. Lignin analyses for the wild type and *BdLAC5* (*Bd4442*) and *BdLAC6* (*Bd5024*) misregulated mutants

Lignin content and structure for extractive-free mature stems were measured by Klason and thioacidolysis methods. Values are means $\pm$ sd from individually analyzed plants ($n = 3$).

Culture and Genotype	Lignin Content	Relative Frequency of Lignin-Derived Thioacidolysis Monomers		
		H	G	S
%				
First culture				
Wild type	17.63 ± 0.01	2.8 ± 0.1	30.9 ± 0.8	66.3 ± 0.9
<i>Bd4442</i>	15.71 ± 0.04 ^a	2.6 ± 0.2	25.3 ± 0.4 ^a	72.2 ± 0.6 ^a
<i>Bd5024</i>	17.53 ± 0.14	2.8 ± 0.1	33.3 ± 0.3	63.9 ± 0.3
Second culture				
Wild type	16.93 ± 0.57	3.0 ± 0.2	30.1 ± 2.5	66.9 ± 2.7
<i>Bd4442</i>	15.60 ± 0.07 ^a	1.6 ± 0.2 ^a	22.2 ± 3.5 ^a	76.3 ± 3.5 ^a
Third culture				
Wild type	17.59 ± 0.49	3.3 ± 0.1	31.0 ± 1.0	65.7 ± 1.0
<i>Bd4442</i>	16.19 ± 0.20 ^a	2.7 ± 0.1	26.5 ± 0.5 ^a	70.8 ± 0.5 ^a

^aSignificantly different from the corresponding wild-type sample (one-way ANOVA, Tukey's honestly significant difference) at $P < 0.05$.

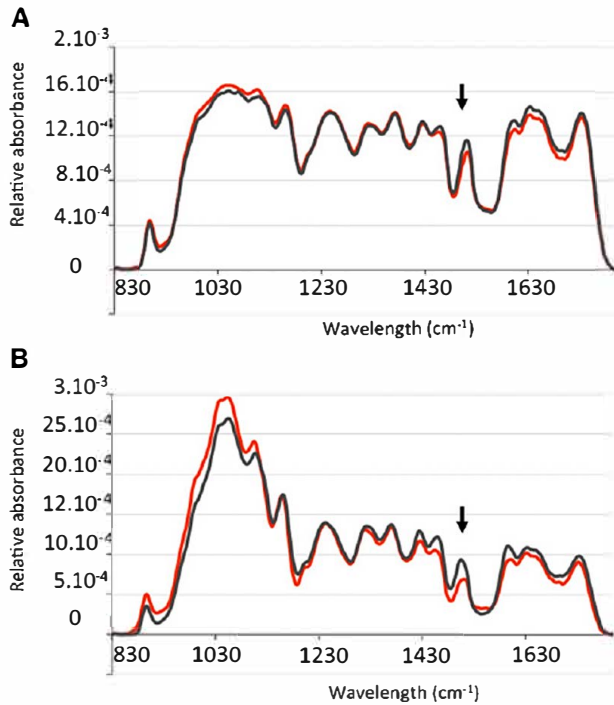


Figure 8. FTIR analysis of interfascicular fibers and vascular bundles. FTIR spectra were acquired from vascular bundles (A) and interfascicular fibers (B) in cross sections of internodes at MS. Black lines indicate wild-type plants, and red lines indicate *Bd4442*. Arrows show absorbance at 1,508 cm^{-1} . Each spectrum corresponds to the normalized means of biological replicates ($n = 6$).

The lignin structure of the wild-type and mutant lines was also investigated by thioacidolysis (Lapierre, 1993). Compared with the corresponding wild-type sample, the frequency of the S thioacidolysis monomers released from *Bd4442* mature stems was found to be systematically higher (Table I). This higher S frequency has been also reported in *Atlac17* (Berthet et al., 2011), an *Arabidopsis* mutant affected in the expression of *AtLAC17* (a presumed *Arabidopsis* ortholog of *BdLAC5*).

We further investigated the impact of the mutation in *Bd4442* at the tissue level by histochemical staining and Fourier transform infrared (FTIR) microspectrophotometry. Compared with the wild type, Wiesner staining of *Bd4442* stems at MS was substantially weaker, particularly in the sclerenchyma (Fig. 7). By contrast, this test did not show obvious differences between the *Bd5024* and wild-type lines. To further investigate the topochemistry of lignification in *Bd4442* culms as compared with control plants, FTIR absorbance spectra of vascular bundle or interfascicular fiber areas were recorded with a spectra-microscope system as described previously (Mouille et al., 2003; Sibout et al., 2005). Relative to the wild type, the most striking impact of the *Bd4442* mutation on the FTIR spectra was a substantial reduction of the lignin-specific aromatic signal at 1,508 cm^{-1} and mainly in the interfascicular fiber area (Fig. 8; Supplemental Fig. S5). By contrast, this lignin peak was

reduced to a much lower extent in the *Bd4442* vascular bundle area. Taken together, the histochemical and FTIR results revealed that the lignification of *B. distachyon* culm was affected by the *Bd4442* mutation, more specifically in interfascicular fibers.

From these analyses, we may conclude that *BdLAC5* disruption results in a decreased lignification of mature stems, mainly in the interfascicular fibers, and that the biosynthesis of G lignin units is affected to a higher extent than that of S lignin units. The latter result might be accounted for either from some substrate specificity of the *BdLAC5* enzyme and/or a spatiotemporally regulated expression of the *BdLAC5* gene at the time when G lignin units are deposited.

It is well established that grass lignins are not only acylated by *p*-coumaric acid (CA) but also are covalently linked to feruloylated arabinoxylans, consisting of a xylan backbone with Ara substituents partially acylated by ferulic acid (FA; Ralph, 2010). At the onset of lignification, these FA esters act as lignin initiation sites by oxidatively driven coupling mainly to coniferyl alcohol (Jacquet et al., 1995). The impact of the *BdLAC5* and *BdLAC6* mutations on the level of CA and FA ester linked to stem cell walls was studied by mild alkaline hydrolysis according to previously published methods (Bouvier d'Yvoire et al., 2013; Petrik et al., 2014). While the level of CA esters was found to be slightly higher in *Bd5024* and unchanged in *Bd4442*, mild alkaline hydrolysis released about 40% more FA from *Bd4442* than from the wild type (Table II). This higher content of FA units only ester linked to *Bd4442* cell walls was accompanied by a lower amount of FA units ether linked to lignins, as revealed by severe alkaline hydrolysis (Barrière et al., 2004a, 2004b). The amount of FA ethers in the *Bd4442* and wild-type samples was 5.89 ± 0.36 and $6.93 \pm 0.48 \text{ mg g}^{-1}$, respectively. According to model studies carried out with artificially lignified maize cell walls, these changes could be linked to the reduced

Table II. Amounts of CA and FA released by mild alkaline hydrolysis of mature stems from the wild type and *BdLAC5* (*Bd4442*) and *BdLAC6* (*Bd5024*) misregulated mutants

Values are means $\pm$ SD from individually analyzed plants ($n = 3$).

Culture and Genotype	Compound	
	CA	FA
	mg g^{-1}	
First culture		
Wild type	7.17 ± 0.04	4.69 ± 0.03
<i>Bd4442</i>	7.50 ± 0.43	6.48 ± 0.41^a
<i>Bd5024</i>	8.20 ± 0.18^a	4.42 ± 0.09
Second culture		
Wild type	6.83 ± 0.52	5.31 ± 0.44
<i>Bd4442</i>	6.52 ± 0.31	7.69 ± 0.15^a
Third culture		
Wild type	9.42 ± 0.21	5.05 ± 0.07
<i>Bd4442</i>	9.48 ± 0.23	7.42 ± 0.11^a

^aSignificantly different from the corresponding wild-type sample (one-way ANOVA, Tukey's honestly significant difference) at $P < 0.05$.

Table III. Lignin and FA analyses for the wild type and the *BdLAC5* (*Bd4442*) mutant line as compared with the *Bd4442* complemented line3 (*CP3*) mutant line complemented with the *BdLAC5* wild-type allele (*T2* transformant)

Lignin content and structure for extractive-free mature stems were measured by Klason and thioacidolysis methods; FA level ester linked to the cell walls was measured by mild alkaline hydrolysis. Values are means $\pm$ sd from individually analyzed plants ($n = 3$). All the plants were obtained from the same culture experiment in the greenhouse.

Genotype	Lignin Content	Relative Frequency of Lignin-Derived Thioacidolysis Monomers			FA Amount
		H	G	S	
		%			mg g^{-1}
Wild type	18.40 ± 0.33	3.0 ± 0.1	27.1 ± 1.1	66.3 ± 0.9	5.05 ± 0.07
<i>Bd4442</i>	16.43 ± 0.19^a	3.1 ± 0.1	23.7 ± 0.5^a	73.4 ± 0.6^a	7.42 ± 0.11
<i>Bd4442 CP3</i>	18.15 ± 0.23	3.1 ± 0.1	32.6 ± 0.5	64.4 ± 0.5	5.88 ± 0.12

^aSignificantly different from the corresponding wild-type sample (one-way ANOVA, Tukey's honestly significant difference) at $P < 0.05$.

lignification of *Bd4442* cell walls (Grabber et al., 2000). In addition and together with peroxidases, *BdLAC5* might participate in the oxidatively driven coupling of FA esters and lignin units at the onset of lignification. Its misregulation together with the reduced lignification might further limit this cross-coupling mechanism.

Complementation of the *Bd4442* Mutant with the *BdLAC5* Wild-Type Allele Restores Lignification and Saccharification Yield

The definitive demonstration that *BdLAC5* is a lignin-specific laccase involved in the lignification of *B. distachyon* culms was provided by complementation experiments. We produced transgenic lines over-expressing a *BdLAC5* wild-type gene copy driven by a maize ubiquitin promoter into *Bd4442*. We obtained few transgenic lines suggesting the recalcitrance of this line to embryogenesis. However, we selected one transformed line with detectable levels of the transgene (Supplemental Fig. S6). This line showed a complete restoration of lignin level, lignin structure, and FA esters (Table III). This line also restored phenotype (Supplemental Table S4) and lignin histochemical staining (Supplemental Fig. S7). Not unexpectedly, the saccharification yield of the extractive-free mature stems was found to be improved for the *Bd4442* mutant and comparable to that of the wild-type sample for the complemented line (Table IV). The weight loss induced by a cellulase treatment without any pre-treatment was found to be $30.5 \pm 1.3\%$ for *Bd4442* samples versus $23.5\% \pm 1.4\%$ for wild-type samples and $20.6\% \pm 0.4\%$ for the complemented line (mean $\pm$ sd values for two independent experiments each performed on three different samples per line).

CONCLUSION

To our knowledge, this study provides the first evidence that a laccase enzyme, *BdLAC5*, is involved in the

lignification of *B. distachyon*, a model plant for important grass crops. Indeed, in the *BdLAC5*-misregulated *Bd4442* mutant line identified in this work, the alteration of the C-terminal domain of *BdLAC5* induces significant alterations to the lignification of mature culms, with a 10% lower lignin level, a slight increase of the frequency of S lignin units, and a substantial increase of measurable FA esters. Despite a similar expression pattern of *BdLAC6* and *BdLAC5*, it is difficult to draw the same conclusion for the role of *BdLAC6* in lignification. This laccase is expressed at a much lower level than *BdLAC5*, and redundancy might prevent any detectable impact on lignin content in the *BdLAC6* mutant. Only a double mutant would reveal a role for both genes in lignin formation. *B. distachyon* possess 29 laccases in its genome, and nine of them belong to the same cluster as *BdLAC5* and *BdLAC6*. More functional experiments are required to determine precisely which other enzymes participate in lignification; however, this study provides evidence that, as in *Arabidopsis*, laccases are involved in the lignification of *B. distachyon*, the model plant for grasses.

Table IV. Saccharification assays of extractive-free mature stem from the wild type and the *BdLAC5* (*Bd4442*) mutant line as compared with the *Bd4442 CP3* mutant line complemented with the *BdLAC5* wild-type allele (*T2* transformants)

Saccharification was evaluated both by the weight loss percentage and by the amount of Glc released from the samples. The data represent means $\pm$ sd from biological replicates (two independent experiments performed each on three different samples per line). Letters indicate significant differences analyzed by one-way ANOVA (Tukey's honestly significant difference, $P < 0.05$).

Genotype	Weight Loss Percentage	Glc
		mg g^{-1}
Bd21-3 wild type	23.5 ± 1.4 a	68.5 ± 3.9 a
<i>Bd4442</i> homozygous	30.5 ± 1.3 b	113.6 ± 4.4 d
<i>Bd4442 CP3</i>	20.6 ± 0.4 c	75.3 ± 3.3 a,b

MATERIALS AND METHODS

Plant Material and Growth Conditions

Mutants were identified in the collection of chemically induced *Brachypodium distachyon* mutants at the Research Center of the Institut National de la Recherche Agronomique-Versailles-Grignon (Dalmais et al., 2013). *B. distachyon* plants (accession Bd21-3) were grown in a greenhouse under long-day conditions (18 h of light, 400-W sodium lamps). Day and night temperatures were 23°C and 18°C, respectively. For sample harvesting, four stages (Supplemental Fig. S8) were chosen as in previous articles (Matos et al., 2013): T5, 17 d after germination, when the plants are tillering and the first true internode is elongating; EFS, approximately 35 d after germination, when an inflorescence appears with immature flowers; MS, approximately 60 d after germination, when seeds are filled and the oldest leaves are turning yellow; and SS, approximately 120 d after germination, when the whole plant is dead and dry.

Histochemical Staining

All the histochemical staining was performed on sections cut in the middle of the second internode from the top, except at T5, where the first internode above the crown was selected. Samples were fixed in 50% (v/v) ethanol, 10% (v/v) formalin, and 5% (v/v) acetic acid solution overnight and then embedded in 7% (w/v) agarose before being transversely sectioned at a thickness of 100 μ m using a vibratome (VT1000S; Leica). Lignin deposition and composition were investigated histochemically by Maile and Wiesner staining as published previously (Bouvier d'Yvoire et al., 2013). All sections were observed with a Zeiss AxioPlan 2 microscope system with automatic exposure times.

Phenotype Measurements and Image Analysis

All the measurements were performed on the tallest stems. Stem height was measured with a ruler, from the crown (the bottom of the stem) to the top of peduncle. The internode diameter was measured with a digital caliper on the second internode from the top or on the first internode above the crown at T5. ImageJ software was used for the quantification of cell wall thickness and vascular bundles. Significant differences were analyzed by one-way ANOVA (Tukey's honestly significant difference, $P < 0.05$).

Lignin Content and Structure Determination

One or two main stems of each plant were collected and ground after removing spikelets and leaves. Ground samples were sequentially extracted at 60°C with 50 mL of ethanol, water, and ethanol. At each step, the samples were vortexed. These steps were repeated twice. Samples were then dried and used for the following analysis.

The protocol of the TGA lignin assay was optimized from Hatfield and Fukushima (2005) and Suzuki et al. (2009). Extractive-free cell wall residues of stem were treated with TGA. Briefly, 10 to 15 mg of plant cell wall residues was treated with TGA at 80°C for 8 h. After washing with water and extracting with NaOH (37°C overnight), the purified lignin complex was precipitated using concentrated HCl. The pellet was dissolved in 1 mL of 1 M NaOH. After 6-fold dilution with 1 M NaOH, the solution was measured by UV spectrophotometry at 280 nm. The calibration curves were made by lignin standards purified from *B. distachyon*. The relative lignin concentration was determined by dividing the amount of lignin calculated based on the calibration curve by the sample weight.

The Klason lignin content was measured according to Dence (1992). The lignin structure and composition were studied by thioacidolysis, as described previously (Lapierre et al., 1999). The lignin-derived thioacidolysis monomers were identified by gas chromatography-mass spectrometry as their trimethylsilylated derivatives. All the analyses were performed with at least three biological replicates. Significant differences were inferred by one-way ANOVA (Tukey's honestly significant difference, $P < 0.05$).

Monosaccharide Composition and Linkage Analysis of Polysaccharides

Neutral monosaccharide composition was determined on 5 mg of dried alcohol-insoluble material after hydrolysis in 2.5 M trifluoroacetic acid for 1.5 h at 100°C as described by Harholt et al. (2006). To determine the cellulose content, the residual pellet obtained after the monosaccharide analysis was

rinsed twice with 10 volumes of water and then hydrolyzed with H₂SO₄ as described by Updegraff (1969). The released Glc was diluted 500-fold and then quantified using high-performance anion-exchange chromatography-pulsed-amperometric detection as described by Harholt et al. (2006).

RNA Extraction and Quantitative RT-PCR

Total RNA was extracted using the Qiagen Plant RNAeasy kit with an additional DNase step. RNA samples were quantified using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). One microgram of total RNA was used for RT using the SuperScript Reverse Transcriptase II kit (Invitrogen). The absence of genomic contamination was confirmed by PCR with primers targeting introns. Quantitative RT-PCR was performed on the Eppendorf Realplex2 Mastercycler using the SYBR Green kit (Bio-Rad) and the following conditions: 95°C for 3 min, followed by 40 cycles of 95°C for 20 s, 59°C for 20 s, and 72°C for 20 s. Information on primers is available in Supplemental Table S4. PCR products were verified by sequencing. The normalized expression levels of target genes were calculated by Q-Gene software (<http://www.biotechniques.com/softlib/qgene.html>) based on the formula for the amplification efficiency of PCR of Muller et al. (2002). The *B. distachyon* ubiquitin gene *BdUBI4* (Hong et al., 2008) was used as the reference gene (Supplemental Table S6). The results were based on three independent biological replicates.

RNA in Situ Hybridization

Primer sequences used for the digoxigenin (DIG)-labeled probe amplification are shown in Supplemental Table S1. Antisense probes were synthesized by in vitro transcription with T7 RNA polymerase using a DIG RNA labeling kit (Roche Applied Science) according to the manufacturer's protocol. A sense probe was used as a negative control.

Second internodes at the flowering stage were fixed, embedded, and cross sectioned as described above. In situ hybridization was carried out as described by Chapelle et al. (2012) with minor changes: after washing and dehydration of the tissues, sections were prehybridized for 1 h at 60°C with hybridization solution (50% [v/v] formamide, 5 $\times$ SSC, 100 μ g mL⁻¹ tRNA, 50 μ g mL⁻¹ heparin, and 0.1% [v/v] Tween). Sections were then hybridized with DIG-labeled antisense or sense probes (1:1,000) overnight at 60°C and washed with a series of SSC solutions. Immunodetection of the DIG-labeled probe was performed with an anti-DIG antibody coupled with alkaline phosphatase using the nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate kit according to the manufacturer's instructions (Roche Applied Science). All sections were observed with a Zeiss AxioPlan 2 microscope system with automatic exposure time.

Phylogeny Tree and Three-Dimensional Structure Prediction

An unrooted phylogenetic tree was constructed with PhyML in the Phylogeny.fr platform (<http://www.phylogeny.fr>) with Blossum62 as a substitution model, with 1,000 bootstraps after loading the protein ClustalW alignment (Dereeper et al., 2008). The final shape of the tree was produced after submitting the Newick format tree to TreeDyn. Multiple sequence alignment was performed with ClustalW software (<http://www.ebi.ac.uk/Tools/clustalw2>).

Genetic Complementation of the BdLAC5 Splicing Mutant Bd4442

The full-length cDNA including the 5'- and 3'-untranslated regions of *BdLAC5* was cloned from *B. distachyon* stem cDNA using specific primers (Supplemental Table S5). The purified PCR product was ligated into the pDONR207 plasmid via the BP Gateway (Invitrogen) reaction and confirmed by sequencing. The resulting pDONR207-BdLAC5 plasmid was used to transfer *BdLAC5* into pIPKb2 (Himmelbach et al., 2007) for *B. distachyon* transformation by the LR Gateway (Invitrogen) reaction. *pIPKb2-BdLAC5* was electroporated into *Agrobacterium tumefaciens* (AGL1 strain). The *BdLAC5* splicing mutant (*Bd4442*) was genetically transformed following the protocol of Vogel and Hill (2008). Transgenic plants were selected on Murashige and Skoog growth medium containing 40 mg mL⁻¹ hygromycin.

FTIR Spectroscopy

Fixed internodes were cut into 50- μ m-thick sections with a vibratome and then were rinsed abundantly with distilled water for 2 min and dried at 37°C for 20 min. FTIR spectra were collected from a 60- $\times$ 60- μ m window targeting

vascular bundles or interfascicular fibers. For each genotype, three sections from three different plants were analyzed. Normalization of the spectral data and statistical analyses were performed as described by Mouille et al. (2003). Student's *t* test was performed to estimate statistical differences between mutants and the wild type.

Cell Wall Saccharification

Saccharification assays were performed as described by Berthet et al. (2011). For each sample, 30 mg of extractive-free cell wall residues was incubated with 4 mL of acetate buffer, pH 4.5, containing 4 mg mL⁻¹ commercial cellulase (Onozuka-R10; Serva) and 0.5 mg mL⁻¹ NaN₃ for 3 d at 45°C on a carousel. After centrifugation, the recovery of the supernatant for Glc was determined via enzymatic assay with the bioMérieux Kit. The pellet was washed twice with water, then freeze dried and weighed to evaluate the weight loss.

Immunolabeling

Based on their amino acid sequences, the C-terminal domains were used to design the potentially antigenic peptide sequences. The peptide sequence 5'-AGGWVAIRFYADNPGVWFMH-3' was used to produce anti-BdLAC5, and peptide sequence 5'-KMFVVENGGKRPSETLI-3' was used to produce anti-BdLAC6. Antibodies were produced by Genescript.

Internodes at flowering and heading stages were collected, fixed, and embedded as described above. After embedding, the internodes were sectioned with a thickness of 30 µm. The immunolabeling was carried out as described by Verherbruggen et al. (2009). Slides were incubated in Evans blue solution (0.001% [v/v] phosphate-buffered saline) for 10 min to decrease the autofluorescence of lignified cells. Sections were labeled with the primary anti-BdLAC5 or anti-BdLAC6 antibody from rabbit (1:1,000), and the secondary antibody was an anti-rabbit antibody conjugated to Alexa Fluor 594 (Invitrogen; 1:500). This dye was found to be the best marker because of its low interference with the autofluorescence of *B. distachyon* cell walls (data not shown). The labeled sections were viewed with a confocal laser-scanning microscope (TCS SP5 Confocal; Leica).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Expression profiles of *BdLAC5* and *BdLAC6* obtained from MaNET database (<http://aranet.mpimp-golm.mpg.de/bradinet/>).

Supplemental Figure S2. Transmembrane segment prediction of *BdLAC5* and *BdLAC6*.

Supplemental Figure S3. The aberrant transcript of *BdLAC5* and its deduced amino acid sequence in *Bd4442*.

Supplemental Figure S4. Hemicellulose and cellulose contents in the extract-free cell wall of wild-type and *Bd4442* mutants.

Supplemental Figure S5. FTIR absorbance of different tissues at 1508 cm⁻¹.

Supplemental Figure S6. Genomic characterization of the transgenic line.

Supplemental Figure S7. Staining of lignified tissues in control plants and the complemented line.

Supplemental Figure S8. Phenotypes of *B. distachyon* (accession no. *Bd21-3*) at different development stages.

Supplemental Table S1. The LACCASE family genes in *B. distachyon*.

Supplemental Table S2. Amino acid sequences of laccases used in this study.

Supplemental Table S3. List of genes coexpressed with *BdLAC5*.

Supplemental Table S4. Anatomical comparison in different genotypes.

Supplemental Table S5. Quantification of thioglycolic acid lignin.

Supplemental Table S6. Primers.

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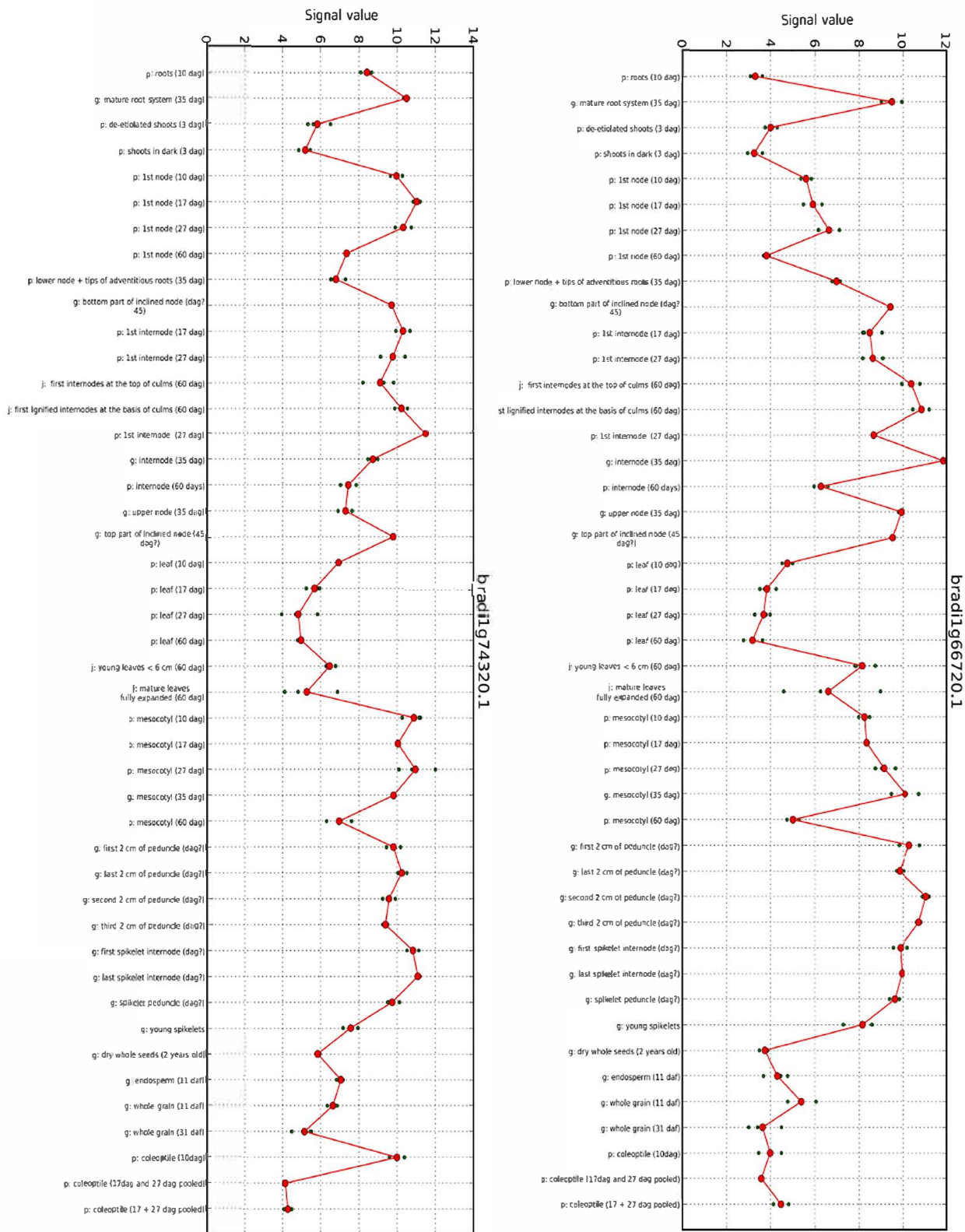


Figure S1. Expression profiles of *BdlAC5* and *BdlAC6* obtained from PLANET database (<http://aranet.mpiimp-golm.mpg.de/bradinet/>)

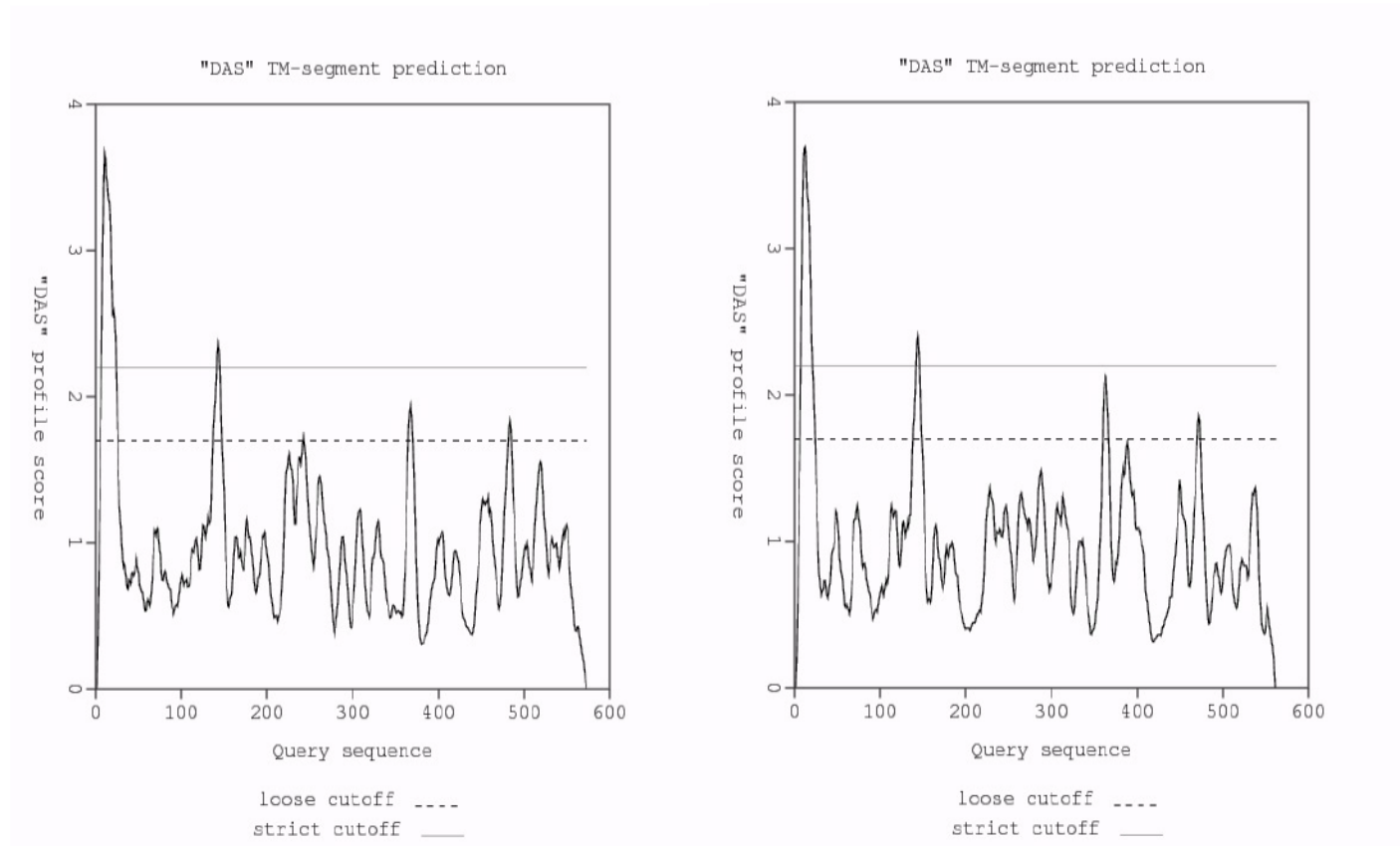


Figure S2. Transmembrane segments prediction of BdLAC5 and BdLAC6. The prediction was performed on DAS platform (Cserzo, 1997). Left: BdLAC5; Right: BdLAC6

Cserző M, Wallin E, Simon I, von Heijne G, Elofsson A (1997) Prediction of transmembrane alpha-helices in prokaryotic membrane proteins: the dense alignment surface method. *Protein Eng* 10: 673–676.

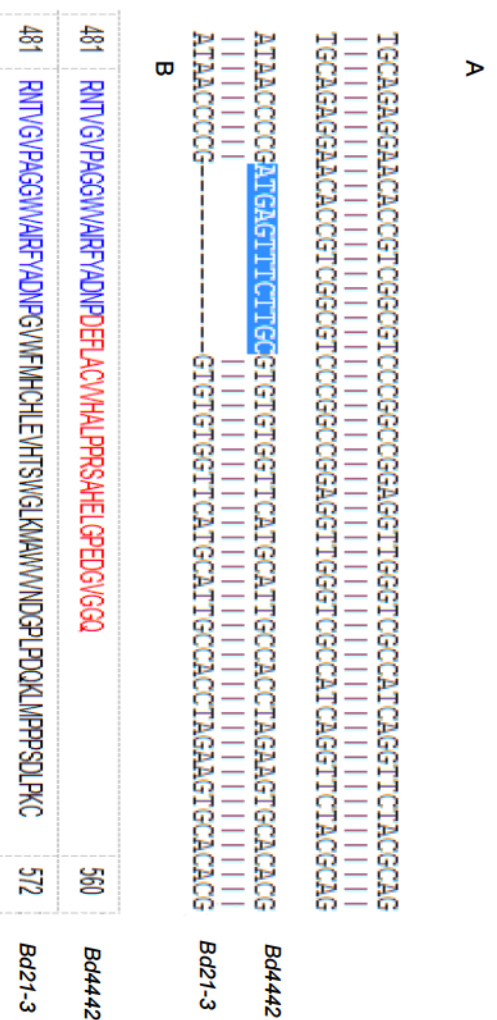


Figure S3. The aberrant transcript of *BdLACS* and its deduced amino acid sequence in *Bd4442*. A, a substitution (G to A) modifies the splicing site of the last intron of *BdLACS* in *Bd4442*. This mutation causes the transcription of thirteen supernumary nucleotides (highlighted in blue) in the mRNA and a shift in the open reading frame. B, the corresponding C-terminal region of *BdLACS* protein is abnormal and truncated in *Bd4442* (red) when compared to wild-type plant (black)

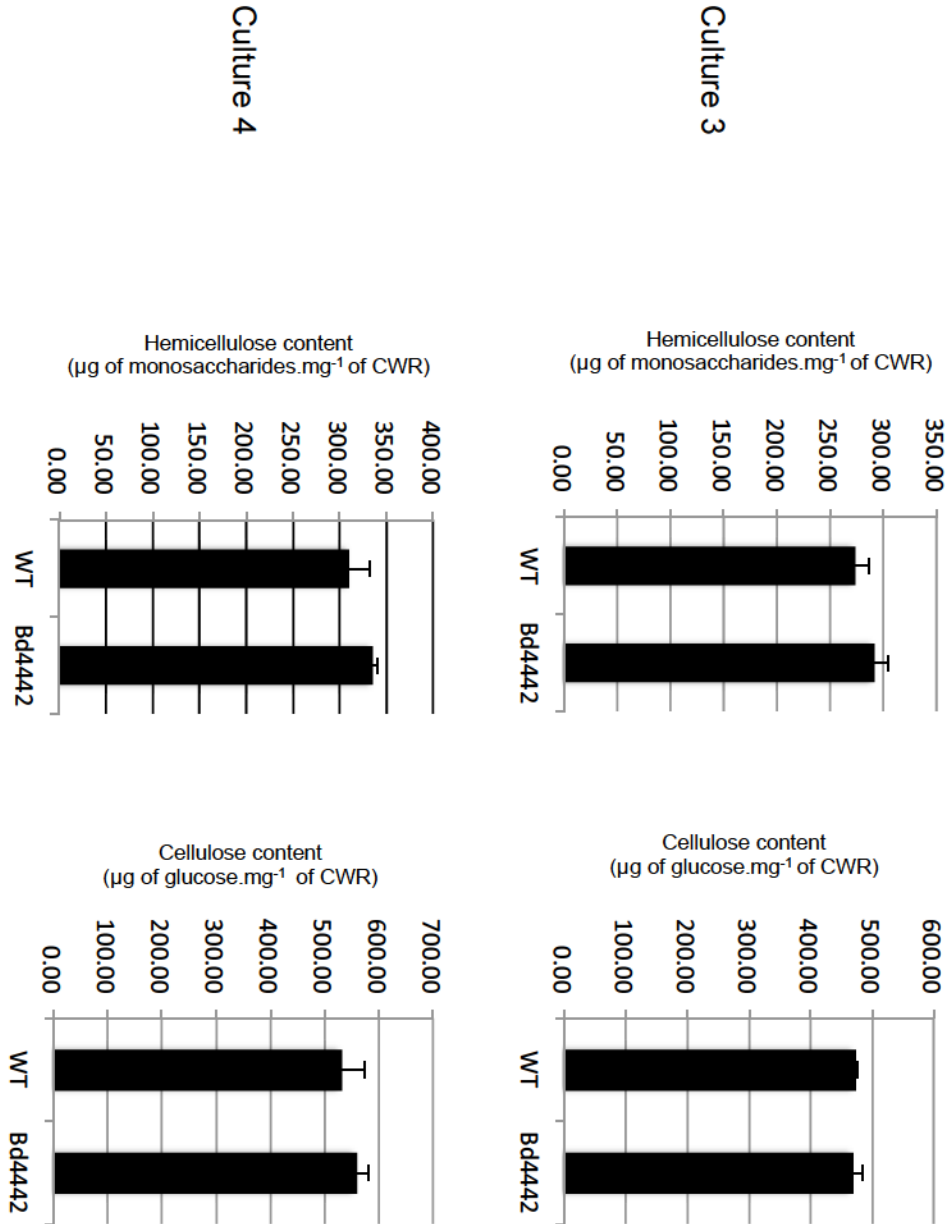
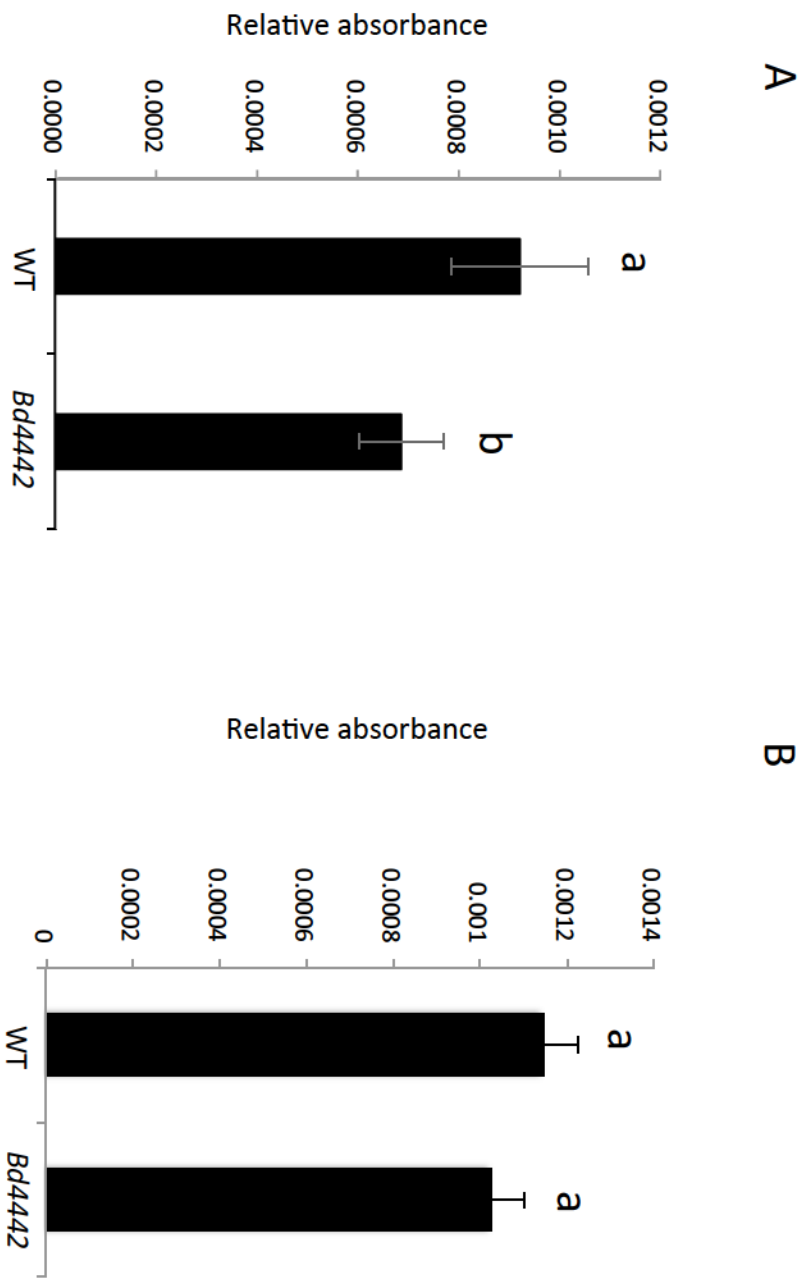


Figure S4. Hemicellulose and cellulose contents in extract-free cell wall (CWR) of wild type and *Bd4442* mutants.



Figures S5. FTIR absorbance of different tissues at 1508 cm⁻¹. **A**, absorbance in interfascicular fibers. **B**, absorbance in vascular bundles. Each bar represents means $\pm$ standard deviation from biological replicates ($n = 6$). Letters indicate significant differences analyzed by one-way ANOVA (Tukey's HSD, $P < 0.05$) between wild-type plant (WT) and *Bd4442*.

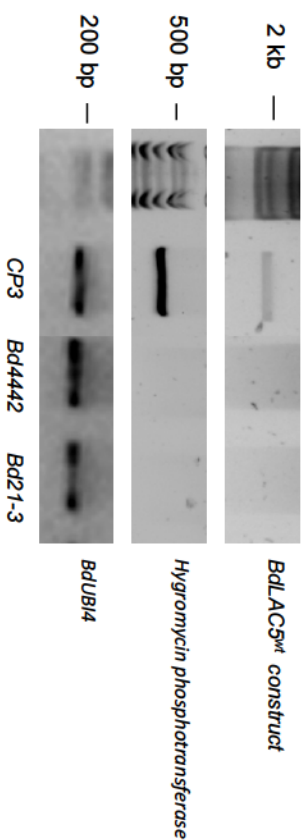


Figure S6. Genomic characterisation of the transgenic line. A genomic PCR was used to verify the presence of the *BdlACS5* wild type allele in CP3 (upper row). Transcript of hygromycin phosphotransferase gene was also amplified in the transgenic line CP3 (middle row). *BdUBI4* was used as reference gene for RT-PCR (bottom row).

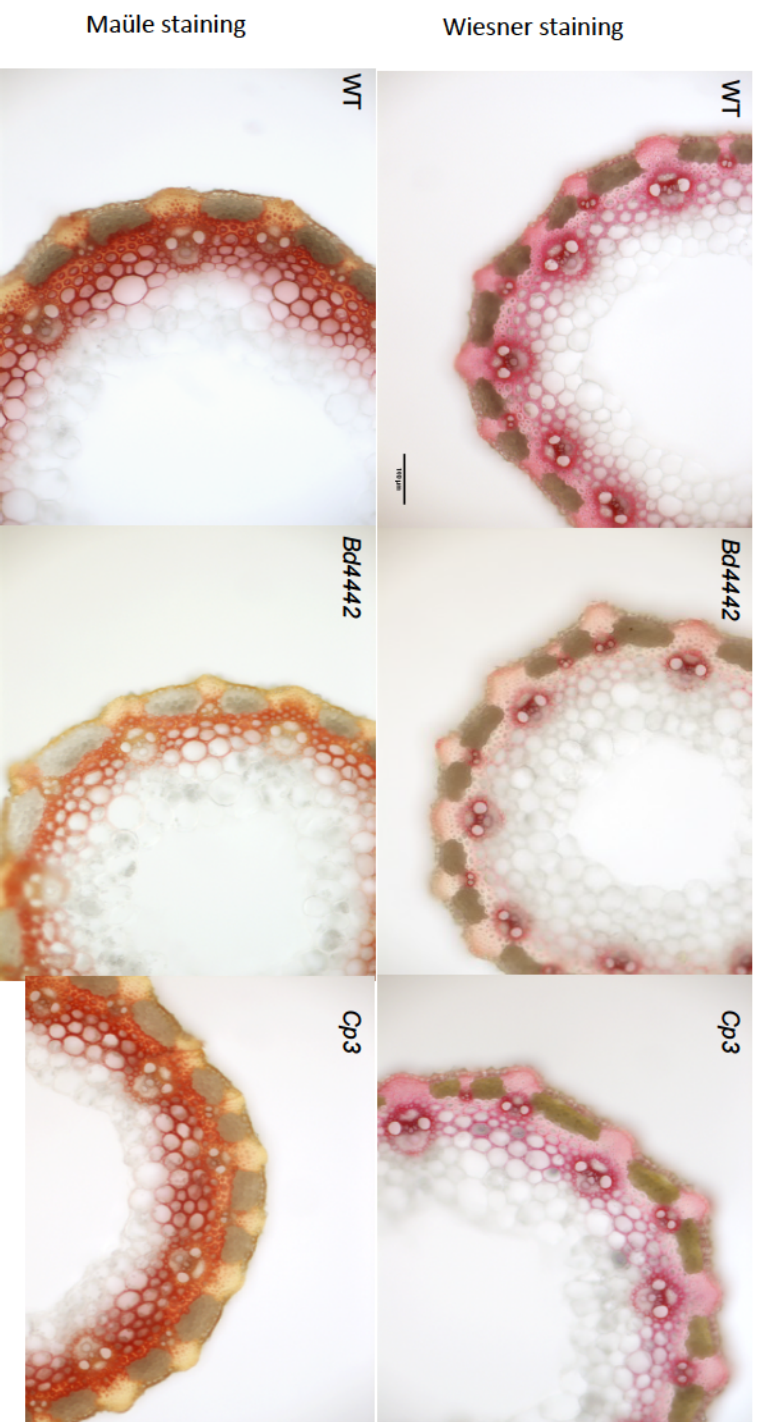


Figure S7. Staining of lignified tissues in control plants and the complemented line. Transverse sections are stained using the Wiesner method (upper row) or the Maüle method (bottom row).

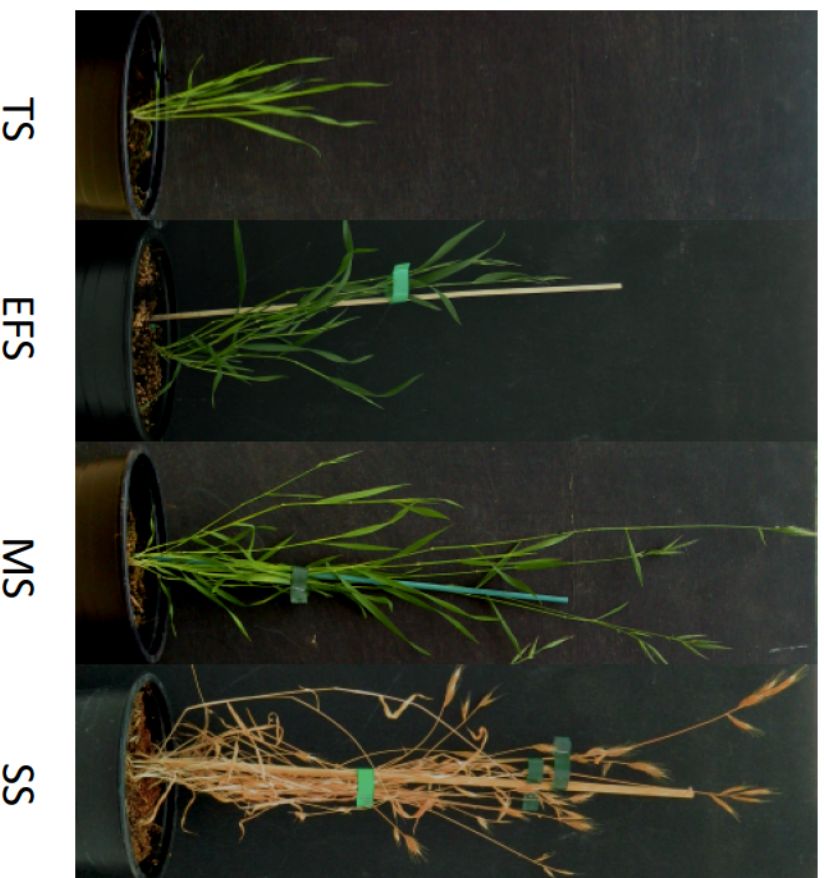


Figure S8. Phenotypes of *Brachypodium distachyon* (accession *Bd21-3*) at different development stages. From left to right: tillering stage (TS, 17 DAG); heading and early flowering stage (EFS, 35 DAG); maturity stage (MS, 60 DAG); senescent stage (SS, 120 DAG).

Table S1 . The LACCASE family genes in *Brachypodium distachyon*

Locus	Name
Bradi1g10570	BdLAC1
Bradi1g24880	BdLAC2
Bradi1g24910	BdLAC3
Bradi1g65100	BdLAC4
Bradi1g66720	BdLAC5
Bradi1g74320	BdLAC6
Bradi2g23350	BdLAC7
Bradi2g23370	BdLAC8
Bradi2g53800	BdLAC9
Bradi2g54680	BdLAC10
Bradi2g54690	BdLAC11
Bradi2g54740	BdLAC12
Bradi2g55050	BdLAC13
Bradi2g55060	BdLAC14
Bradi3g02290	BdLAC15
Bradi3g02300	BdLAC16
Bradi3g22320	BdLAC17
Bradi3g45190	BdLAC18
Bradi3g59180	BdLAC19
Bradi3g59190	BdLAC20
Bradi3g59210	BdLAC21
Bradi4g11830	BdLAC22
Bradi4g11840	BdLAC23
Bradi4g11850	BdLAC24
Bradi4g11860	BdLAC25
Bradi4g11770	BdLAC26
Bradi4g11780	BdLAC27
Bradi4g39330	BdLAC28
Bradi4g44810	BdLAC29

Supplemental Table S2. Amino acid sequences of laccases used in this study

>GaLAC1

MGLQQGLVTWVFGVLFSTLLLSNADVHHYEFFVRESNFTKLCNTTLLVVNDSYPGPEIRVH
RGDTVFNVHNQGNYGFTIHWGKQPRNPWSDGPEFVTQCPIQPGTNFTYEIVLSDEIGTL
WWHAHSDWTRGSVHGAFVILPAKKETYPFPTPDADQTHILESWDYDGYKQIIDDALAAGVSP
RQPSAYAISGHVGDYGCNPDITFRMQVDSEKIYLLRIINAAMNEHFFFTIANHTLTVVAQDAS
YVRRFTRDYILISPGQTMVLSANRNVGQYYMAIRPFSDSSAAPVDNITTGIFEYTNSEGLNA
SLITLPMNDTDAMINFLNQIRNTKVSQNPRINVPADKDIKRRVFMFLAVNNLPCNTCVVGSR
LVLASLNNVSYVSPSIDILQAYYNNRMSGVYTEDFPLNPPVIYDFTGNLTNLNTPVEEGTRVIVV
NYGEGVEMVLQATQMGAGGSHPIHLHGSSFYWVGTGFGNFNNKTDPRTYNLVDPPLINTVHV
PGRRWVAIRFFATNPGVWFMHCHLERHSSWGMDTVLRNGKTKKTSIRPPPSTMPRCPGT

>ZmLAC3

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EIVLQNPTAVPAENHPIHLHGFNFFVLAQGLGTFTPGNASAYNLVDPVSRNTLAVPTGGWAVI
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>Bradi2g54740

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PCPSNQTCCQPNGTKFAASINNNFSVRPKTALLEAHYQSRVAGVLMANFPTTPHPFNYTGTP
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>Bradi2g54690

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CAGANGTCQGPNGSRFAAAVNNVSVFLPTTALLQSHFTGMSNGVYESNFPAMPSSPFNYTGTP
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>Bradi2g54680

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LPCPKNMTCQGPNGTQFAAAVNNVSLVLPKALLQSHFTGLTTGVYASDFPAMPLSPFNYTGT
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>Bradi2g53800

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>Bradi2g23350

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WHAHVSWLRATVHGPIVILPPLGVYPFSPVPYKEVPLMFGGEWWKNDTEAVIAQALQTGGGP
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>Bradi1g65100

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>Bradi1g24910

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GTLWWHAHISWIRATVYGAIVILPELGIPYLAAPHEEVPIIFGEWWKANTEAVVKQALQTGG
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>Bradi1g24880

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TLWWHAHISWLRLATVYGAIVILPEFGVPYPFAAPHEEVPIIFGEWWKADTEAVVKQALQTGG
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>Bradi1g10570

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DVGDQVGTLLWWHAHVSLRATLHGIIIVIRPKSVAYPFLKPDMDVPVISEWWQKDLIKVDKNF
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> AT1G18140 (Lac1)

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> AT2G29130 (Lac2)

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KC

> AT2G30210 (Lac3)

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IKGTKAYKLKYKSNVQIVLQDTSIVTPENHPMHLHGYYQFYVVGSGFGNFNPRTDPAFNLFD
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> AT2G38080 (Lac4)

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> AT2G40370 (Lac5)

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> AT2G46570 (Lac6)

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AIVFEYGSRIQIIFQNTGTLTTENHPIHLHGHSFYVIGYGTGNYDQQTAKFNLEDPPYLNTIGVP
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> AT3G09220 (Lac7)

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SMSNHSFVLPPKLSILEAVFHDVKGIFTADFPDQPPVKFDYTNPNTVTQTNPGLLFTQKSTSAKI
LKFNTTVEVVLQNHALIAAESHMHLHGFFNFHVLAAQGFNYDPSRDRSKLNLVDPQSRNTLA
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> AT5G01040 (Lac8)

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KIRFNSTVEIVLQNTAISPE SHPMHLHG FNFYVLGYGFGNYDPIRDARKLNL FNPQMHN TVGV
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> AT5G01050 (Lac9)

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NNRTFMIPERISMQEAYFYNITGVYTD DFPDQPPLKFDFTKFEQHPTNSD MEMMFPERKTSV
KTIRFNSTVEIVLQNTGILTPESH PMHLHG FNFYVLGYGFGNYDPIRDARKLNL FNPQMHN TV
GVPPGGWVVLRFIANNPGIWLFHCHMDAHLPLGIMMAFIVQNGPTRETS LPSPPSNLPQCTRD
PTIYDSRTTNVDMSY

> AT5G01190 (Lac10)

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VLWLRATVHGAIVILPKLGLPYFPKPHREEVILGEWWKSDTETVVNEALKSGLAPNVSDAH
VINGHPGFVPNCPSQGNFKLAVESGKTYMLRLINAALNEELFFKIAGHRFTVVEVDAVYVKPFN
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PPPQNATSVANTFVNSLRSLNSKTYPANVPITVDHDL LFTVGLGINRCHSCKAGNFSRVAAIN
NITFKMPKTALLQAHYFNLTGIYTTDFPAKPRRVFDFTGKPPSNLATMKATKLYKLPYNSTVQ
VVLQDTGNVAPENHPIHLHG FNFVVG LGTGNYSKKDSNKFNLVDPVERNTVGVPSGGWAA
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> AT5G03260 (Lac11)

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WHAHILWLRATVYGAIVILPAPGKPYFPQP YQESNIILGEWWNKDVETAVNQANQLGAPPP
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NNITFIMPKTALLKAHYSNISGVFRTDFPDRPPKAFNYTGVPLTANLGTSTGTRLSRVKFNTTI
ELVLQDTNLLTVESHPPFHLHGYNFFVVG TGVGNFDPKKDPAKFNLVDPPERNTVGVPTGGWA
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> AT5G05390 (Lac12)

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DAYTINGQPGDLYNCSTKETVVVPINSGETSLLRVINAALNQPLFFT VANHKLTVVGADASYLK
PFTTKVLMLGPGQTTDVLLTADQPPKRYIIAARAYQSAQNAPFDNTTTT TAILQYKKT TTTTSKPI
MPVLP AFNDTNTVTVSFSRKFKSLRN VVPKTIDDNLFFTIGLGLDNC PKKFPKSRQCGLNGTRF
TASMNNVSFVLPSNFSLQAH SNGIPGVFTTDFPSKPPVKFDYTGNNISRALFQPVKGTKLYKL

KYGSRVQVVLQDTNIVTSENHPIHLHGDFYIVGEGFGNFNPKKDTSKFNLVDPPLRNTVAVP
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> AT5G07130 (Lac13)

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SRWLRATVYGALIIRPPLSSPHYFPVIPKREITLLLGEWWDRNPMDVLNLAQFTGAAPNISDA
FTINGQPGDLYRCSSQETLRLVLGSGEIVLLRVINSALNQELFFGVANHKLTVVAADASYTKPFS
TNVIMLPGGQTTDVLLTADQPPAHYYMAAHAYNSANAAFNTTTTAILKYKDASCVTLQAKS
QARAIPAQLPGFNDTATAAFTAQMKSPSKVKVPLEIDENLFFTIVGLGLFNCPTPNTQRCQGP
NGTRFTASINNVSFVFPKQNSIMQAYYQGTPTGVFTTDFPPTPPVTFDYTGNSRGLWQPTRG
TKAYKLKFNQVQIILQDTSIVTTENHPMHLHGYEFYVVGTVGVGNFNPNTDTSSFNLDPPRRN
TIGTPPGGWVAIRFVANNPGAWLMHCHIDSHIFWGLAMVFLVENGEHLQSVQSPPLDLPQC

> AT5G09360 (Lac14)

MEFKLNIPNTIHKTLQTIVFFLVLLAFQIAEAEIHHHTFKIKSKAYTRLCNTNKILTVNGEFPGP
TLKAYRGDKLIVNVINNANYNITLHWHGARQIRNPWSDGPEYVTQCPIRPGESYVYRIDLKVEE
GTIWWHAHSQWARATVHGAFIVYPKRGSSYPFKPHREIPLILGEWWKKENIMHIPGKANKT
GGEPASDSYTINGQPGYLYPCSKPETFKITVVRGRRYLLRIINAVMDEELFFAIANHTLTVVAK
DGFYLKHFKSDYLMITPGQSMDEVLLHANQRPNHYFVAARAYSSAFGAGFDKTTTTAILQYKGD
TLNRIKPILPYLPYNRTEASTRFTNQFRSQRPVNPVKINTRLLYAISVNLNMCSDDRPCTGPF
GKRFSSINNISFVNPSVDILRAYRRHIGGVFQEDFPRNPPTKFNYTGENLPFPTRFGTKVVLD
YNSSVELILQGTTVWASNIHPIHLHGYNFYVVGSGFGNFDRRKDPLRYNLVDPPEETTVGVPR
NGWTAVRFVANNPGVWLLHCHIERHATWGMNTVFIVKDGP TKSSRMVKPPPDLPC

> AT5G48100 (Lac15)

MSHSFFNLFLISLFLYNNCIAHHYTFTVREVPTYTKLCSTKAILTVNSQFPGPIIKVHKGDTIYVNV
QNRASENITMHWHGVEQPRNPWSDGPEYITQCPIRPGSDFLYKVIFSIEDTTVWWHAHSSWT
RATVHGLIFVYPRPPQILPFPKADHEVPILGEWWKRDVREVVEEFVRTGGAPNVSDALTINGH
PGFLYPCSKSDTFHLTVEKGKTYRIRMVNAAMNLPLFFAIANHSLTVVSADGHYIKPIKATYITI
SPGETLDMLLHADQDPERTYYMAARAYQSGNIDFNNSTTIGILSYTSSCKAKTSSFSGYPTLPF
YNDTSAAFGFFT KIKCLFSGQVPVQISRRITTVSINLRMCQNSCEGPNGSRLAASMNNISFVTP
SHVDILKAYYYHIKGVYGRFPEFPPLIFNFTAENQPLFLETPLATEVKVIEFGQVVELVIQGS
LVGGGLDHPMHLHGFSFYVVGVGFGNYNISEEDPSSRYNLYDPPYKNTMTVPRNGWIAIRFVA
DNPGVWFMHCHLDRHQTWGMNVVFIVKNGREPQQILPPPDDLPPCYE

> AT5G58910 (Lac16)

MTNTTKLCSSKPIVTVNGQFPGPTIVAREGDTILIKVVNHVKYNVSIHWTGWADGPAYITQCPI
QPGQNYLHNFTLTGQRGTLWWHAHILWLRATVHGAIVILPKLGVYPFPKPYKEKTIVLSEW
WKSDVEELINEASRIGTAPSASDAHTINGHSGSISNCPSQSSYGLPVRAKTYMLRIINAALNEE
LFFKIAGHVLTVVEVDAVYTKPYKTDTVFIAPGQTTNVLLTANANAGSNYMVAATTTDAHIP
YDNVTATATLHYIGHTSTVSTSKKTVLASLPPQNATWVATKFTSLRLSLNSLEYPARVPTTVE
HSLFFTIVGLGANPCQSCNNGVRLVAGINNVFTMPKTALLQAHFFNISGVFTDDFPAKPSNPY
DYTAPVKLGVNAATMKGTKLYRLPYNATVQIVLQNTAMILSDNHPFHLHGFFNFVGRGLGN
FNPEKDPKAFNLVDPVERNTPVGPAGGWTAIRFIADNPGVWFMHCHLELHTTWGLKMAFVV
DNGHGPDPQSLPPPADLPKC

> AT5G60020 (Lac17)

MALQLLLAVFSCVLLLPQPAFGITRHYTLEIKMQNVTRLCHTKSLVSVNGQFPGPKLIAREGDQ
VLIKVVNQVPNNISLHWHGIRQLRSGWADGPAYITQCPIQTGQSYVYNYTIVGQRGTLWYHAH
ISWLRSTVYGPLIILPKRGVPYPFAKPHKEVPMIFGEWFNADTEAIIRQATQTGGGPNVSDAYTI
NGLPGPLYNCSAKDTFRLRVKPGKTYLLRLINAALNDELFFSIANHTVTVVEADAIYVKPFETE
TILIAPGQTTNVLLKTKSSYPSASFFMTARPYVTGQGTFDNSTVAGILEYEPPKQTKGAHSRTSI
KNLQLFKPILPALNDTNFATKFSNKLRLNSKNFPANVPLNVDRKFFFTVGLGTNPCNHKNNQ
TCQGPTNTTMFAASISNISFTMPTKALLQSHYSGQSHGVYSPKFPWSPIVPFNYTGTPPNNTM
VSNGTNLMVLPYNTSVELVMQDTSILGAESHPLHLHGFFVVGQGFGNFDPNKDPRNFNLV
DPIERNTVGVPSGGWAAIRFLADNPGVWFMHCHLEVHTSWGLRMAWLVLVDG
DKPDQKLLPPPADLPKC

Supplemental Table S3. List of genes co-expressed with *BdLAC5*

TranscriptID	Annotation
Bradi1g66720.1	laccase precursor protein, putative, expressed
Bradi5g25090.1	IQ calmodulin_binding motif family protein, putative, expressed
Bradi5g04540.1	zinc finger, C3HC4 type domain containing protein, expressed
Bradi2g55340.1	transmembrane amino acid transporter protein, putative, expressed
Bradi2g46410.1	glycosyltransferase family 43 protein, putative, expressed
Bradi2g00880.1	No annotation
Bradi4g25540.1	BTB9 _ Bric_a_Brac, Tramtrack, Broad Complex BTB domain
Bradi2g54680.1	laccase precursor protein, putative, expressed
Bradi2g17067.1	auxin_responsive protein, putative, expressed
Bradi1g67870.1	expressed protein
Bradi4g23910.1	KIP1, putative, expressed
Bradi4g29290.1	serine/threonine_protein kinase, putative, expressed
Bradi1g74320.1	laccase_22 precursor, putative, expressed
Bradi5g20130.1	MYB family transcription factor, putative, expressed
Bradi1g28366.1	alpha_1,2_fucosidase, putative, expressed
Bradi1g21300.1	dehydrogenase, putative, expressed
Bradi3g59730.1	HEAT repeat family protein, putative, expressed
Bradi2g23300.1	expressed protein
Bradi3g16530.1	O_methyltransferase, putative, expressed
Bradi1g67460.1	phospholipase A2, putative, expressed
Bradi4g37980.1	cell cycle control protein, putative, expressed
Bradi1g09460.1	endoglucanase, putative, expressed
Bradi1g25117.1	CSLF2 _ cellulose synthase_like family F
Bradi5g15490.1	ARPC2B, putative, expressed
Bradi2g46197.1	no apical meristem protein, putative, expressed
Bradi4g22250.1	dirigent, putative, expressed
Bradi2g54240.1	respiratory burst oxidase, putative, expressed
Bradi3g37530.1	ferric reductase, putative, expressed
Bradi3g47950.1	expressed protein
Bradi3g48730.1	DUF869 domain containing protein, expressed
Bradi3g32180.1	expressed protein
Bradi2g47330.1	MDR_like ABC transporter, putative, expressed
Bradi4g06680.1	expressed protein
Bradi4g31130.1	ferric reductase, putative, expressed
Bradi3g36887.1	cinnamoyl_CoA reductase, putative, expressed
Bradi2g08790.1	Cupin domain containing protein, expressed
Bradi5g14720.1	transferase family protein, putative, expressed
Bradi5g23460.1	shikimate kinase, putative, expressed
Bradi5g12460.1	uncharacterized Cys_rich domain containing protein
Bradi2g49040.1	respiratory burst oxidase, putative, expressed
Bradi3g05750.1	AMP_binding domain containing protein, expressed
Bradi1g35477.1	STRUBBELIG_RECEPTOR FAMILY 7 precursor, putative
Bradi1g75350.1	RING finger and CHY zinc finger domain_containing protein 1
Bradi2g21300.1	cytochrome P450, putative, expressed

Bradi1g54420.1	expressed protein
Bradi2g52060.2	membrane protein, putative, expressed
Bradi3g37230.1	GDU1, putative, expressed
Bradi1g06290.1	fasciclin domain containing protein, expressed
Bradi2g55730.1	cytochrome P450, putative, expressed
Bradi1g31820.1	vacuolar_sorting receptor precursor, putative, expressed
Bradi1g14050.1	uncharacterized protein At4g06744 precursor, putative
Bradi5g16060.1	expressed protein
Bradi5g09130.1	expressed protein
Bradi2g55720.1	hemerythrin family protein, expressed
Bradi4g21790.1	peptide transporter PTR2, putative, expressed
Bradi4g28260.1	hydroxyproline_rich glycoprotein family protein, putative
Bradi4g44860.1	PMR5, putative, expressed
Bradi2g26770.1	annexin, putative, expressed
Bradi5g02460.1	cytochrome P450 93A2, putative, expressed
Bradi3g09500.1	sulfotransferase domain containing protein, expressed
Bradi1g03940.1	leaf senescence related protein, putative, expressed
Bradi3g39800.1	citrate transporter, putative, expressed
Bradi1g68710.2	microtubule associated protein, putative, expressed
Bradi2g37970.1	glycosyltransferase family 43 protein, putative, expressed
Bradi2g23370.1	laccase precursor protein, putative, expressed
Bradi1g32850.1	RIC10, putative, expressed
Bradi1g57607.1	Homeobox domain containing protein, expressed
Bradi1g60750.1	phospho_2_dehydro_3_deoxyheptonate aldolase, expressed
Bradi2g12150.2	S_adenosylmethionine synthetase, putative, expressed
Bradi3g28350.1	CESA7 _ cellulose synthase, expressed
Bradi2g10302.1	flavin monooxygenase, putative, expressed
Bradi4g21240.1	plant_specific domain TIGR01627 family protein, expressed
Bradi4g21220.1	auxin_induced protein 5NG4, putative, expressed
Bradi2g59410.1	exostosin family domain containing protein, expressed
Bradi4g33490.1	fasciclin domain containing protein, expressed
Bradi5g15527.1	Os4bglu14 _ monolignol beta_glucoside homolog
Bradi1g59880.2	COBRA_like protein precursor, putative, expressed
Bradi2g56970.1	amino acid transporter, putative, expressed
Bradi5g01737.1	peptide transporter PTR2, putative, expressed
Bradi2g16337.1	CGMC_MAPKCMGC_2.8 CGMC
Bradi1g17242.1	serine hydrolase domain containing protein, expressed
Bradi3g49260.1	phenylalanine ammonia_lyase, putative, expressed
Bradi2g47600.1	zinc finger, C3HC4 type domain containing protein, expressed
Bradi2g19160.1	anthocyanidin 5,3_O_glucosyltransferase, putative, expressed
Bradi1g00710.1	expressed protein
Bradi1g07060.1	expressed protein
Bradi4g30437.1	methyladenine glycosylase, putative, expressed
Bradi1g10470.1	MYB family transcription factor, putative, expressed
Bradi1g34670.1	glycosyltransferase, putative, expressed
Bradi2g24010.1	protein kinase, putative, expressed
Bradi2g34790.1	GDSL_like lipase/acylhydrolase, putative, expressed
Bradi1g65530.1	expressed protein

Bradi3g38950.1	methyladenine glycosylase, putative, expressed
Bradi5g18150.1	expressed protein
Bradi2g12370.1	GDSL_like lipase/acylhydrolase, putative, expressed
Bradi2g47590.1	MYB family transcription factor, putative, expressed
Bradi3g56787.1	polygalacturonase, putative, expressed
Bradi3g00377.1	rhodanese_like domain containing protein, putative, expressed
Bradi3g42430.1	MYB family transcription factor, putative, expressed
Bradi4g27720.1	expressed protein
Bradi3g41210.1	microtubule associated protein, putative, expressed
Bradi1g22980.1	formin, putative, expressed
Bradi2g17680.3	CPuORF26 _ conserved peptide uORF_containing transcript
Bradi3g40820.1	membrane protein, putative, expressed
Bradi3g04080.1	endoglucanase, putative, expressed
Bradi2g49912.1	CESA4 _ cellulose synthase, expressed
Bradi4g34300.1	membrane protein, putative, expressed
Bradi4g30540.1	CESA9 _ cellulose synthase, expressed
Bradi3g06480.1	dehydrogenase, putative, expressed
Bradi1g59160.1	flavin_containing monooxygenase family protein, putative
Bradi2g11500.1	kelch motif family protein, putative, expressed
Bradi1g23620.1	expressed protein
Bradi2g18447.1	sulfotransferase domain containing protein, expressed
Bradi1g72350.1	glycosyl transferase, putative, expressed
Bradi1g17830.1	potassium transporter, putative, expressed
Bradi1g10660.1	histidine kinase, putative, expressed
Bradi3g49250.2	phenylalanine ammonia_lyase, putative, expressed
Bradi3g05860.1	flavin_containing monooxygenase family protein, putative
Bradi2g23530.1	homeodomain protein, putative, expressed
Bradi1g17680.1	B3 DNA binding domain containing protein, putative
Bradi1g69870.1	ATMAP70 protein, putative, expressed
Bradi1g65430.1	chloroplast unusual positioning protein, putative
Bradi4g16560.1	cytochrome P450, putative, expressed
Bradi2g45090.1	zinc finger C_x8_C_x5_C_x3_H type family protein
Bradi2g11010.1	TsetseEP precursor, putative, expressed
Bradi1g64560.1	glycosyltransferase, putative, expressed
Bradi2g58490.1	rho GDP_dissociation inhibitor 1, putative, expressed
Bradi4g36240.1	endoglucanase, putative, expressed
Bradi5g15850.1	transporter_related, putative, expressed
Bradi4g42400.1	RCN1 Centroradialis_like1 homologous to TFL1 gene
Bradi1g04750.1	expressed protein
Bradi2g16560.1	fasciclin domain containing protein, expressed
Bradi1g51960.1	RGH2B, putative, expressed
Bradi5g08907.1	heparan_alpha_glucosaminide N_acetyltransferase, putative
Bradi4g40400.1	plant_specific domain TIGR01627 family protein, expressed
Bradi3g56290.1	bZIP transcription factor domain containing protein, expressed
Bradi3g33070.1	expressed protein
Bradi4g05450.1	isoflavone reductase homolog

This list was generated by PlaNet platform using BdLAC5 as bait with default parameters.
Laccases were labeled in red , some other lignin related genes were in blue.

Supplemental Table S4. Anatomical comparison in different genotypes.

Genotype	Stem Height (cm)	Internode Diameter (μm)	Vascular Bundles Area (μm^2)	Cell Wall Thickness (μm)
Bd21-3 WT	37.7 $\pm$ 3.7 a	946 $\pm$ 56 a	5134 $\pm$ 474 a	2.53 $\pm$ 0.19 a
Bd4442 HO	31.3 $\pm$ 1.4 b	811 $\pm$ 79 b	4634 $\pm$ 337 b	2.41 $\pm$ 0.33 a
Bd4442 CP3	37.4 $\pm$ 1.6 a	923 $\pm$ 31 a	5214 $\pm$ 162 ab	2.47 $\pm$ 0.15 a
Bd5024 HO	39.7 $\pm$ 2.3 a	973 $\pm$ 64 a	5536 $\pm$ 441 a	2.53 $\pm$ 0.17 a

The data represent means $\pm$ standard deviation from biological replicates (n = 6). Letters indicate significant differences analyzed by one-way ANOVA (Tukey's HSD, $P < 0.05$).

Supplemental Table S5. Quantification of thioglycolic acid lignin.

Genotype	Relative TGA Lignin Content (%)
Bd21-3 WT	100.0±2.5
Bd4442 HO	86.0±3.5
Bd4442 CP3	93.3±2.8
Bd5024 HO	97.5±3.7

TGA lignin content are shown in percentages of control plants (*Bd21-3*). The data represent means ± standard deviation from biological replicates (n = 3).

Supplemental Table S6. Primers

Name	Primer (5' --- 3')	Anotation
QBdUbi4-R	GAGGGTGGAATCCTTCTGGA	qRT-PCR of BdUBI4
QBdUbi4-F	TGACACCATCGACAACGTGA	
QBdLAC5-3F	ACACCTTCAATGTGACGATGAAG	qRT-PCR of BdLAC5
QBdLAC5-3R	CTCAGCCGTGCCAGTGGATGGT	
QBdLAC6-3R	CTTACCCCATGCCAGTGGATGGTA	qRT-PCR of BdLAC6
QBdLAC6-3F	TACAAGTTCAATGTGGTTATGAGG	
BdLAC5probe-3F	GATAACCCCGGTGTGTGGTTCATG	synthesis of BdLAC6 anti-sense probe for in situ hybridization
BdLAC5probe-3R	TGTAATACGACTCACTATAGGGCTTCGA ACAGAACTTCGATCGA	
BdLAC5probeCK-3F	TGTAATACGACTCACTATAGGGCGATAA CCCCGGTGTGTGGTTCATG	synthesis of BdLAC5 sense probe for in situ hybridization
BdLAC5probeCK-3R	TTCGAACAGAACTTCGATCGA	
BdLAC6probe-3F	TACAAGTTCAATGTGGTTATGAGG	synthesis of BdLAC6 anti-sense probe for in situ hybridization
BdLAC6probe-3R	TGTAATACGACTCACTATAGGGCTTCGA ACCCATCTTGTGAGGAAGC	
BdLAC6probeCK-3F	TGTAATACGACTCACTATAGGGCTACAA GTTCAATGTGGTTATGAGG	synthesis of BdLAC6 sense probe for in situ hybridization
BdLAC6probeCK-3R	CTTGAACCCATCTTGTGAGGAAGC GGGGACAAGTTTGTACAAAAAGCAGGC	
GWBdLAC5cdnaF	TTCCACGAGCGCACGCTGTGGCTC	clone of BdLAC5 full length cDNA
GWBdLAC5cdnaR	GGGGACCACTTTGTACAAGAAAGCTGG GTCGCTTCGAACAGAACTTCGATCG	
HPT-F	CGCACAATCCCACTATCCTTCGCAA	verification of BdLAC5 complementation lines
HPT-R	GGCAGTTCGGTTTCAGGCAGGTCTT	
proZmUBI-F	GGCACCTCCGCTTCAAGGT	verification of BdLAC5 complementation lines
4442cp-R	CTCAGCCGTGCCAGTGGATGGT	

2.3 BdLAC8 plays a role in lignification of Brachypodium stems

2.3.1 *BdLAC8* is a new candidate gene involved in lignification

According to the phylogenetic tree constructed with the deduced 29 Brachypodium laccases and 17 Arabidopsis laccases (Wang et al., 2015), three laccases, BdLAC5 (protein encoded by Bradi1g66720), BdLAC8 (protein encoded by Bradi2g23370) and BdLAC12 (protein encoded by Bradi2g54740) gathered together in a sub-cluster including AtLAC17 and AtLAC2. BdLAC8 shares 62% identity and 77% similarity with AtLAC17 and AtLAC2. BdLAC12 shares 65% identity and 79% similarity with AtLAC2 and 64% identity and 77% similarity with AtLAC17. PlaNet tools (<http://aranet.mpimp-golm.mpg.de/>), allow to determine that *BdLAC8* and *BdLAC12* have similar expression patterns (Figure 2.3-1). Both genes were highly expressed in lignified tissues (such as internode, node and mesocotyl) and poorly expressed in organs with low lignin content or non-lignified tissue (such as young spikelets and endosperm). However, *BdLAC8* transcript level is high in the peduncle whereas *BdLAC12* is less expressed. Peduncle is a part of the stem supporting the inflorescence, and it is a highly lignified organ. It is important to note that the transcription of the lignin specific laccase identified to date in grasses, i.e. *BdLAC5* was found substantial in the peduncle (Wang et al., 2015). At last, *BdLAC5*, *BdLAC8* and *BdLAC12* are all expressed in the second internode of the culm. In order to compare the level of their respective transcript level more precisely, quantitative RT-PCR were performed (Figure 2.3-2A). *BdLAC8* is the most expressed gene after *BdLAC5*. In consequence, we focused our attention on *BdLAC8* and investigate its putative role in lignification.

2.3.2 *BdLAC8* is expressed in the lignified tissues of the Brachypodium culm

The expression levels of *BdLAC8* were analyzed by quantitative RT-PCR on RNA isolated from different organs at three developmental stages (see Materials and Methods in 2.2), namely tillering stage (TS), early flowering stage (EFS) and maturity stage (MS). *BdLAC8* transcripts were detected in all tested organs and stages, but with considerable variations (Figure 2.3-2B). At TS, the transcription level of *BdLAC8* is higher in root and mesocotyl than in leaves and young stems, consistently with the extent of lignification in these organs. Similarly, the transcription level of *BdLAC8* is higher in roots and in different parts of stem than in young spikelets and leaves at EFS and MS (Figure 2.3-2B).

To localize *BdLAC8* transcripts more accurately, *in situ* hybridization experiments were performed on sections of internodes collected at the EFS. At this stage, the sclerenchyma and a few parenchyma cells in the interfascicular area (IF) and vascular bundles (VB) are lignified (Wang et al., 2015). The intense signal of the *BdLAC8* antisense probe was detected mainly in IF and bundle sheath (Figure 2.3-3). It was also detected in cells around phloem. By contrast, the metaxylem and collenchyma cells with thickened secondary walls were poorly labeled. This phenomenon could be accounted for by the fact that metaxylem cells are cytosol-free and quickly dead after their formation (Bollhöner et al., 2012; Schuetz et al., 2013). In conclusion, *BdLAC8* transcripts were found in lignifying tissues at different developmental stages, the timing and space of the *BdLAC8* transcripts accumulation show a strong correlation with the deposition of lignins.

2.3.3 *BdLAC8* proteins might be secreted into the apoplasm

The *BdLAC8* gene (1,743 bp coding sequence) encodes a predicted 580-aa polypeptide (theoretical molecular mass: 63.6 kDa and isoelectric point: 9.27).

According to a protein prediction database (Motif Scan, http://myhits.isb-sib.ch/cgi-bin/motif_scan), BdLAC8 is a hydrophilic protein that could be N-glycosylated which may contribute to the high stability of the enzyme. TargetP (Emanuelsson et al., 2000) allows predicting BdLAC8 to be secreted as it may be the case for lignin related laccases (Berthet et al., 2011; Cesarino et al., 2013; Wang et al., 2015). The cleavage sites of signal peptides are in the N-terminal region between 32 and 33 amino acid, according to SignalP program (Bendtsen et al., 2004).

2.3.4 Identification of *BdLAC8* mutants affected in the highly conserved C-terminal domain

A TILLING (Targeting Induced Local Lesion IN Genome) screen was carried out in the Versailles Brachypodium mutant collection in order to identify mutations in *BdLAC8* gene by reverse genetics. This screen led to the identification of one stop codon mutant *Bd5731* and two missense mutants *Bd5000* and *Bd12*. The mutant *Bd12* possesses a single G to T nucleotide substitution at position 1388 of the *BdLAC8* cDNA, which introduces a substitution of threonine to isoleucine at position 463 in the amino acid sequence. The mutant *Bd5000* possesses a single G to A nucleotide substitution at position 1649 of the *BdLAC8* cDNA, which introduces a substitution of serine to asparagine at position 550 in the amino acid sequence. The stop codon mutant *Bd5731* possesses a single G to A nucleotide substitution at position 1653 of the *BdLAC8* cDNA, which introduces a premature stop codon at tryptophan 551. Consequently, the *Bd5731* should have a truncated laccase protein, which lack the highly conserved and essential C-terminal domain. The C-terminal domain may have functions in recognizing and binding substrates (McCaig et al., 2005; Reiss et al., 2013). The 3D-structure of BdLAC8 predicted from the crystallized ascorbate oxidase from *Cucurbita pepo* var. melopepo (38% sequence similarity)

(Figure 2.3-4) suggests that the C-terminal domain extends to the outer parts of the protein like a tail. Structures of laccase and ascorbate oxidase are predicted to be similar (Gromov et al., 1999). It is well known that laccase activity depends on four copper atoms distributed among the three different binding sites, named as Type 1 (T1), Type 2 (T2), and Type 3 (T3) (Durão et al., 2006). The T1 Cu localized at C-terminal domain can be oxidized by reducing substrates, and then the electron is transferred from T1 Cu to the trinuclear cluster made up of the T2 and T3 Cu atoms where oxygen binds to and is reduced to water (Durão et al., 2006). We, therefore, focused on the stop codon mutant (*Bd5731*), which lacks a large part of the C-terminal domain.

2.3.5 Impact of mutations on the lignification

2.3.5.1 FTIR and histochemical analysis show that cell wall composition of *Bd5731* interfascicular fibers is impacted

Mañile and Wiesner assays were used to locate the presence of lignin polymer in plant cell walls. Mañile staining ($\text{KMnO}_4\text{-HCl}$) is based on the oxidation of lignin by KMnO_4 . It is interesting to note that the syringyl units are detected in red when G units remain orange. It is thus possible to estimate any substantial change in S to G ratio in any mutant or transgenic. The phloroglucinol (Wiesner test) can react with hydroxycinnamyl aldehydes (coniferyl aldehyde, sinapyl aldehyde) and hydroxybenzaldehydes (vanillin and syringaldehyde) in ethanol-hydrochloric acid to yield pink-red color (in the case of hydroxycinnamyl aldehydes) or red-brown color (in the case of hydroxybenzaldehydes). Despite these particularities, phloroglucinol-HCl is often used to detect the presence of lignins whatever its composition. When subjected to Wiesner staining, cross sections of the *Bd5731* mutants displayed a slight but distinguishable decrease of staining in the interfascicular fibers. There were no detectable differences in the staining of xylem

vessels of *Bd5731* compared to wt (Figure 2.3-5). Maïle staining revealed a similar result. *Bd5731* showed less overall staining than the wild type (Figure 2.3-5). Based on these results, we concluded that the lignification in *Bd5731* culm may be affected.

For further characterization of the cell wall composition, Fourier Transform Infrared absorbance (FTIR) spectromicroscopy in transmission mode was used to compare the absorbance spectra from cell walls. Spectroscopic methods are usually considered as qualitative and semi-quantitative, since cellulose, hemicelluloses and lignin can share many common absorbances at specific wavelength. As previously described, we focused our attention on the spectral differences at the wavenumbers which are characteristic of lignin, such as the bands around 1427 cm^{-1} and 1508 cm^{-1} . These wavelengths are related to vibrations of aromatic ring. The absorbance at 1458 cm^{-1} is related to C-H deformation of CH₂ and CH₃ groups (Mouille et al., 2003; Sibout et al., 2005). The FTIR spectra were acquired separately from the sclerenchyma cells in interfascicular fibers and the metaxylem cells inside the VB of the second internode at MS (Figure 2.3-6). The results showed significant decreases of absorption at wavelengths characteristic of lignin in interfascicular fibers of *Bd5731* compared with WT (13.3 % reduction at 1508 cm^{-1} , 7.0%reduction at 1458 cm^{-1} and 6.7% reduction at 1427 cm^{-1}). In vascular bundles, we did not observe any reduction in lignin absorbance. These results are in agreement with histochemical staining and suggest that the lignification is affected in interfascicular fibers of *Bd5731* but not in its vascular bundles.

FTIR spectroscopy can be used to investigate the changes in cellulose and hemicellulose structure. For instance, the band at 900 cm^{-1} represents the β -(1-4) glycosidic linkages of cellulose. $1000\text{-}1200\text{ cm}^{-1}$ indicates changes in cellulose and hemicellulose in general. For instance 1037 cm^{-1} indicates C-O stretching and 1164 cm^{-1} the C-O-C asymmetrical stretching (Colom et al., 2003; Oh et al., 2005). 1247 cm^{-1} (C-O stretching) is a feature of both hemicellulose and lignin

(Pandey and Pitman, 2003). 1316 cm^{-1} could be due to vibration of the CH_2 group of cellulose and may also be due to C-O syringyl ring in lignin (Colom et al., 2003; Pandey and Pitman, 2003). The absorption around $1363\text{-}1370\text{ cm}^{-1}$ could be related to the presence of C-H in cellulose and hemicelluloses. At last, the absorption at 1733 cm^{-1} is mainly attributed to C=O unconjugated stretching of hemicellulose (Chandel et al., 2013). Significant decreases of absorptions between $1000\text{-}1200\text{ cm}^{-1}$ and as well as around 1247 cm^{-1} in vascular bundles of *Bd5731* was found compared with WT (Figure 2.3-6). These results suggest there were a reduction of cellulose and hemicellulose content in vascular bundles of *Bd5731*, but not in interfascicular fibers.

Taken together, the histochemical and FTIR results display that the lignification of the *Brachypodium* culm is affected in the *BdLAC8* stop codon mutant and more specifically in interfascicular fibers.

2.3.5.2 Crossing *BdLAC5* and *BdLAC8* mutants and identification of double homozygous lines

In order to confirm the role of *BdLAC8* in lignification and to assess the possible biological redundancy between *BdLAC5* and *BdLAC8*, we crossed *Bd4442* and *Bd5731*. Five F1 lines with confirmed heterozygous alleles for each gene were produced. Plants were grown until enough F2 seeds were obtained. Screenings for the double homozygous mutants were carried out by sequencing of the genotyping PCR products (Figure 2.3-7). We thus isolated plants with all possible genotypes from a segregating population. To carry on further analysis, we chose some lines with different genotypes:

- *5ho8az*: lines sharing homozygous mutation in *BdLAC5* (as detected in *Bd4442*) and wild-type allele for the *BdLAC8* gene.
- *8ho5az*: lines sharing homozygous mutation in *BdLAC8* (as detected in *Bd5731*) and wild-type allele for *BdLAC5* gene.

- *5ho8ho*: lines sharing homozygous mutation in *BdLAC5* (as detected in *Bd4442*) and homozygous mutation in *BdLAC8* (as detected in *Bd5731*).
- *5az8az*: lines sharing wild-type allele for *BdLAC5* and wild-type allele for *BdLAC8* gene.

We will perform lignin analysis of these segregating lines in future.

2.3.5.3 Lignin formation is significantly affected in homozygous double mutants of *BdLAC5* and *BdLAC8*

The lignin content of extractive-free dry culms was quickly screened by thioglycolic acid (TGA) method (Wang et al., 2015) (Figure 2.3-8) and confirmed by the Klason lignin method (Table 2.3-1). As expected, there is a significant decrease of lignin content in the homozygous *Bd4442* and *Bd5731* single mutants as well as in the double mutant, *5ho8ho* compared with the lignin content levels in wild type and other segregating lines. Using the Klason lignin method, *Bd4442* has 10.5% reduction of lignin content, *Bd5731* has 9.7% reduction of lignin content and *5ho8ho* has 34% reduction of lignin content compared to the wild type. Not unexpectedly, *5ho8az* has less lignin content than the WT as detected in *Bd4442*. A similar result was obtained by the TGA lignin assay (Figure 2.3-8). Consistently with the histochemical staining and FTIR spectra (see above), the single mutants of *BdLAC5* and *BdLAC8* have modest but significant reduction of lignin content and distribution in culm. More strikingly, the homozygous double mutant of *BdLAC5* and *BdLAC8* shows a dramatic decrease of lignin content.

Lignin structure in mutant lines and the WT was also investigated by thioacidolysis (Lapierre, 1993), since this method can identify H, G and S thioethylated monomers from arylglycerol- β -ether-linked H, G and S units. Compared to WT, the frequency of the S thioacidolysis monomers released from the homozygous double mutant culms (*5ho8ho*) was found to be significantly higher (Table 2.3-1). This phenomenon was already detectable in the single *LAC5*

mutant, *Bd4442* (Wang et al., 2015). Interestingly enough, a higher S to G ratio was also reported in the mutants of *Atlac17* and *AtLAC4xAtLAC17* (Berthet et al., 2011).

We further investigated the lignin distribution in the double mutants of *BdLAC5* and *BdLAC8* by histochemical staining (Figure 2.3-9). Compared to WT, Wiesner staining of *5ho8ho* internode at MS stage was significantly weaker in overall tissues, and particularly in the interfascicular fiber. In *5ho8ho* internode, the color of staining in sclerenchyma cells is yellow and on the contrary, the sclerenchyma cells in WT are stained as pink-red. At MS stage, some parenchyma cells in the interfascicular fiber of WT are lignifying, but there was no clear staining in *5ho8ho*. However the lignin staining of VB in *5ho8ho* is only slightly decreased and mainly in the mestome layer. Surprisingly enough, the staining of metaxylem cell walls is not distinguishable from those of the WT. The Wiesner staining of other segregants generated from the crossing of *Bd5731* and *Bd4442* provide additional information. Indeed, *5ho8az* shows comparable staining patterns to *Bd4442* as well as to *5az8az* and WT. These encouraging results provide an evidence that *BdLAC8* is also involved in lignin formation as well as *BdLAC5* and work together to play additional roles.

Table 2.3-1. Lignin analyses for the dry culms of selected segregants from the cross between *Bd4442* and *Bd5731*. Lignin content and structure from extracted mature stems are measured by Klason and thioacidolysis methods

Genotype	Klason content (%)	Lignin	Relative frequency of lignin-derived thioacidolysis monomers		
			%H	%G	%S
WT	18.29 ± 0.09		3.4 ± 0.2	33.9 ± 0.8	62.7 ± 0.9
<i>5ho8ho</i>	12.10 ± 0.04 ^a		3.4 ± 0.2	24.8 ± 1.5	71.8 ± 1.5
<i>5ho8az</i>	15.86 ± 0.46 ^a		2.7 ± 0.0	33.5 ± 1.6	63.8 ± 1.6
<i>5az8az</i>	16.74 ± 0.26		3.0 ± 0.2	31.1 ± 1.1	66.0 ± 0.9

<i>Bd4442</i>	16.37 ± 0.10 ^a	2.3 ± 0.1	25.7 ± 1.8	72.0 ± 1.9
<i>Bd5731</i>	16.52 ± 0.22 ^a	2.5 ± 0.1	31.4 ± 0.6	66.2 ± 0.6

Values are means ± standard deviations (SD) from individually analyzed plants (n=3). Significant difference (^a) is compared with the corresponding WT sample (one way ANOVA, Tukey's HSD) at p<0.05.

2.3.6 Homozygous double mutants of *BdLAC5* and *BdLAC8* release more hydroxycinnamic acids from cell walls after hydrolysis

The hydroxycinnamic acids (*p*-coumaric acid and ferulic acid) cross-link lignin and hemicellulose with ester and/or ether bonds (Hatfield et al., 1999). In monocots, most of *p*-coumaric acids (CA) are primarily ester-linked to syringyl units of lignin polymer (Ralph et al., 2004), but they are also present on arabinoxylans. It is well recognized that most ferulic acids (FA) are ester-linked to arabinoxylan and some of them are ether-linked to lignin (Jacquet et al., 1995). Ester- and ether-linked hydroxycinnamic acids were determined by mild and severe alkaline hydrolysis, respectively in order to evaluate the impact of laccases deficiency on these cell wall components (Table 2.3-2). Mild alkaline hydrolysis revealed that homozygous double mutant *5ho8ho* released remarkably higher amounts of FA than single mutants, *5az8az* and wild type. There was about 91% increase of FA in *5ho8ho* compared with *5az8az* (which harbor wt alleles for both genes), 141% increase compared with wild type, 70% increase compared with *BdLAC5* mutants (*Bd4442* and *5ho8az*) and 121% increase compared with *Bd5731* (Table 2.3-2). In accordance with the previous report (Wang et al., 2015), the single mutant of *BdLAC5* (*Bd4442*) can released more FA from cell walls by mild alkaline hydrolysis, while the single mutant of *BdLAC8* (*Bd5731*) does not release more FA compared with the wild type. Therefore, the tremendous increase of released FA content in homozygous *5ho8ho* compared with *Bd4442* and *Bd5731* is probably accounted for by the decreased lignin content (Table 2.3-2)

and also suggests that the level of cross-linking between lignins and arabinoxylans is reduced in the double mutant of *BdLAC5* and *BdLAC8*. Based on these results, *BdLAC5* may play the primary role.

Another hydroxycinnamic acid, *p*-coumaric acid (CA) is also bound with lignin, mainly by ester-linkage with S units in grass cell walls (Ralph, 2010), although a proportion of CA is ester-linked to arabinoxylans (Faulds et al., 2004). In contrast with FA, no obvious change in the released CA content was observed in *Bd4442*, *Bd5731* and *5ho8ho* compared to WT.

Table 2.3-2. Amount of *p*-coumaric acid and ferulic acid released by mild alkaline hydrolysis

Genotype	Compound (mg g ⁻¹)	
	<i>p</i> -coumaric acid	ferulic acid
WT	6.01 ± 0.26	5.24 ± 0.79
<i>5ho8ho</i>	4.81 ± 0.39 ^a	12.98 ± 0.5 ^a
<i>5ho8az</i>	5.31 ± 0.15	7.45 ± 0.71 ^a
<i>5az8az</i>	5.82 ± 1.16	6.80 ± 0.58 ^a
<i>Bd4442</i>	5.71 ± 0.51	7.86 ± 0.18 ^a
<i>Bd5731</i>	5.08 ± 0.19 ^a	5.88 ± 0.03

Values are means ± standard deviations (SD) from individually analyzed plants (n=3). Significant difference (^a) is compared with the corresponding WT sample (one way ANOVA, Tukey's HSD) at p<0.05.

2.3.7 Mutations in *BdLAC8* and *BdLAC5* increase saccharification yields

Lignin content is the main factor determining the saccharification yield, there is usually a negative correlation between lignin content and saccharification efficiency of plant cell walls (Chen and Dixon, 2007; Jorgensen et al., 2007). Strikingly, cellulose content did not significantly affect saccharification yield (Van Acker et al., 2013b). In this study, enzymatic saccharification efficiency was evaluated both by the weight loss percentage and by the amount of glucose released from the extractive-free dry stems (Table 2.3-3). The saccharification

yield was assessed for the single mutants of *BdLAC8* (*Bd5731*), *BdLAC5* (*Bd4442*) and for the double mutant (*5ho8ho*). As expected, the highest saccharification yield was found in the double mutant (*5ho8ho*), up to 160 mg·g⁻¹ of extracted cell wall residues, more than twice as many as WT (67 mg·g⁻¹), and the weight loss after the cellulase treatment was 49% more than that of wild-type (22%). The saccharification yield of *Bd4442* is also higher compared with WT. However *Bd5731* is not significantly different from WT.

At last, we propose that decreasing the lignin content and altering its structure by the simultaneous deficiency of *BdLAC8* and *BdLAC5* orthologues can be a promising strategy to improve saccharification in grasses.

Table 2.3-3. Saccharification assays of cell wall residues

Genotype	Weight Loss (%)	Anhydroglucose (mg·g ⁻¹)
WT	22.4±1.3	67.4±2.6
<i>Bd5731</i>	20.4±1.0	64.7±0.4
<i>Bd4442</i>	28.1±1.3 ^a	97.9±5.8 ^a
<i>5ho8ho</i>	48.8±0.9 ^b	160.1±26.8 ^b

The data represent means ± standard deviation from biological replicates (2 independent experiments performed each on 3 different samples per line). Letters indicate significant differences analyzed by one-way ANOVA (Tukey's HSD, P< 0.05).

2.3.8 The decrease of lignin content in cell walls of the double laccase mutant (*5ho8ho*) is compensated by an increased amount of cellulose

Because higher saccharification yields were found in the extracted cell wall of laccase mutants, we are curious about the cellulose and hemicellulose content in the same plant materials. Polysaccharide analysis were used for comparing *Bd4442*, *Bd5731* and *5ho8ho* with the WT. Although no significant variations in cellulose and hemicellulose content were observed among the wild type, *Bd4442*

and *Bd5731* (Figure 2.3-10), the average of cellulose content in *Bd4442* is slightly higher than that of wild type. Moreover, *5ho8ho* had significantly increased cellulose amount. Given the severe lignin decrease in *5ho8ho* detected by Klason, thioglycolic acid methods and acetyl bromide assays (data not shown), the cell walls of the double mutants contain more cellulose that compensate for the reduction in lignin. Interestingly, hemicellulose does not seem to be impacted. Although the mechanism is not clear, some studies have displayed the reduction of lignin content could be compensated by an increased amount of cellulose and/or hemicellulose in plant cell walls (Hu et al., 1999; Novaes et al., 2010; Vermerris et al., 2010). By contrast, Van Acker et al., (2013) found that low-lignin mutants were not compensated by increased cellulose amount. Our latest results support the former point of view, but we suppose the compensation mechanism will occur only when the lignin content in cell walls are severely reduced.

2.4 Materials and Methods

Plant growth conditions, histochemical staining, lignin content and structure determination, RNA extraction and quantitative RT-PCR, RNA *in situ* hybridization, 3D-structure prediction, fourier transform infrared (FTIR) spectroscopy and cell wall saccharification (cellulolysis) were performed as described previously (see above, Wang et al., 2015). Detailed information of primers used for the quantitative RT-PCR and DIG-labelled probe amplification are available at (Table 2.4-1).

Crossing of *Brachypodium distachyon*

Crossing the laccase mutant *Bd5731* with *Bd4442* homozygous plants was performed as described as the manual available at

<http://www.ars.usda.gov/SP2UserFiles/person/1931/BrachypodiumCrossing.pdf>.

Mild alkaline hydrolysis

The determination of ester-linked CA and FA was via mild alkaline hydrolysis, following procedures published previously (Ralph et al., 1994; Bouvier d'Yvoire et al., 2013).

Cellulose content determination

The Updegraff method was used for cellulose content estimation in the extracted cell wall residues (Updegraff, 1969). Briefly, samples are first hydrolyzed with acetic acid/nitric acid solution to remove matrix polysaccharides and amorphous cellulose. The glucose content is measured using the anthrone reagent and quantified using an HPAEC-PAD chromatography as described (Harholt et al., 2006).

Table 2.4-1. Information of used primers				
Gene Locus	Primer Names	Sequence	Length	Usage
Bradi3g 04730	QBdUbi4-R	GAGGGTGGACTCCTTCTGGA	142 bp	qRT-PCR
	QBdUbi4-F	TGACACCATCGACAACGTGA		
Bradi2g 23370	QBdLAC8-2R	CTCACGCCGTGCCAGTGGAAAG GTG	176 bp	
	QBdLAC8-2F	TACACGTTCAATGTGACAATG GCG		
Bradi1g 66720	QBdLAC5-3R	CTCACGCCGTGCCAGTGGATG GT	175 bp	
	QBdLAC5-3F	ACACCTTCAATGTGACGATGA AG		
Bradi1g 74320	QBdLAC6-3R	CTTACCCCATGCCAGTGGATGG TA	176 bp	

	QBdLAC6-3F	TACAAGTTCAATGTGGTTATGAGG		
Bradi2g 54740	QBdLAC12-F	GGTGCCTGCGTGGTCTCCGA	221 bp	
	QBdLAC12-R	CGATCGCCTTCCCTCGCGACC		
Bradi2g 23370	BdLAC8probe-3R	TGTAATACGACTCACTATAGG GCCCGACAATCCCGGTGTATG GATCA	500 bp	<i>in situ</i> hybridization of mRNA
	BdLAC8probe-3F	ACCGGCACTTCTTCTTCACTGT TG		
	BdLAC8probeCK-3R	CCGACAATCCCGGTGTATGGA TCA	500 bp	
	BdLAC8probeCK-3F	TGTAATACGACTCACTATAGG GCACCGGCACTTCTTCTTCACT GTTG		

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Figure 2.3-1. Expression profiles of *BdLAC8* (Bradi2g23370) and *BdLAC12* (Bradi2g54740).

Figure 2.3-2. Laccase expression levels in different organs of *Brachypodium* as determined by qRT-PCR.

Figure 2.3-3. Tissue localization of *BdLAC8* transcripts *Brachypodium*.

Figure 2.3-4. The predicted 3D-structure of the *BdLAC8* protein.

Figure 2.3-5. Histochemical staining of lignin in the *BdLAC8* stop codon mutant (*Bd5731*) and the WT.

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Figure 2.3-7. Identification of a double *LAC5 LAC8* homozygous mutant.

Figure 2.3-8. Lignin content of extractive-free dry culms in different mutants using ThioGlycolic Acid (TGA) method.

Figure 2.3-9. Detection of lignin distribution in different genotypes.

Figure 2.3-10. Polysaccharide content in the cell wall of *Bd4442*, *Bd5731*, *Bd4442XBd5731* (double homozygous) and WT (*Bd21-3*).

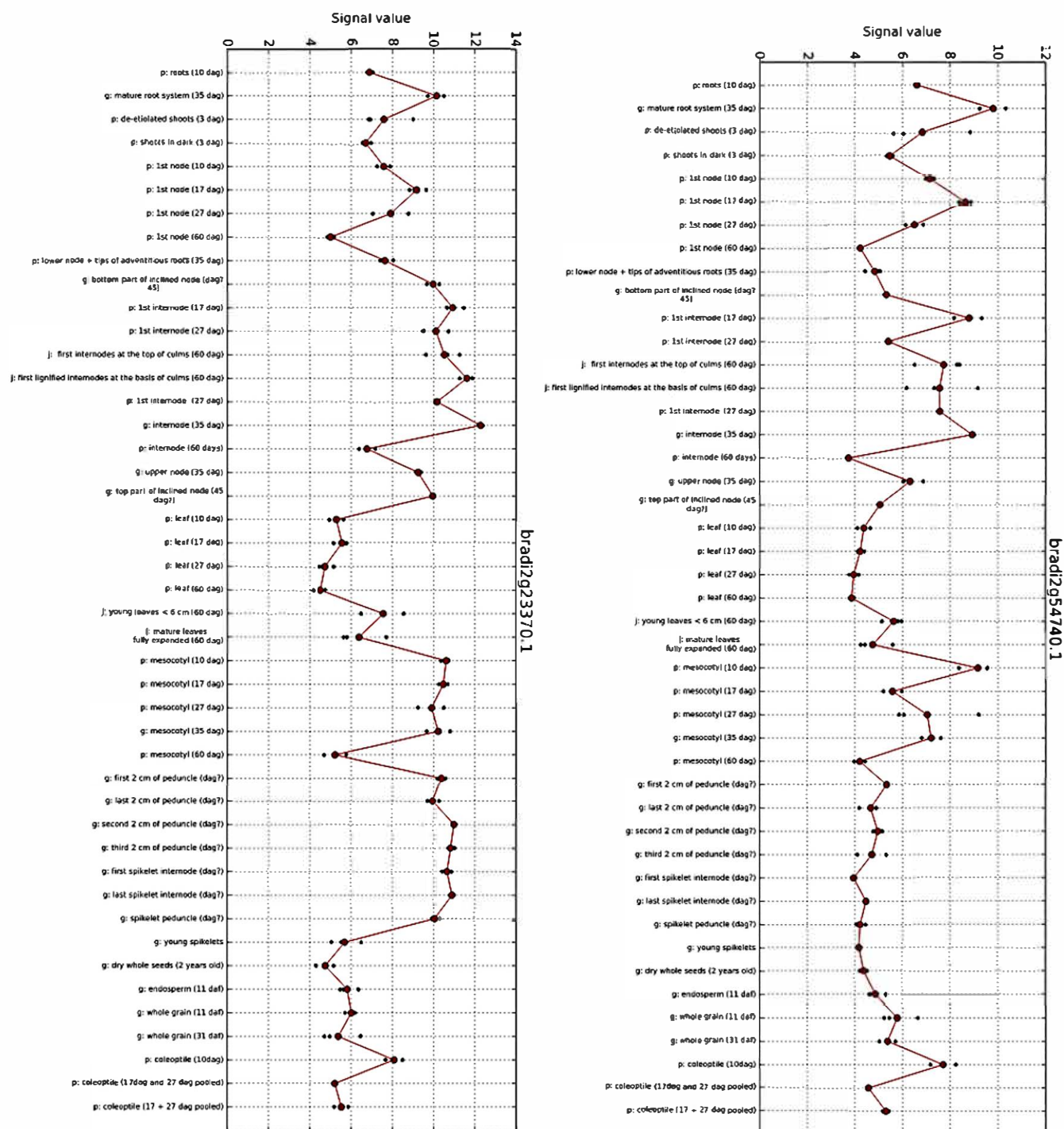


Figure 2.3-1. Expression profiles of *BdLAC8* (Bradi2g23370) and *BdLAC12* (Bradi2g54740).

Expression data were adapted from PlaNet (<http://aranet.mpimp-golm.mpg.de/>). Y-axis: expression values, x-axis: tissues/treatments, red dots: average expression, black dots: expression from individual microarray/RNAseq experiments.

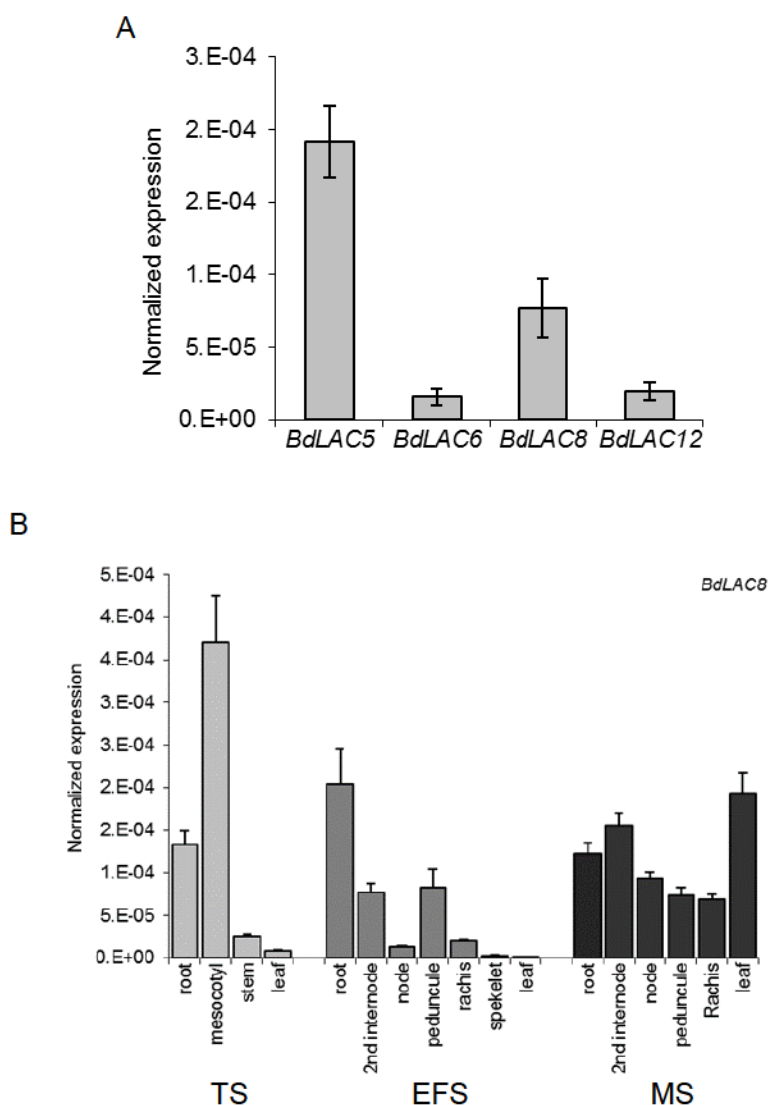


Figure 2.3-2. Laccase expression levels in different organs of *Brachypodium* as determined by qRT-PCR.

A: Normalized transcript levels of *BdLAC5*, *BdLAC6*, *BdLAC8* and *BdLAC12* in the second internode of the culm at early flowering stage and under longer day condition (18 h light/ 6 h dark, 35 days after germination).

B: Expression pattern of *BdLAC8* in different organs at three developmental stages, tillering stage (TS), early flowering stage (EFS) and maturity stage (MS).

All data represent the mean with the standard deviation (error bars) from three biological replicates.

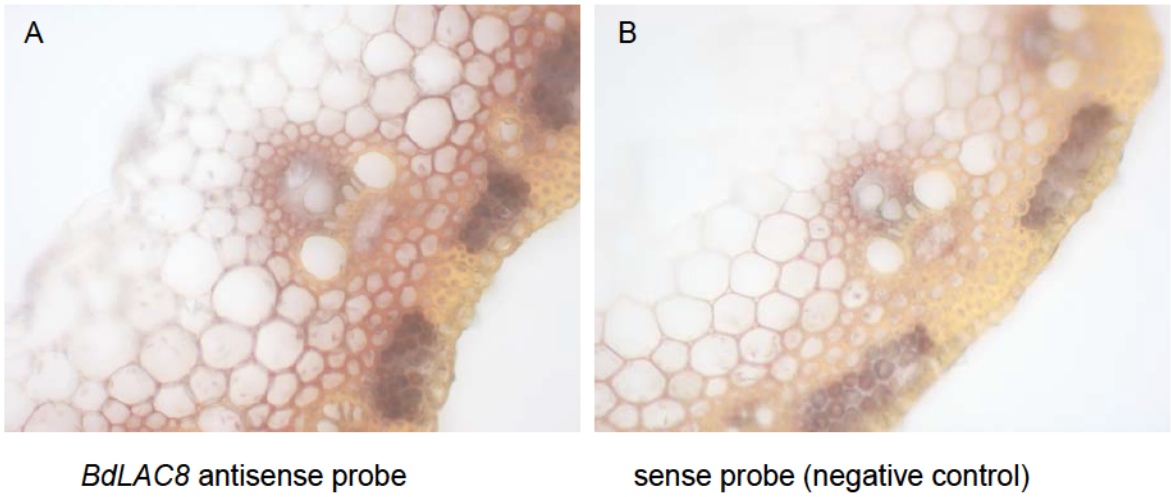


Figure 2.3-3. Tissue localisation of *BdLAC8* transcripts brachypodium.

in situ hybridization experiments were performed on sections of internodes at early flowering stage. A: *BdLAC8* antisense probe; B: *BdLAC8* sense probe used as the control.

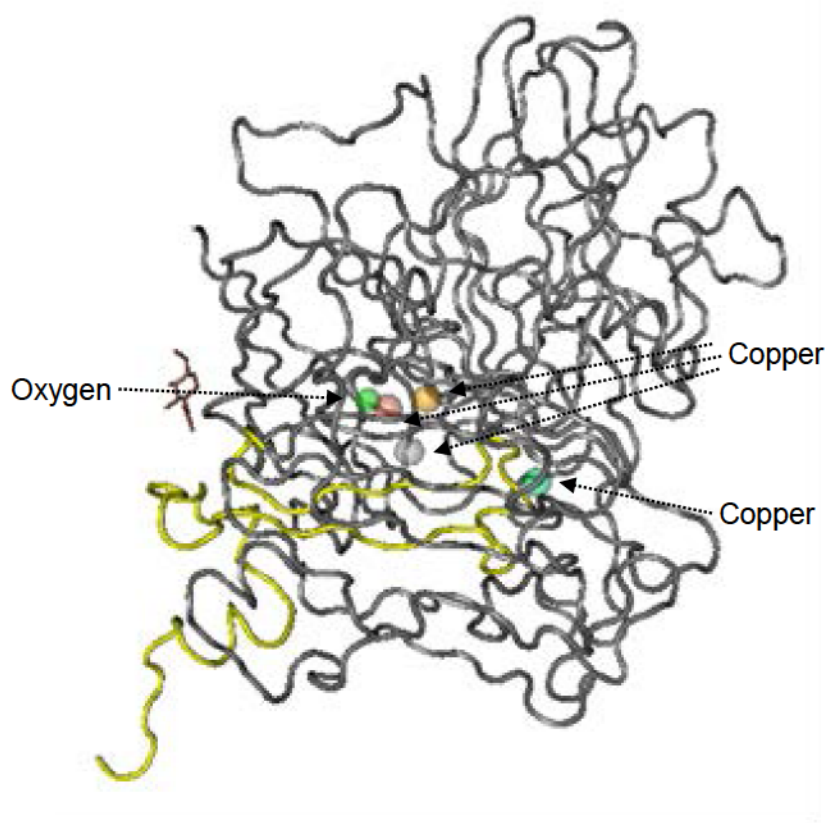


Figure 2.3-4. The predicted 3D-structure of the BdLAC8 protein.

The predicted 3D-structure of BdLAC8 is based on the structure of crystallized ascorbate oxidase from *Cucurbita pepo* var. *melopepo* (Zucchini) (PDB sum identification: 1ASO, 38% sequence similarity) using the molecular modelling program Geno3D (<http://geno3d-pbil.ibcp.fr/>). The conserved C-terminal domain is shown in yellow.

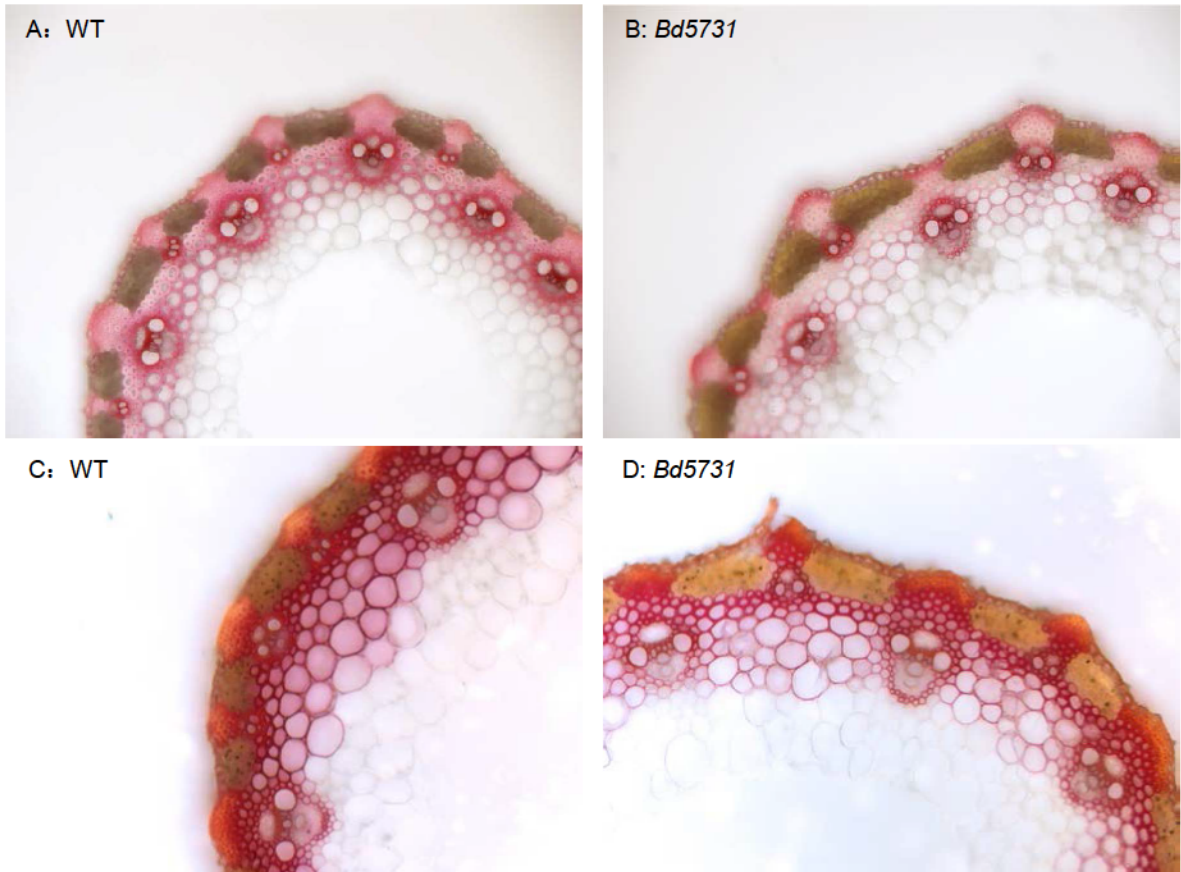


Figure 2.3-5. Histochemical staining of lignin in the *BdLAC8* stop codon mutant (*Bd5731*) and the WT.

Cross sections of the second internode at mature stage were stained. A and B: WT (*Bd21-3*) and *Bd5731* by Wiesner staining; C and D: WT (*Bd21-3*) and *Bd5731* by Mäule staining.

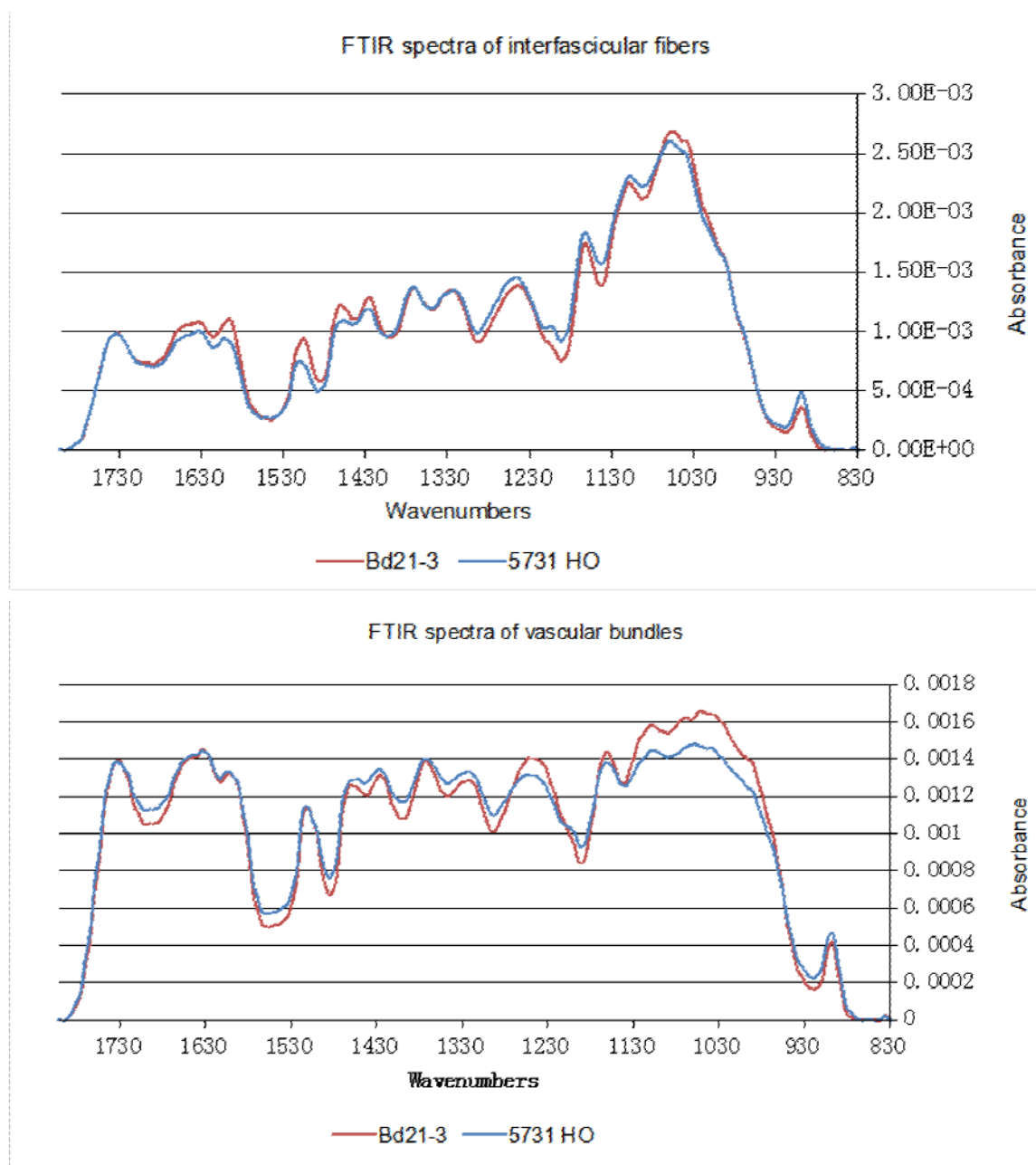


Figure 2.3-6. FTIR spectra obtained from internodes of *Bd5731* and WT (*Bd21-3*). The FTIR spectra were acquired in transmission mode separately from the sclerenchyma cells in interfascicular fibers (A) and the metaxylem cells inside the vascular bundle of the second internode at mature stages (B). Each spectrum corresponds to the normalized means of biological replicates (n=6).

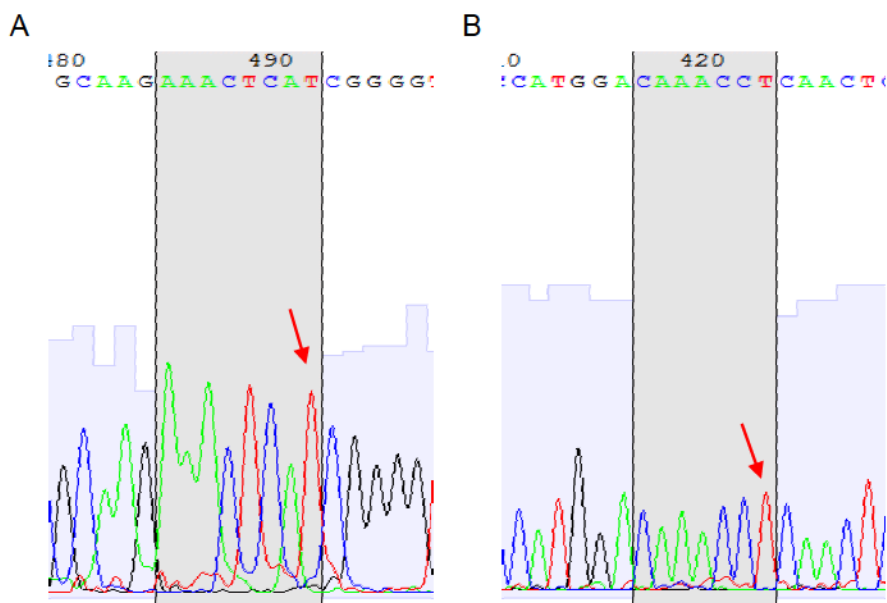


Figure 2.3-7. Identification of a double *LAC5 LAC8* homozygous mutant.

PCR products were amplified from genomic DNA of F2 (*Bd5731XBd4442*) lines and sequenced. A: using the specific primers of *BdLAC5*; B: using the specific primers of *BdLAC8*. Arrows indicate the mutation site in *BdLAC5* and *BdLAC8* PCR products respectively. In wild type allele, the same position has cytosine not thymine.

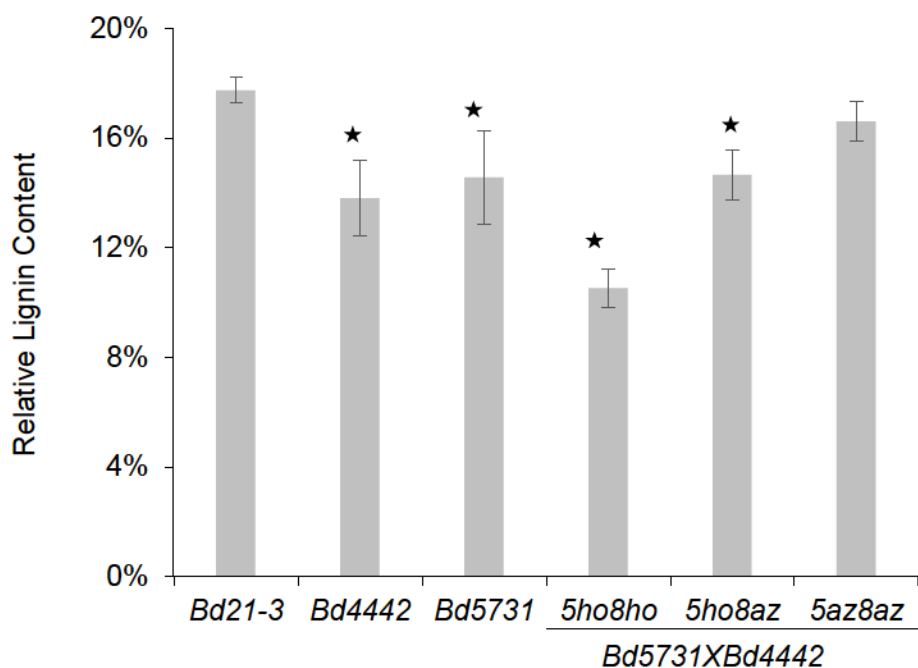


Figure 2.3-8. Lignin content of extractive-free dry culms in different mutants using ThioGlycolic Acid (TGA) method.

Samples are WT (*Bd21-3*), single mutants of *BdLAC5* (*Bd4442*) and *BdLAC8* (*Bd5731*) and three F2 genotyped progenies from the crossing between *Bd4442* and *Bd5731*. *5ho8ho*: *BdLAC5* homozygous X *BdLAC8* homozygous; *5ho8az*: *BdLAC5* homozygous X *BdLAC8* azygous; *5az8az*: *BdLAC5* azygous X *BdLAC8* azygous. Note: azygous lines have wild type allele for the gene of interest.

The relative lignin concentration shown in percentage was determined by dividing the amount of lignin calculated based on the calibration curve by the sample weight. All data represent the mean with the standard deviation (error bars) from three biological replicates. Stars indicate significant differences analyzed by one-way ANOVA (Tukey's HSD, $P < 0.05$).

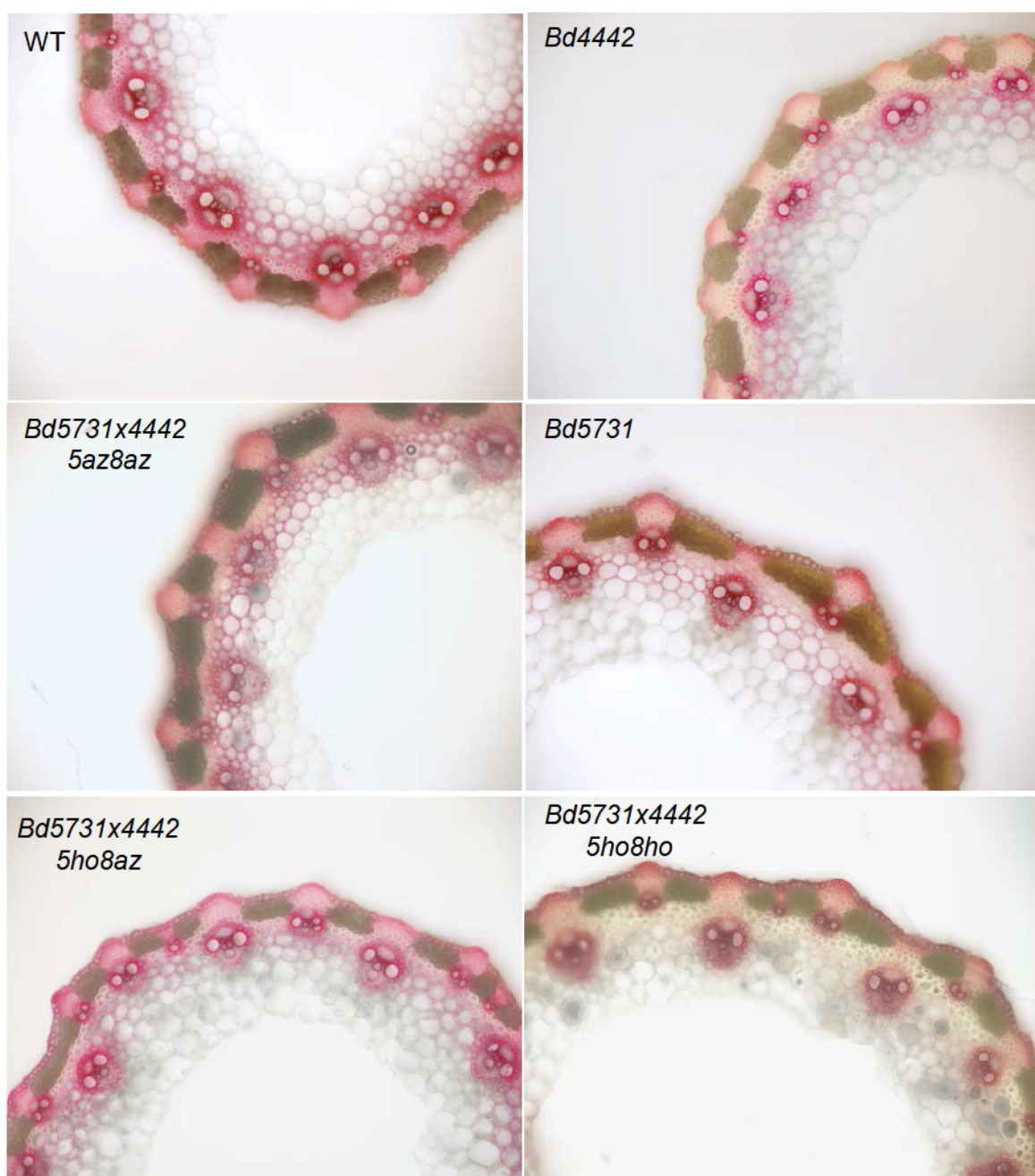


Figure 2.3-9. Detection of lignin distribution in different genotypes.

Genotypes are described in the legend of **Figure 2.3-8**. Transverse sections of the second internode were stained with Wiesner reagent. All pictures were taken at the same magnification (10x objective).

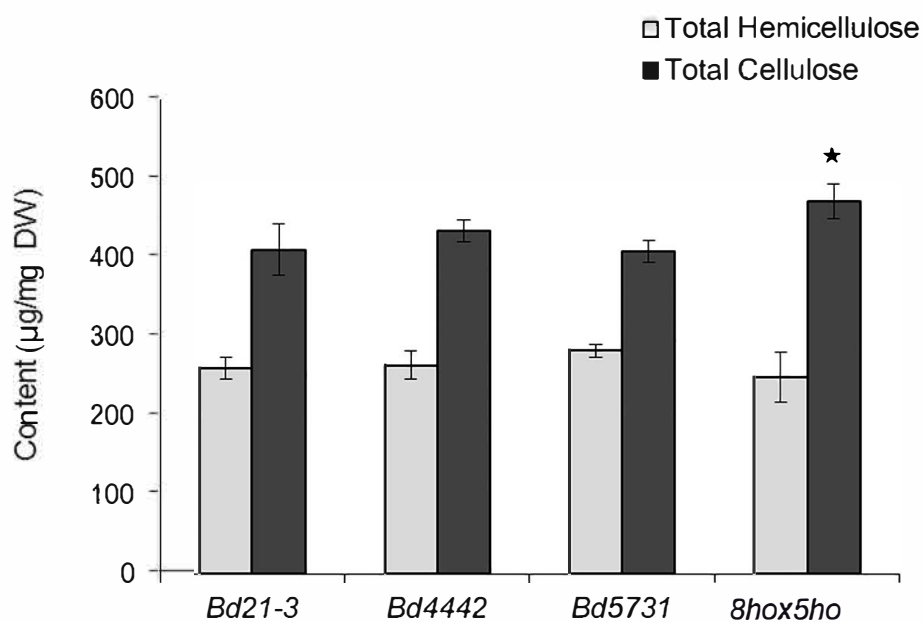


Figure 2.3-10. Polysaccharide content in the cell wall of *Bd4442*, *Bd5731*, *Bd4442XBd5731* (double homozygous) and WT (*Bd21-3*).

Soluble-free stems samples were used for both lignin and polysaccharide analyses in this experiment. All data represent the mean with the standard deviation (error bars) from three biological replicates. Stars indicate significant differences analyzed by one-way ANOVA (Tukey's HSD, $P < 0.05$).

Chapter 3 The regulation of laccases in *Arabidopsis thaliana* stems

During this PhD work, I was interested in exploring some transcription factors which would regulate lignin biosynthesis. However, I always kept in mind, that my objective was to identify genes that would not impact too much on the expression of upstream genes in the phenylpropanoid metabolism, but would only (or mainly) modify the last enzymatic reaction in lignin formation, i.e. the oxidation of monolignols. Ideally, it would be worth identifying genes that would target preferentially interfascicular fibers than xylem cells which are more important for plant growth and development.

Because our group identified some specific laccases responsible for lignification in the interfascicular fibers of *Arabidopsis* and *Brachypodium* stems, the last purpose of this PhD thesis was to identify specific regulators of laccases in the two model plants.

3.1 Introduction

Transcription factors (TFs) are proteins that typically bind specific DNA sequences or cis-elements in the promoter region, normally localized upstream of the coding sequence, through their DNA-binding domain and consequently activate or repress the spatial and temporal expression pattern of the target genes (Dubos et al., 2010a).

Secondary cell wall regulatory network is now partially understood and seems to be conserved across many species from dicot to monocot plants (Handakumbura and Hazen, 2012; Ruprecht and Persson, 2012). NAC domain TFs (VNDs, SNDs, VNDs and NSTs) are master switches that trigger the

secondary cell wall formation and cell differentiation into vessel or fiber cells (Kubo et al., 2005; Zhong et al., 2006; Mitsuda et al., 2007; Caño-Delgado et al., 2010; Ohtani et al., 2011; Wang and Dixon, 2012; Zhou et al., 2014). These regulators control the genes involved in the biosynthesis of the three major secondary cell wall polymers: cellulose, hemicelluloses and lignin. The second cascade includes MYB46, MYB83 and MYB85. The third cascade includes MYB103, MYB52, MYB54, MYB42, MYB43, MYB20, MYB58, MYB63, MYB61 and MYB69 (Rogers et al., 2004; Zhong et al., 2008; McCarthy et al., 2009; Zhou et al., 2009; Zhong and Ye, 2012; Ko et al., 2014; Öhman, 2014). However it is still not clearly understood which MYBs act in the upstream of other MYBs, and the biological functions of MYB20, AtMYB69, MYB52, MYB54, MYB42 and MYB43 in secondary cell wall formation have not been fully characterized. So far, based on the studies of these secondary wall related TFs, many deficient mutants and/or overexpression lines were produced and analyzed. Interestingly, there are no obvious phenotypes observed in single loss-of-function mutants in general. By contrast, double or multiple mutants, dominant repression or simultaneous RNA interference inhibition lines show dwarf phenotype or disability of standing upright (Rogers et al., 2004; Kubo et al., 2005; Mitsuda et al., 2007; Zhong et al., 2008; Ko et al., 2009; McCarthy et al., 2009; Zhou et al., 2009; Ko et al., 2014; Zhou et al., 2014; Zhong et al., 2014). Furthermore, overexpressors of NACs or MYBs have ectopic lignin distribution, curly leaves and also dwarf / semi-dwarf phenotype (Zhong et al., 2011; Romano et al., 2012; Zhou et al., 2014). The unchanged phenotypes of single mutants could be attributed to the functional redundancy of NACs and MYBs. Indeed, NACs are highly similar in protein sequences and MYBs belong to a superfamily. The dwarf phenotypes are probably induced by the simultaneously and continuously activated biosynthesis of cellulose, hemicelluloses and lignin in wrong tissues or organs and/or at an improper developmental stage. Therefore, it is challenging to manipulate cell wall composition or content of lignified organs

or tissues without severely impacting cell wall integrity and plant development (Anterola et al., 2002; Brown et al., 2005; Voelker et al., 2010).

Considering the complexity of the MYBs mentioned above, we decided to target some of the known interactors of MYBs. The physical interaction and regulatory synergy between specific R2R3-MYB and bHLH, together with WD repeat protein (MBW complex), is well studied in the biosynthesis of proanthocyanins, the root hair development, the seed mucilage biosynthesis and the trichome development (Xu et al., 2013; Xu et al., 2014). Similar MBW complexes have been also characterized in other plant species such as maize, apple, strawberry or petunia (Xu et al., 2014).

The basic helix-loop-helix (bHLH) proteins are a superfamily of transcription factors. In *Arabidopsis* 162 bHLH protein-encoding genes classified into 21 subfamilies were identified (Toledo-Ortiz et al., 2003). They bind as homodimers or heterodimers with other bHLH proteins to specific DNA target sites. N-terminal domain is a basic region involved in DNA binding. The HLH region at the C-terminus, functions as a dimerization domain (Toledo-Ortiz et al., 2003). Outside of the conserved bHLH domain, these proteins exhibit considerable sequence divergence (Atchley et al., 1999). The biological functions of some bHLHs have already been identified. These studies brought out that biological roles of bHLHs are extremely diverse in plants (Sun et al., 2012; Xu et al., 2013; Xu et al., 2014).

Some MYCs are involved in the stress responses of *Arabidopsis*. MYC2 is a key bHLH TF regulating the expression of different subsets of JA-responsive genes (Chini et al., 2007; Dombrecht et al., 2007). It was suggested that MYC2, MYC3 and MYC4 may form homo- or heterodimers to regulate specific JA-dependent responses (Fernández-Calvo et al., 2011). Yeast two-hybrid and pull-down experiments indicated that MYC2/MYC3/MYC4 interact directly with glucosinolate-related MYBs. A triple mutant lacking MYC2, MYC3, and MYC4 was shown to have a dramatic decrease of glucosinolate biosynthesis gene

expression and content in leaves. Moreover, the triple mutant *myc2myc3myc4* (*myc234*) is impaired in the activation of JA-mediated responses such as the defense against bacterial pathogens and insect herbivory. Additionally, MYC2 acts as a positive regulator of flavonoids biosynthesis in JA-treated Arabidopsis plants (Dombrecht et al., 2007).

Some bHLHs are involved in xylem formation. LONESOME HIGHWAY (LHW) and LONESOME HIGHWAY LIKE 1–3 (LHL1–LHL3) play an essential role in regulating the earliest steps of xylem development (Ohashi-Ito and Bergmann, 2007). The *lhw lhl3* double mutant displayed severe phenotypic defects in the vasculature of the cotyledons and roots in Arabidopsis. Only one xylem vessel was formed at the metaxylem position in *lhw lhl3* roots, whereas the *lhw* root formed one protoxylem and one or two metaxylem vessels. Overexpression of *LHL3* enhanced xylem development in the roots. It was also suggested a relationship between polar auxin transport and LHW function during vascular development (Ohashi-Ito and Bergmann, 2007; Ohashi-Ito et al., 2014).

As a last example, it has been shown that few bHLHs are involved in light signaling. For instance, PHYTOCHROME INTERACTING FACTOR4 and 5 (PIF4 and PIF5) promote elongation of hypocotyls (Huq and Quail, 2002). PIF4 and PIF5 are capable of integrating light/GA/clock signals to properly regulate the elongation of hypocotyls during early photomorphogenesis. This regulation is most prominent under short day conditions (Niwa et al., 2009).

Unlike the MYB and bHLH domains, it is difficult to clearly define a family of proteins based on the presence of a WD-repeat motif (Smith, 2008). In general, WD-repeat proteins do not bind DNA, but the WD-repeat generally functions as a scaffolding or adaptor between other proteins within the complex. The plant WD-repeat proteins are commonly referred to small proteins consisting of four or five WD-repeats (De Vetten et al., 1997). In Arabidopsis, TRANSPARENT TESTA 1 (TTG1) is a famous WD-repeat proteins. Three-hybrid experiments in yeast have demonstrated that TTG1 is able to interact

simultaneously with TT2 (a MYB protein) and TT8 (a bHLH protein) (Baudry et al., 2004). This interaction increased the activity of the TT2/TT8 complex after induction of BAN transcription *in planta*, but did not seem to modify the specificity of the complex for DNA binding. This implies that TTG1 could affect the functionality of the bHLH partner, and might participate in the activation, modification or stabilization of this TF (Payne et al., 2000; Baudry et al., 2004).

LWD1 and LWD2 are circadian clock proteins that regulate Arabidopsis photoperiodic flowering and circadian clock in Arabidopsis (Wang et al., 2011). LWD1 shares approximately 90% amino acid sequence identity with LWD2. The Arabidopsis double mutant *lwd1 lwd2* showed an early-flowering phenotype under short day and long day conditions.

During my PhD, I tried to explore some uncharacterized transcription factors from the MYB/bHLH/WD40 families in Arabidopsis and Brachypodium which could specifically regulate the monolignol polymerization process and therefore that would have less effects on the monolignol synthesis.

3.2 Results and discussion

3.2.1 Selection of candidate bHLHs involved in lignin biosynthesis according to their expression profiles

To explore TFs that could regulate the monolignol polymerization process, we first analyzed the expression patterns of the genes encoding enzymes responsible for monolignol oxidation such as laccases and compared them with those of TFs. According to previous results (Berthet et al., 2011), LAC4 and LAC17 play major role in the lignification of Arabidopsis stem. Promoter fused with β -glucuronidase (*GUS*) assay showed *LAC4* is highly expressed in the vasculature of young seedlings, rosette leaf vessels, vascular bundles (xylem and cambium) and

interfascicular fibers in mature stems and anthers. *LAC17* is more specifically expressed because no visible GUS staining was detected in young seedlings and vascular bundles of mature stem. However *LAC17* is observed in the interfascicular fibers, leaf vessels and anthers (Koizumi et al., 2009; Berthet et al., 2011). To summarize, *LAC4* and *LAC17* transcripts are present in lignified tissues (Berthet et al., 2011). In order to extend our knowledge about expression of these two genes and TFs, we referred to the transcriptomic database: Arabidopsis eFP Browser (Winter et al., 2007). The dataset come from different microarray experiments and the data were normalized by the GCOS method, TGT value of 100. First, we analyzed the expression patterns of the lignin specific genes *AtLAC4* and *AtLAC17* by extracting their normalized data from eFP Browser (Figure 3.2-1). In young seedlings, *LAC17* expression is very low in cotyledon (~2), hypocotyl (~14) and root (~96). In mature plant, the highest expression level (~1577) is in the bottom of primary stem, while it is a bit lower (~149) in the epidermis of the bottom stem. The expression level in the bottom stem is also much higher than in the top of primary stem (~570), as well as shoot apex (~7), rosette leaf (~37), young flower (~32) and mature root (~125). In other non-lignified tissues at different stages, the expression levels of *LAC17* are very low. *AtLAC4* show very similar expression patterns. As observed previously in the literature, the expression profiles of *AtLAC4* and *AtLAC17* are almost perfectly consistent with the lignin distribution in plant/organs and with their predicted level of lignification.

We then checked the expression patterns and transcription levels of the whole bHLH family (162 members) in Arabidopsis (Toledo-Ortiz et al., 2003). Only a few genes displayed similar expression patterns as *AtLAC17/4* and interestingly, none of them has been reported to be involved in lignin regulation so far. Nonetheless we first focused our attention on one *bHLH*, *MYC4* (At4gl 7880) for further investigation. *MYC4* has a conserved N-terminal (named as pfam14215: bHLH-MYC_N) which has been proved to bind with MYB

protein (Schweizer et al., 2013) and possesses also a Helix-loop-helix DNA-binding domain.

3.2.2 MYC4 is specifically express in the vasculature

According to eFP Browser (Figure 3.2-1), *MYC4* expression is very low in the cotyledons (~60), the roots of young seedlings (~53) and a little higher (~146) in the hypocotyl. In the secondary thickened hypocotyl of mature plant, the expression of *MYC4* is high in xylem (~530) as well as in the cortex (~370). The eFP Browser allowed to detect a high expression level in the bottom of primary stem (~300). This expression level is much higher than in the top of primary stem (~83), as in rosette leaf (~35) and root (~53). In other non-lignified tissues (and at different stages), the expression levels of *MYC4* are very low. Promoter-GUS assay showed *MYC4* is specifically expressed in the vasculature of mature plants at different developmental stages (Fernández-Calvo et al., 2011). This provide a possibility that MYC4 is a positive TF for lignin biosynthesis in Arabidopsis.

MYC3 (59% identity and 67% similarity with MYC4) and MYC2 (57% identity and 72% similarity with MYC4) are close homologs of MYC4, and they are all nuclear proteins (Lorenzo et al., 2004; Chini et al., 2007; Fernández-Calvo et al., 2011). Therefore we might assume that these transcription factors may have redundant functions. MYC3 is expressed ubiquitously in most organs of the young and mature plant, including stems, siliques, flowers, and leaves. In young roots, expression of MYC3 and MYC4 was very weak, whereas the expression of MYC2 is ubiquitous and high in roots but quite low in aerial tissues of young seedlings (Fernández-Calvo et al., 2011; Schweizer et al., 2013).

3.2.3 *MYC4* overexpressors have increased lignification via upregulation of *LAC4* and *LAC17*

Because of the expression pattern of *MYC4* in the vasculature of wild-type plants, we assumed that this gene might control lignification. Therefore, the full cDNA of *MYC4* was cloned under the control of the CaMV35S promoter and introduced into wild-type col-0 and ws. As expected, when *AtMYC4* was overexpressed in ws background and grown under long day condition (18 h light/ 6 h darkness), the lignin content was increased and distributed ectopically (Figure 3.2-2). The *MYC4* overexpressor plants showed more intense Wiesner staining in the overall of interfascicular fibers and vessels in addition to ectopic staining in parenchyma cells around protoxylem and epidermis cells which are usually known to be non-lignified cells in Arabidopsis. The increased and ectopic lignification in overexpressors of *MYC4* is probably caused by the upregulation of lignin biosynthetic genes and we thus carried out quantitative RT-PCR in order to compare the transcription level of lignin biosynthesis genes in overexpressors with the control. We selected three independent transformed lines for which overexpression of *MYC4* was confirmed. We assessed the transcript levels of *LAC4*, *LAC17* and *CCR1*, a gene encoding cinnamoyl CoA reductase. As shown in Figure 3.2-3, all genes were found upregulated in the transgenic lines (only one transgenic line is shown in Figure 3.2-3). The transcription levels of *LAC4* and *LAC17* were significantly increased in overexpressors of *MYC4* while the transcription of *CCR1* was moderately upregulated compared to wild type. We did not observe any dramatic morphological phenotype in the overexpressors of *MYC4* unlike other overexpressors of TFs related to lignin biosynthesis and/or secondary wall formation. For instance, the overexpression of MYBs (MYB46, MYB83, MYB58, MYB63 and AtMYB85) and NACs (SND1, VND6 and VND7) often led to plants with stunted shape accompanied with some phenotypes usually attributable to stress, such as curly leaves and dwarfism (Rogers et al., 2004; McCarthy et al., 2009; Zhou et al., 2009; Zhong et al., 2011; Romano et al., 2012; Ko et al., 2014; Zhou et al., 2014). This absence of severe phenotype is not completely unexpected since the overexpression of *LAC4* and *LAC17* did not

cause any strong phenotypic nor any increase of total lignin content in the same growth conditions (data not shown).

In conclusion, *MYC4* overexpressors showed enhanced lignification and normal growth and development. This is an interesting feature. It would be interesting to further investigate *MYC4* overexpressors and check whether they display enhanced resistance to biotic stress such bacteria and insects for which lignins are usually detrimental.

3.2.4 *myc234* show less JA-induced lignification and anthocyanin accumulation

MYC2 is a master regulator within the jasmonate (JA) signaling pathway including gene expression, inhibition of root growth and pathogen and insect resistance (Kazan and Manners, 2013). Fernández-Calvo et al., (2011) suggested that MYC2, MYC3, and MYC4 may form a cluster of TFs regulating specific JA-dependent responses. Because of the high similarity between MYC2, MYC3 and MYC4 protein sequences, the triple mutant *myc2myc3myc4* (named as *myc234*) is insensitive to JA signaling (Fernández-Calvo et al., 2011). Schweizer et al., (2013) showed the triple mutant *myc234* was almost completely devoid of glucosinolates. Interestingly enough, it has been shown that lignin biosynthesis can be induced by exogenous JA signaling (Denness et al., 2011), so we may suppose MYC2/3/4 participate in this process.

The seedlings of *myc234* (seeds were kindly provided by Dr.Philippe Reymond) and WT (col-0) were grown on media with or without methyl jasmonate (MeJA, 50 μ M) during 12 days. The lignin content analysis was carried out using TGA lignin method after extracting the soluble compounds from the seedlings (Figure 3.2-4). Interestingly, the lignin content was increased by the MeJA treatment in the WT. By contrast, there was no significant increase of lignin content in *myc234* by the MeJA treatment compared with the WT. The β -O-4 substructures of lignins in these plantlets were also analyzed by

thioacidolysis (Figure 3.2-5). Only a few S and H units linked with β -O-4 were detected in WT seedlings in non-treated media whereas we observed the accumulation of a huge amount of S units when WT were treated with MeJA. The triple mutant *myc234* behaved differently and no significant changes were observed with or without MeJA. Actually basic levels of S and H units were found already high prior any MeJA treatment.

MeJA treatment is usually used for mimicking stress, such as wounding. It was previously shown that stressed plants could produce lignins enriched in H units (Lange et al., 1993) but the lignin composition of plants treated with MeJA is poorly described.

It is well known that MYC2 is responsible for the activation JA signaling and it has been proposed that MYC3 and MYC4 act synergistically to MYC2 (Fernández-Calvo et al., 2011; Guo et al., 2013). Indeed the single mutants of *MYC2*, *MYC3* and *MYC4* do not show a complete loss of JA sensitivity (Fernández-Calvo et al., 2011). Our results strongly suggest that the TF cluster of MYC2, MYC3 and MYC4 are required in activating JA-induced lignin biosynthesis despite lignin structure / composition is naturally changed in *myc234* compared with *WT*.

We checked microarray data of Arabidopsis WT seedlings treated with MeJA treatment (Goda et al., 2008). We noted that some genes in the lignin biosynthetic pathway are upregulated by MeJA treatment herein *PAL1* (AT2G37040), *CCR1* (AT1G15950), *CAD5* (AT4G34230), *F5H* (AT4G36220) and *CSE* (AT1G52760) (Rebecca Van Acker 2013). F5H is the most important enzyme for S-units production and its upregulation might thus explain the increase of the S/G ratio in wt seedlings treated with MeJA. On one hand, we also noticed from the expression data that *MYC2* and *MYC3* are upregulated by MeJA treatment, *MYC2* being the most overexpressed. On the other hand, *MYC4* is not significantly induced in Arabidopsis WT seedlings. This means that in MeJA treated WT plants, *MYC2* and *MYC3* may be the TFs which activate lignin genes.

However in a *myc2* deficient background, *MYC4* can be strongly induced by MeJA treatment (Guo et al., 2013), which means *MYC4* could be a substitute for JA response. Fernández-Calvo et al., (2011) showed MYC2 and MYC3 have almost identical DNA binding specificities. Both proteins therefore, likely recognize similar targets *in vivo* and by contrast, *MYC4* recognize a slightly different subset of target genes. Moreover, MYC2 has a major role in root growth inhibition, whereas MYC3 and MYC4 seem to be more important for the response to herbivory in aerial parts. We may hypothesize that a role in lignin biosynthesis would be one of the function of MYC4.

The lignin in plant cell wall matrix is essential for biotic and abiotic stress. A hypothesis has been proposed suggesting plants possess a monitoring mechanism for maintenance of cell wall integrity by changing cell wall composition and/or structure (Seifert and Blaukopf, 2010; Denness et al., 2011). In response to cell wall damage, lignin production is initiated as a putatively compensatory response, so that cell walls could be reinforced to maintain functional integrity (Seifert and Blaukopf, 2010). While lignin biosynthesis itself is well characterized, the mechanisms regulating lignin biosynthesis and deposition in response to environmental stimuli are not well understood. Cell wall damage-induced lignin biosynthesis is regulated by a reactive oxygen species- and jasmonic acid-dependent process in *Arabidopsis* (Denness et al., 2011). The plant hormones jasmonates (JAs) are fatty acid-derived oxylipins required for the regulation of defense against mechanical injury, pathogen and insect attack (Fürstenberg-Hägg et al., 2013). They are also important regulators of growth and developmental programs in many plant species (Zhang and Turner, 2008). We may thus hypothesize that MYC2, MYC3, MYC4 are corner stones in the crosstalk between hormones, cell wall biosynthesis and stresses.

3.2.5 *myc234* show less JA-induced anthocyanin accumulation

Under MeJA treatment, the WT seedlings accumulated more anthocyanin especially in hypocotyl-cotyledon junction and the primary root elongation was inhibited. Consistently with previous reports, this phenotype is observed during JA response (Fernández-Calvo et al., 2011) and also as a features by ultraviolet light irradiation (Tong et al., 2008; Li et al., 2013). As expected, *myc234* did not hyperaccumulates anthocyanin under MeJA treatment compared with WT (Figure 3.2-6) and the growth of roots was not significantly inhibited (data not shown). Niu et al., (2011) found that transgenic plants overexpressing *MYC2*, *MYC3* and *MYC4* had higher levels of anthocyanin, but *myc3* and *myc4* single mutants showed no differences with WT. We also produced *MYC4* overexpressors, but no significant increase of anthocyanin can be detected (Figure 3.2-7). Because in WT seedlings *MYC2* and *MYC3*, but not *MYC4* are induced by MeJA treatment, we suppose *MYC4* is not involved in the regulation of anthocyanin biosynthesis, at least it is not a primary regulator. Although some evidences support *MYC2/3/4* may be involved in anthocyanin biosynthesis (Niu et al., 2011), they may only control some steps of the anthocyanin biosynthetic pathway, and consequently their overexpressing can not produce so large amounts of anthocyanin as *PAP1* overexpressors (Teng et al., 2005).

3.2.6 *myc234* does not display any decrease of lignin content but a slightly irregular xylem shape

The expected reduction of TGA lignin content in *myc234* compared with wild type was not observed (Figure 3.2-8). This result was confirmed by Wiesner staining on stem sections (Figure 3.2-9C). Moreover, the transcription levels of *LAC4*, *LAC17* and *CCR1* were astonishingly not significantly decreased in mature stem of *myc234* (Figure 3.2-10). However, we noticed that the diameter of *myc234* stem was smaller than that of WT (*col-0*), this phenotype was observed for three different culture (Figure 3.2-9). Moreover, when plants were grown

under longer day condition (18h light/ 6h dark), *myc234* plants were slightly shorter than WT (Figure 3.2-9B). We did not observe this phenotype in single mutant of *MYC2*, *MYC3* or *MYC4* (data not shown). It suggested the simultaneous lacking of *MYC2*, *MYC3* and *MYC4* could have some influence in stem growth and development.

Furthermore, the shape of some xylem vessels in *myc234* mutants was irregular (Figure 3.2-9C). Although this phenotype was not found in all vascular bundles. This result suggests the stiffness of xylem cell walls is decreased in *myc234* in a similar manner than for other mutants with irregular xylem phenotype. These irregular xylem phenotypes are generally caused by perturbation of cellulose (IRX1/2/3/5), hemicellulose (IRX8/9/10/14) or lignin biosynthesis (IRX4/12, CADC/D) (Persson et al., 2007; Keppler and Showalter, 2010; Berthet et al., 2011; Lefebvre et al., 2011; Bensussan et al., 2015). It is impossible to explain this result as our investigation stands at present and further analyses are needed. However based on all results cited above, we may hypothesized that *MYC2/3/4* play a role in the stiffness of xylem vessels but their major role would be related to stress-induced lignins.

3.2.7 Specific binding sites for MYBs and bHLH are present on *LAC4* and *LAC17* promoters Arabidopsis

The potential binding site of TFs in the promoters of target genes can be predicated *in silico* by some internet database produced from computational and experimental resources. We used three different softwares to predict TFs binding site in the promoter of *AtLAC4* and *AtLAC17*, AGRIS (<http://arabidopsis.med.ohio-state.edu/>), PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) and PLACE (<http://www.dna.affrc.go.jp/PLACE/>). These tools predicated that some known R2R3-MYB binding motifs, such as MYB-core (CNTTR, R=A/G) and AC-

elements [ACC(T/A)(A/C)(A/C)C] and some bHLH binding motifs such as E-box (CANNTG) and G-box (C/G)ACGT(A/G) are present on both promoters (1 kb upstream of CDS). It is worthwhile to point out that AC elements were previously shown to be present in promoters of many lignin related genes in *A. thaliana* (Raeiks et al., 2003) and other plant species (Hauffe et al., 1993; Hatton et al., 1995; Lacombe et al., 2000).

A bunch of MYBs (MYB19/31/46/49/50/58/60/61/63/86/92/94/103/107) were shown to bind to AC element *in vitro* (Dubos et al., 2014) and some interactions were confirmed *in vivo* (Zhong et al., 2007; Zhong et al., 2008; Zhong et al., 2011; Zhong and Ye, 2012). Likewise some studies established that MYC2, MYC3 and MYC4 show similar DNA-binding specificities to E-box and G-box (Fernández-Calvo et al., 2011).

To uncover some putative partners of MYC4 that could bind to *LAC4* and *LAC17* promoters and significantly activate their transcription by the MBW protein complex, I selected some candidates MYB and WD40 genes what were expressed in the same tissues than both laccases and MYC4.

3.2.8 AtMYB61 is a positive regulator of lignin biosynthesis

Several results suggested that MYB TFs play an important role in regulating lignin biosynthetic genes (Zhong et al., 2007; Zhong et al., 2008; Zhong et al., 2011; Zhong and Ye, 2012). However most of these researches are based on chimeric repressors (overexpression of TFs fused with EAR-motif) or overexpressors, but only one report showed a double mutants had less lignin phenotype (Ko et al., 2014), probably because the family of MYB TFs is one of the most abundant classes of TFs in plants (Dubos et al., 2010b) and functional redundancy may occur.

We analyze the expression patterns and transcription levels of the complete R2R3-MYB family (126 members) in *Arabidopsis* (Dubos et al., 2010b).

According to eFP-browser, some genes display similar expression patterns as *LAC17* and *LAC4*, including MYB58/52/54/103/20/63/48/83/85/69/46/43/61. Among them, MYB58/103/63/83/85/46/61 have been reported to be involved in lignin regulation. As mentioned above, the overexpression of MYB58, MYB63, MYB85, MYB83 and MYB46 has very strong influence on phenotypes. Because we are planning to co-express genes in plants for complementation or overexpression experiments, we chose to study MYB61 as a potential partner of MYC4 for further investigation.

The AtMYB61 gene was shown to participate in lignin biosynthesis in Arabidopsis (Romano et al., 2012)(Newman et al., 2004) and it is involved in establishing the ratio of xylem to phloem during secondary growth (Newman et al., 2004). *AtMYB61* expression was restricted to the developing vascular cells (protoxylem and metaxylem) in the inflorescence stem, leaves, hypocotyls and roots, but poorly in fibers of elongating internodes (Romano et al., 2012). The AtGenExpress Visualization Tool (Schmid et al., 2005) confirms MYB61 is highly expressed in shoots and seeds. Its expression in leaf, silique, flower and shoot apex is very low.

3.2.9 MYB61 mutants show IRX phenotype and slight decreased lignin

The *MYB61* mutants have morphological phenotypes indistinguishable from wild type and we did not observed any severe decrease of lignin staining (Wiesner reaction) in vessels or interfascicular fibers in the basal part of stem (Figure 3.2-11). However, the shape of xylem vessels in *myb61* is irregular (Figure 3.2-11). Again, it suggests the stiffness of xylem cell walls is decreased in *myb61* as often noted for other mutants with irregular xylem phenotype. This results suggests a role of MYB61 in lignification of xylem vessels, but maybe not in interfascicular fibers of mature stems. Unfortunately, the transcription levels of *LAC4* and *LAC17* are not significantly affected in *myb61* (data not shown). It is not

surprising that the deficiency of *MYB61* alone cannot cause significant influences to the transcription laccase genes, because of the redundant functions among the lignin related MYBs. A previous study has already point out that *myb61* mutant has less lignified xylem in hypocotyls (Romano et al., 2012) and it was shown by the same group that the root growth is inhibited in this mutant. In contrast, gain of *MYB61* function has the opposite effect on these traits. Overexpressors of *MYB61* are semi-dwarf and produced ectopic accumulation of lignin. Suppression of *MYB61* expression in the de-etiolated 3 mutant (*det3*), partially rescued the phenotypes of *det3* which develops morphologically as a light-grown plant when grown in the dark. Interestingly, *MYB61* misexpression was account for the ectopic lignification phenotype in the *det3* mutant (Newman et al., 2004).

3.2.10 *WDR* genes may be involved in the regulation of laccases

3.2.10.1 Identification of three WDR proteins putatively involved in lignification

WD40 repeat domain containing (WDR) proteins may function as a coregulator to control target gene expression by interacting with other transcription factors and/or binding to the target promoters it regulates (Xu et al., 2014). Baudry et al., (2006) proposed that the role of WDR proteins is probably to stabilize the complex of the MYB and bHLH.

There are three WDR proteins which share high similarity in Arabidopsis, TRANSPARENT TESTA GLABRA1 (TTG1), LIGHT-REGULATED WD1 (LWD1) and LWD2. TTG1 shares 62% identity and 77% similarity with LWD1 and LWD2 shares 91% identity and 95% similarity with LWD1. LWD1, LWD2 and TTG1 form a distinct group of WD-repeat proteins in Arabidopsis as described previously by (van Nocker and Ludwig, 2003). Other proteins containing WD40 repeat domain share very low similarities with TTG1, LWD1 and LWD2. The subcellular localization of TTG1 and LWD1 were shown by

fusing with YFP or GFP respectively (Balkunde et al., 2011; Wang et al., 2011). Both of them are preferentially localized in the nucleus and also in the cytoplasm. Balkunde et al., (2011) show that a bHLH (GL3) mediates the nuclear localization of TTG1 by direct interaction with TTG1. In *gl3* mutants, the *TTG1-YFP* distribution appears to be diffuse from the nucleus to the cytoplasm, by contrast in the GL3 overexpressors TTG1-YFP is localized mainly in the nucleus. In summary, WDR proteins (TTG1, LWD1 and LWD2) could bind together with bHLH and/or MYB to form a complex which directly control the transcription of target genes (Balkunde et al., 2011; Wang et al., 2011) and a role in the regulation of the flavonoid biosynthesis has already been suggested (Xu et al., 2014).

3.2.10.2 *LAC4* and *LAC17* transcription is synchronized with expression of LWD1

Transcriptomic data from Arabidopsis eFP-browser revealed that the *TTG1*, *LWD1* and *LWD2* genes are ubiquitously expressed in various organs at different development stages (Figure 3.2-12). The overall transcription levels of *TTG1* and *LWD1* are much higher than *LWD2*, especially in the mature stem. None of them show “lignin specific” expression patterns as we mentioned previously. Interestingly, although there is high similarity in their protein sequences and overlapped expression patterns, their functions are not redundant. The knock-out mutant of *TTG1* shows distinct phenotypes with *lwd1* and *lwd2* (Baudry et al., 2006; Wang et al., 2011). For example, *ttg1* has transparent testa, no trichome formation and cannot produce anthocyanin. Despite Wu et al., (2008) suggested the functional redundancy between LWD1 and LWD2, TTG1 should have different roles with LWD1 and LWD2 *in planta*.

In Arabidopsis seedlings, the three *WDR* genes (*LWD1*, *LWD2* and *TTG1*) display similar circadian expression patterns under long day condition (16h light/ 8h dark) but not under short day (8h light/ 16h dark) (Figure 3.2-13). LWD1,

LWD2 and TTG1 together A: short and B: long) according to Diurnal database (Mockler et al., 2007). The transcription of LWD1 fluctuates much more sharply than LWD2 and TTG1 do. Interestingly, *LWD1* transcription is repressed by light and strongly induced by darkness. Then we checked the diurnal variation of *LAC4* and *LAC17* transcripts level, strikingly, their expression patterns were synchronized with LWD1 under long day and short day conditions (Figure 3.2-14). We thus might hypothesize LWD1 may be involved in the circadian control of *LAC4* and *LAC17* transcription.

Despite *LWD1*- and *LWD2*-promoter GUS fusion were not available we attempted to check GUS staining in lines carrying *TTG1* promoter-GUS fusion which were kindly provided by Dr. Loic Lepiniec (Figure 3.2-15). The GUS staining in the cross section of basal stem showed that TTG1 would be express in most of tissues, except protoxylem and pith, the same expression patterns was observed in two different transformed lines. TTG1 was thus expressed in both lignified and non-lignified tissues. For *LWD1* and *LWD2* expression, because there was no established experimental results, I aimed at producing promoter-GUS transgenic lines or perform mRNA *in situ* hybridization for these genes in the future plan.

3.2.10.3 Single mutants of *TTG1* or *LWD1* do not show significant decrease of lignification in mature stem

The homozygous T-DNA insertion mutant of *TTG1* (GK-580A05-021290) and *LWD1* (SALK_006874C) were identified and genotypes (Figure 3.2-16A). The phenotype of *ttg1* is consistent with previous reports (Baudry et al., 2004), such as absence trichome and yellow seeds were observed. The *lwd1* does not show any phenotype compared with wild type, consistently with the previous reports (Wang et al., 2011). The lignin distribution in the bottom of mature stems was characterized by Wiesner staining (Figure 3.2-16C). No obvious decreased

density of coloration or any special pattern were noticed in vascular bundles or interfascicular fibers of *ttg1* or *lwd1*. By TGA lignin method (Figure 3.2-16B), the lignin content in the mature stems of *ttg1* and *lwd1* were not significantly different with the WT control, although the means of TGA lignin content in *lwd1* is slightly lower than the WT. Considering the overlap of expression patterns and high similarity between their protein sequences, we suppose that LWD1, LWD2 and TTG1 may have redundant functions. To overcome this problem, we are currently producing the triple mutants of the three *WDR* genes.

3.2.10.4 The MYB61–MYC4–TTG1/LWD1 complex can activate the promoter of *LAC4* in *Physcomitrella patens* protoplasts

The *Physcomitrella patens* (*P. patens*, moss) has recently emerged as a powerful model system, allowing fundamental questions of plant biology to be addressed (Prigge and Bezanilla, 2010). It was recently shown that this organism can be easily used as an efficient heterologous system to study protein interactions (Thevenin et al., 2012). This new approach takes advantage of the distinct but close phylogenetic relationship between moss and higher plants. High relationship between plant models could indeed be the source of false positive but conversely, it could also increase the robustness of the system by providing the necessary partners for suitable expression or stabilization of a given complex. Although some higher plant-specific developmental aspects cannot be directly studied in moss, cellular processes and signaling can be successfully dissected in this system, establishing this moss as the yeast equivalent for plant research (Thevenin et al., 2012).

Taking advantage of the establishment of this method in the group of Loïc Lepiniec (Thevenin et al., 2012). We investigated whether the bHLH–MYB–WDR (BMW) transcription factor complex can activate the promoter of laccases or not. This one hybrid-like plant system was successfully employed to

quantitatively assess the activity of BMW complex binding the promoters of proanthocyanidins biosynthesis genes and identify its target recognition sites on the promoters (Thevenin et al., 2012). Briefly, the method is based on the recognition of a target promoter fused to a reporter gene (green fluorescent protein GFP in this case) by one (or more) heterologous transcription partners that consequently act on the transcription of the reporter. The promoter activity can be assessed directly with flow cytometry quantification of the fluorescence signal emitted by GFP in cells.

A 2020 bp fragment in the upstream of *LAC4* coding sequence was cloned from Arabidopsis genome and fused with GFP. *LAC4* promoter activity was assessed directly with flow cytometry quantification of the GFP fluorescence signals. Protoplasts transfected with *proLAC4::GFP* alone displayed a low level of background fluorescence. This result suggests that a single member of MBW complex cannot activate the promoter of *AtLAC4* significantly. A tiny increase of the fluorescent signal was detected when *MYC4* and *MYB61* were separately transfected with *proLAC4-GFP*. Fortunately, when the MBW complex of MYB61–MYC4–TTG1 were transfected together, the promoter of *LAC4* was activated in *P. patens* protoplasts (Figure 3.2-17). When TTG1 was exchanged by LWD1 in the complex, the later still activated the promoter. We then tested whether this functional redundancy could be extended to the MYB transcription factors. When the *MYB61* was changed to MYB103, the complex did not work anymore or at least did not activate transcription of the GFP. This suggests the specificity of MYBs are very important for the function of MBW complex to activate the promoter of *LAC4*. The same strategy for analyzing the promoter of *AtLAC17* will be performed in the future.

3.2.11 Identification of the bHLH–MYB–WDR (BMW) complex which can activate the promoter of laccases in *Brachypodium*

Relatively few studies have identified TFs responsible for lignin biosynthesis in grasses. After a quick survey of databases, we found that most R2R3 MYBs that regulate secondary cell wall formation in Arabidopsis are likely conserved in grass genomes (data not shown and Handakumbura and Hazen, 2012). Based on our preliminary data on interaction between MYB, bHLH and WD40 in Arabidopsis we decided to investigate whether such mechanism could be present in Brachypodium.

3.2.12 *In silico* analysis of laccase promoter in Brachypodium

In silico promoter analysis of Brachypodium laccases (BdLAC5, BdLAC6 and BdLAC8, previously studied in the current PhD work and cited above) were carried out with PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) and PLACE (<http://www.dna.affrc.go.jp/PLACE/>) databases. Not unexpectedly, we were able to identify some known R2R3-MYB and bHLH binding motifs within 1 kb upstream of the coding sequences. We thus hypothesize there are similar regulation mechanism between Brachypodium and Arabidopsis.

3.2.13 *In silico* identification of MYB, bHLH and WD40 putatively involved in lignification of Brachypodium

We hypothesized that mechanisms of regulation of lignin biosynthesis genes (herein the laccase genes) are similar between Arabidopsis and Brachypodium. To test this hypothesis, we intended to find potential members of MBW complex involved in lignification of Brachypodium stems. First, we tried to find some orthologues of Arabidopsis TFs related to lignin biosynthesis in Brachypodium genome. The protein sequences of several MYBs, one bHLH (MYC4) and three WDR (TTG1, LWD1 and LWD2) in Arabidopsis were used to perform BlastP

search in PGSB database (<http://pgsb.helmholtz-muenchen.de/plant/search.jsp>) which include Brachypodium protein libraries (Nussbaumer et al., 2013). Some candidates were selected (Table 3.2-1). We thus consulted the Brachypodium transcriptomic database PlaNET (<http://aranet.mpimp-golm.mpg.de/>) to estimate their transcriptomic level in lignified tissues. Some genes (Bradi2g11080, Bradi3g49190, Bradi3g28830, Bradi3g56917, Bradi3g56917 and Bradi4g063 1 7) were then excluded because they showed very low level of expression or were found expressed ubiquitously. Some of them showing tremendous expression in non-lignified tissues were removed from our list of candidates. This screen resulted in a short list of genes herein Bradi3g52260 (the putative ortholog of AtMYB58), Bradi5g20130 (the putative ortholog of AtMYB63), Bradi2g36730 (the putative ortholog of AtMYB61 and AtMYB83), Bradi3g42430 (the putative ortholog of AtMYB85), Bradi2g1 7982 and Bradi2g47590 (the putative orthologues of AtMYB46). We used PLaNET as a tool to identify coexpressed genes with *BdLAC5*, a laccase gene known to be responsible for culm lignification in Brachypodium (Wang et al., 2015). Most of genes selected previously showed up herein Bradi2g47590, Bradi5g20130, Bradi3g42430 and Bradi1g10470 (a putative ortholog of MYB52 and MYB54, which have not been proved to positively regulate lignin biosynthesis). At last and based on the combination of sequence homology, expression patterns and coexpression analysis, we selected: Bradi2g47590 (the putative ortholog of AtMYB46), Bradi5g20130 (the putative ortholog of AtMYB63), Bradi3g42430 (the putative ortholog of AtMYB85) and Bradi2g36730 (the putative ortholog of AtMYB61 and AtMYB83) for further investigation in this study. Expression profiles from PlaNET are shown in (Figure 3.2-18).

We used the same strategy to select putative bHLH involved in lignification. MYC4 was used as bait to search gene candidate in Brachypodium. We selected only one ortholog of AtMYC4 (Bradi3g34200 named BdMYC4 in this study).

Likewise TTG1, LWD1 and LWD2 were used as baits to search putative WDR involved in lignification in *Brachypodium*. The best ortholog of TTG1, LWD1 and LWD2 is Bradi3g51820, (named BdWD1 in this study). According to PlaNET, *BdMYC4* (Bradi3g34200) is expressed in lignified organs, such as internode, node and peduncle, but lower in non-lignified organs such as leaf and endosperm. By contrast, *BdWD 1* is expressed ubiquitously, with similar level of transcription in lignified and non-lignified tissues (Figure 3 .2-18).

In conclusion we selected four MYBs (Bradi2g47590, Bradi5g20130, Bradi3g42430 and Bradi2g36730), one bHLH (Bradi3g34200) and one WDR (Bradi3g51820) for further investigations.

Table 3.2-1. Identification of Arabidopsis TFs' orthologues in *Brachypodium* with BlastP program in PGSB database

Arabidopsis TFs	Locus	Close orthologues in <i>Brachypodium</i>
AtMYB61	AT1G09540	Bradi2g36730, Bradi2g11080
AtMYB58	AT1G16490	Bradi3g49190, Bradi3g52260, Bradi3g28830
AtMYB63	AT1G79180	Bradi5g20130
AtMYB85	AT4G22680	Bradi3g42430, Bradi3g56917
AtMYB46	AT5G12870	Bradi4g06317, Bradi2g17982, Bradi2g47590
AtMYB83	AT3G08500	Bradi4g06317, Bradi2g36730
AtMYB103	AT1G63910	Bradi3g17165
AtMYB52	AT1G17950	Bradi1g10470
AtMYC4	AT4G17880	Bradi3g34200
AtTTG1	AT5G24520	Bradi3g51820, Bradi3g44580
AtLWD1	AT1G12910	Bradi3g44580, Bradi3g51820

3.2.14 MBW complex can activate *BdLAC5* promoter

To study the MBW complex interactions with laccase promoters in *Brachypodium*, transient expression system with *Physcomitrella patens* protoplasts was used in a similar manner than for *Arabidopsis* (see above). About 1 kb fragment in the upstream of coding sequences of *BdLAC5*, *BdLAC6* and *BdLAC8* respectively, were cloned from *Brachypodium* genome (Bd21-3) and fused with the GFP. All of candidate TFs, herein four *MYBs* (Bradi2g47590, Bradi5g20130, Bradi3g42430 and Bradi2g36730), one *bHLH* (Bradi3g34200) and one *WDR* (Bradi3g51820) were cloned from the reverse transcripts of mRNA isolated from internodes of *Brachypodium* plants at early flowering stage and integrated into plant expression vector under the control of Maize ubiquitin promoter (see Materials and Methods). *BdLAC5* promoter activity was assessed directly with flow cytometry quantification of the GFP fluorescence signals (Figure 3.2-19).

Protoplasts transfected with *proBdLAC5::GFP* alone displayed a low level of background fluorescence. As previously observed in *Arabidopsis*, a single member of MBW complex cannot activate the promoter of laccase in *Physcomitrella*. No significant increase of fluorescent signal was detected when BdMYC4 and BdMYB58 (Bradi2g47590) were separately transfected with *proBdLAC5::GFP*. Fortunately, when the MBW complex of BdMYC4 and BdWD1 and BdMYB58 (Bradi2g47590 or Bradi2g36730) were transfected together, the promoter of *BdLAC5* was activated in *Physcomitrella* protoplasts (Figure 3.2-19). However, in our hands, the BdMYBs Bradi5g20130 and Bradi3g42430 did not activate the transient expression of GFP. Surprisingly, the promoters of *BdLAC6* and 8 were not activated by the complex of BdMYC4, BdWD1 and BdMYB (Bradi2g47590 or Bradi2g36730) suggesting that other specific MYB or bHLH could be involved in the interaction with these laccase promoters.

3.2.15 identification of TILLING mutants of BdMY58 (Bradi2g47590), BdMYC4 and BdWD1

In order to assess the function of the MBW complex *in vivo* we identified TILLING mutants so far for *BdMYB58* (Bradi2g47590), *BdMYC4* (Bradi3g34200) and *BdWD1* (Bradi3g51820). Most of these lines share missense mutations which cause substitutions in the amino acid sequence or stop codons (Table 3.2-2). The analysis using the SIFT software (using 0.05 as significant threshold) indicated that the substitutions caused by the mutations in lines *Bd3701* may partially or totally disrupt the BdWD1 protein activity. Likewise, the substitutions caused by the mutations in lines *Bd5615* and *Bd5361* may also partially or totally disrupt the BdMYC4 protein function. At last, stop codon mutation in lines *Bd5615* and *Bd5361* were identified in *BdMYB38* (Bradi2g47590). These mutants will be characterized for lignin phenotypes in the future.

Table 3.2-2. TILLING mutants of BdWD1 (Bradi3g51820), BdMYC4 (Bradi3g34200) and BdMYB38 (Bradi2g47590).

Genotype	Nucleic acid transition ^a	Amino acid substitution ^b	Type of Mutation	Family	SIFT Score ^c	Prediction
BdWD1 (Bradi3g51820)	C229T	L77F	Missense	3453	0.43	Tolerated
	C275T	P92L	Missense	3701	0	Damaging
	C388T	L130F	Missense	8763	0.08	Tolerated
BdMYC4 (Bradi3g34200)	G1597A	A533T	Missense	5615	0	Damaging

	G1681A	A561T	Missense	5361	0	Damaging
BdMYB (Bradi2g47590)	G57A	W19*	STOP	8678	-	Damaging
	G110A	G37A	Missense	7480	0.03	Damaging
	G161A	S54N	Missense	3983	0	Damaging
	G272A	W91*	STOP	6101	-	Damaging
	C395T	S132F	Missense	7399	0.02	Damaging
	G700A	G234R	Missense	3487	0	Damaging

a: Position of transition in mutants are relative to the starting ATG on the coding sequence.

b: Position of substitution in the encoded protein. Mutants are relative to the starting methionine of the encoded protein. c: numbers are predictive score from the SIFT software (<http://sift.bii.a-star.edu.sg/>).

3.3 Materials and Methods

Plant material and growth conditions

Arabidopsis seeds were cold-treated for 3 days to synchronize the germination. Plants were grown in soil under long day conditions (16 h light / 8 h dark), unless otherwise indicated.

For in vitro culture, seeds were liquid sterilized with 10% commercial bleach and 0.1% Triton X-100 solution for 10 min, and then rinsed five times with autoclaved deionized water. Seeds were sown in petri dishes on sterile Murashige and Skoog (MS) 1% agarose medium (supplemented with 2% sucrose, and then cold stratified for 3 days. Petri plates were then placed in a culture chamber under 16 h of light ($150 \mu\text{mol m}^{-2} \text{s}^{-1}$) and 8 h of dark, with temperatures of 22°C. Seedling age was counted starting from the light exposure.

Arabidopsis thaliana (L.) Heynh accessions Columbia (col-0) and Wassilewskija (ws) were used as wild type. The T-DNA insertion lines SALK_006874 (*lwd1*) and GK-580A05-021290 (*ttg1*), were obtained from the

Arabidopsis Biological Resource Center. Homozygous T-DNA lines were obtained by PCR screening using the left border T-DNA primer and a right border gene-specific primer. The insertion site in these mutants are localized in the exon. The homozygous T-DNA insertion mutant of *ttg1* and *lwd1* were confirmed by RT-PCR (Figure 3.2-16A). The *myb61* mutants (col background) were generously provided by Dr. Christian Dubos (INRA) and described by (Newman et al., 2004). *TTG1* promoter: GUS lines were kindly provide by Dr. Loic Lepienic and described by (Baudry et al., 2004). The seeds of *myc234* (col background) were kindly provided by Dr. Philippe Reymond and described by (P Fernández-Calvo - 2011).

MeJA treatment

After melting the sterile Murashige and Skoog (MS) 1% agarose medium (supplemented with 2% sucrose), wait until the liquid medium has cooled down to 40~50°C. Then add MeJA (final concentration 50 µM, Sigma-Aldrich) into the medium. The final concentration (50 µM) of MeJA is chosen according to (Kim HJ et al 2014), without severely negative effects for plants development.

RNA extraction and quantitative RT-PCR

Total RNA extraction, reverse transcription reaction and quantitative RT-PCR analysis were performed as previously described (Wang et al 2015). Arabidopsis Actin 2 (At3g18780) was used as the reference gene. Information of primers are available in (Table 3.3-1), PCR products were verified by sequencing. The normalized expression levels of target genes were calculated by Q-Gene software (<http://www.biotechniques.com/softlib/qgene.html>) based on the formula with amplification efficiency of PCR reaction (Muller et al., 2002). The results included three independent biological replicates.

Genetic transformation

The full-length CDS of target genes was cloned from *Arabidopsis* or *Brachypodium* cDNA producing from stem. Information of the specific primer are available in (Table 3.3-2). The purified PCR product was ligated into the pDONR207 vector via BP Gateway (Invitrogen) reaction and confirmed by sequencing. Then the target gene cassette in the Gateway Entry clone was transferred into Gateway compatible plant expression vector by LR Gateway (Invitrogen) reaction. For *Arabidopsis* genes, pGWB2 (with the Cauliflower Mosaic Virus 35S promoter) was used, for *Brachypodium* pIPKB2 (with the Maize ubiquitin promoter) was used. The constructions can be used for both of transient expression in moss protoplasts and stable transformation in plant. For stable transformation in *Arabidopsis*, *Agrobacterium tumefaciens* (C58 strains) was used. Transgenic plants were selected on Murashige and Skoog medium containing 50 mg/ml hygromycin. At least 12 independent transformed lines were obtained, for each construct.

Histochemical analysis of GUS expression

The sections of transgenic plants expressing promoter:GUS were incubated in GUS staining solution containing 1 mM 5-bromo-4-chloro-3-indoxyl-b-D-glucuronide (X-Gluc) and 0.1 M Na₂HPO₄–NaH₂PO₄ buffer (pH 7.0) at 37°C overnight, then followed by washing in 70% ethanol and observed under microscopy.

Histochemical staining

All the histochemical staining were performed on hand-cut sections of the primary shoot. Samples were fixed in the FAA (50% ethanol, 10% formalin, 5% Acetic Acid) solution overnight. Lignin deposition were investigated by Wiesner or Mañile staining according to standard protocols as previously published (Wang et al 2015). All sections were observed under Zeiss AxioPlan 2 microscope system with automatic exposure time.

Lignin content and structure determination

The primary shoot of each plant was collected after removing leaves and inflorescence. Ground samples were sequentially extracted at 60 °C with 50ml (~10X volume of samples) of ethanol, water and ethanol. At each step the samples were vortexed. These steps were repeated twice. Samples were thus dried and used for the following analysis.

The protocol of thioglycolic acid lignin assay was described as previous (Wang et al 2015). Extractive-free cell wall residues of stem were subjected to thioglycolic acid (TGA). The calibration curves were made by lignin standard purified from the same materials with the tested samples. The relative lignin concentration was determined with the amount of lignin calculated from the calibration curve divided by the sample weight.

The lignin structure and composition were studied by thioacidolysis, as previously described (Lapierre et al., 1999). The lignin-derived thioacidolysis monomers were identified by gas chromatography–mass spectrometry as their trimethylsilylated derivatives.

Relative quantitation of anthocyanins

Relative quantitation of anthocyanins was performed as described by Ronchi et al. (1997) with minor modifications. In brief, 5 seedlings grown consistently were selected randomly and removed the germinated seeds. Immersed the whole plantlets in 500 ul HCl 1% (v/v) in methanol for 4 hours at room temperature, then all the red colors were removed from plantlets. Add 330 ul distilled water, vortex and wait for 1 hour at room temperature. 600 ul of chloroform was added to remove chlorophylls through mixing and centrifugation (10 min at 10,000g). Take 200 ul of the supernatant aqueous phase, which contains anthocyanins and the absorption at 535nm was determined spectrophotometrically (A₅₃₅). Relative anthocyanin content were calculated per seedling of (A₅₃₅).

Promoter cloning

All the cloning PCR products were obtained using high-fidelity Phusion DNA polymerase with the HF buffer (Thermo Scientific-Finnzymes, Waltham, MA, USA). For cloning the promoters of laccases *LAC4* in *Arabidopsis* (*col-0*), 2020 bp upstream of ATG (including the 5' UTR) was amplified with specific primers (Table 3.3-2). For cloning the promoters of laccases, *BdLAC5*, *BdLAC6* and *BdLAC8* in *Brachypodium* (*Bd21-3*), 1 kb upstream of ATG (including the 5' UTR) were amplified respectively using specific primers (Table 3.3-2). PCR products were cloned into pDONR207. Then the Entry clones were recombined into the pBS Tpp-B GFP vector (Thevenin et al., 2012) by LR Gateway (Invitrogen) reaction. The integrity of the constructs was checked by sequencing.

Coding sequence (CDS) cloning and expression

The CDS of *MYB61*, *MYB103*, *MYC4*, *TTG1* and *LWD1* were amplified from cDNA of *Arabidopsis* (*col-0*) mature stem. The fragments obtained were then recombined first into the pDONR207 vector and subsequently into pGWB2 expression vector.

Transient expression assays in *Physcomitrella patens* protoplasts

The Gransden wild-type strain of *P. patens* was used in this study. Moss culture, protoplast preparation, protoplast transformation and flow cytometry measurement analysis were carried out as previously described (Thevenin et al., 2012). Flow cytometry of live protoplasts expressing GFP was performed on a PARTEC CyFlow Space instrument (Partec S.A.R.L., France), using FLOMAX (Partec S.A.R.L., France) acquisition and analysis software, and a 488-nm solid sapphire 20-mW laser for excitation. GFP fluorescence was detected with an FITC 527 nm / 30 nm band-pass filter (FL1 channel). Red chlorophyll-based

fluorescence from living protoplasts was detected with a 610 nm / 30 nm band-pass filter in the FL2 channel.

The weighted GFP fluorescence per population of cells was calculated as the product of the average fluorescence intensity of the population of cells above the background threshold. This threshold was set based on no DNA transformed control compared with a positive control. Each experiment was repeated at least three times.

Statistical analysis

All the analyses were performed with at least three biological replicates. Significant differences were analyzed by one-way ANOVA (Tukey's HSD, $P < 0.05$).

Table 3.3-1. Information of used primers

Gene Locus	Primer Names	Sequence	Length	Usage
upstream of At2g38080	GWproLac4R	GGGGACCACTTTGTAC AAGAAAGCTGGGTCTC CCTCTCTATCTTTCTCT TC	2020 bp	cloning of promoter
	GWproLac4F	GGGGACAAGTTTGTAC AAAAAAGCAGGCTTGC CAAATAAAGCGACCAC ATT		
	GWproLac17F	GGGGACAAGTTTGTAC AAAAAAGCAGGCTTCC	2000 bp	

		ACTTTACTACGTAGTAA TTG		
	GWproLac17R	GGGGACCACTTTGTAC AAGAAAGCTGGGTTGA AGTGAGCTTGGACACG GAG		
At4g17880	GWATMYC4-R	GGGACCACTTTGTACA AGAAAGCTGGGTCGCT CATGGACATTCTCCAA C	1770 bp	
	GWATMYC4-F	GGGGACAAGTTTGTAC AAAAAAGCAGGCTTCA TGTCTCCGACGAATGTT C		
At1g09540	GWMyb61CDS- R	GGGGACCACTTTGTAC AAGAAAGCTGGGTCCT AAAGGGACTGACCAAA	1101 bp	
	GWMyb61CDS- F	GGGGACAAGTTTGTAC AAAAAAGCAGGCTTCA TGGGGAGACATTCTTG C		
At1g63910	cMYB103-GW-R	GGGGACCACTTTGTAC AAGAAAGCTGGGTCTT AAAACGAAGAAGGGA AAGAAG	1113 bp	
	cMYB103-GW-F	GGGGACAAGTTTGTAC AAAAAAGCAGGCTTCA TGGGTCATCACTCATGC TGC		
At1g12910	cLWD1-GW-R	GGGGACCACTTTGTAC AAGAAAGCTGGGTCTC AAACCCTGAGAATTG CAGC	1041 bp	

Cloning of the full length of CDS

	cLWD1-GW-F	GGGGACAAGTTTGTAC AAAAAAGCAGGCTTCA TGGGAACGAGCAGCGA TCCG		
At5g24520	cTTG1-FullB1	GGGGACAAGTTTGTAC AAAAAAGCAGGCTTCA TGGATAATTCAGCTCC AGA	1026 bp	qRT-PCR
	cTTG1-FullB2	GGGGACCACTTTGTAC AAGAAAGCTGGGTCTC AAACTCTAAGGAGCTG CATT		
At5g60020	QAtLAC17-R	GAAAACAGAGCTGTGC AACAGTA	262 bp	
	QAtLAC17-F	TGGCTTGTTCTTGATGG AGATAA		
At2g38080	QAtLAC4-R	ATCCACGCATTAAATC ACCACT	224 bp	
	QAtLAC4-F	GCAGATAATCCCGGGG TTTGGT		
At1g15950	QCCR1R	ACTTAAGGGGGATACT TGAGGGAATTAATAA	149 bp	
	QCCR1F	GCCTCTACGACACAGT CAAGAGCTTACAA		
At3g18780	QAtACT2-R	CAATGGGACTAAAACG CAAAACG	247 bp	
	QAtACT2-F	GGTCCAGGAATCGTTC ACAGAAA		

Table 3.3-2. Information of used primers

Gene Locus	Primer Names	Sequence	Length	Usage
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Bradi5g20130	GWbMY B20130R	GGGGACCACTTTGTACAAG AAAGCTGGGTCGCTCACAA ATCCATCCGGTTATC	1098 bp	Cloning of the full length of CDS
	GWbMY B20130F	GGGGACAAGTTTGTACAAA AAAGCAGGCTTCATGGGGA AAGGCCGGGCACCG		
Bradi3g51820	GWbWD5 1820F	GGGGACAAGTTTGTACAAA AAAGCAGGCTTCATGGACC AGCCGCAGCCGCAG	1056 bp	
	GWbWD5 1820R	GGGGACCACTTTGTACAAG AAAGCTGGGTCGCTCAGAC CCTGAGAAGTTGAAC		
Bradi3g34200	GWbMY C34200F	GGGGACAAGTTTGTACAAA AAAGCAGGCTTCATGAACC TGTGGACGGACGAC	2121 bp	
	GWbMY C34200R	GGGGACCACTTTGTACAAG AAAGCTGGGTCGCTTACCG GATTGGCACTGGGGC		
Bradi3g42430	GWb3g42 430-F	GGGGACAAGTTTGTACAAA AAAGCAGGCTATGGGGAG GCAGCCGTGCTGTG	807 bp	
	GWb3g42 430-R	GGGGACCACTTTGTACAAG AAAGCTGGGTCCTAAAATT TTGATTCTTCCGAG		
Bradi2g47590	GW2g4759 0-F	GGGGACAAGTTTGTACAAA AAAGCAGGCTATGGGGCGG CACGCGGGCACTGGCG	852 bp	
	GW2g4759 0-R	GGGGACCACTTTGTACAAG AAAGCTGGGTCTCAAAAGT ACTCGAGGTTGAAGTCA		
Bradi2g36730	GW2g3673 0-F	GGGGACAAGTTTGTACAAA AAAGCAGGCTATGGGGAG GCATTCTTGTTGCTACA	1284 bp	

	GW2g3673 0-R	GGGGACCACTTTGTACAAG AAAGCTGGGTCCTACATAT GCTCAAAAGACAAGGGC		
upstream of Bradi1g66720	GWPROLA C5-F	GGGGACAAGTTTGTACAAA AAAGCAGGCTCTGCCTTCT GCATCGTTTTTC	1000 bp	cloning of promoter
	GWPROLA C5-R	GGGGACCACTTTGTACAAG AAAGCTGGGTCCTACAATA CGAGAGAGGACA		
upstream of Bradi1g74320	GWPROLA C6-F	GGGGACAAGTTTGTACAAA AAAGCAGGCTCTCGAGAAA GAATCGTGGTA	1000 bp	
	GWPROLA C6-R	GGGGACCACTTTGTACAAG AAAGCTGGGTCTTTGCTTC ACACAGATTAGGT		
upstream of Bradi2g23370	GWproBdla c8R	GGGGACCACTTTGTACAAG AAAGCTGGGTCTTTATGCA AGTCTTC	1000 bp	
	GWproBdla c8F	GGGGACAAGTTTGTACAAA AAAGCAGGCTTCAAAGGCT AAATAGTTT		

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Figure 3.2-19. Transient expression of TFs and *BdLAC5/6/8* promoter-GFP in *P. patens* protoplasts.

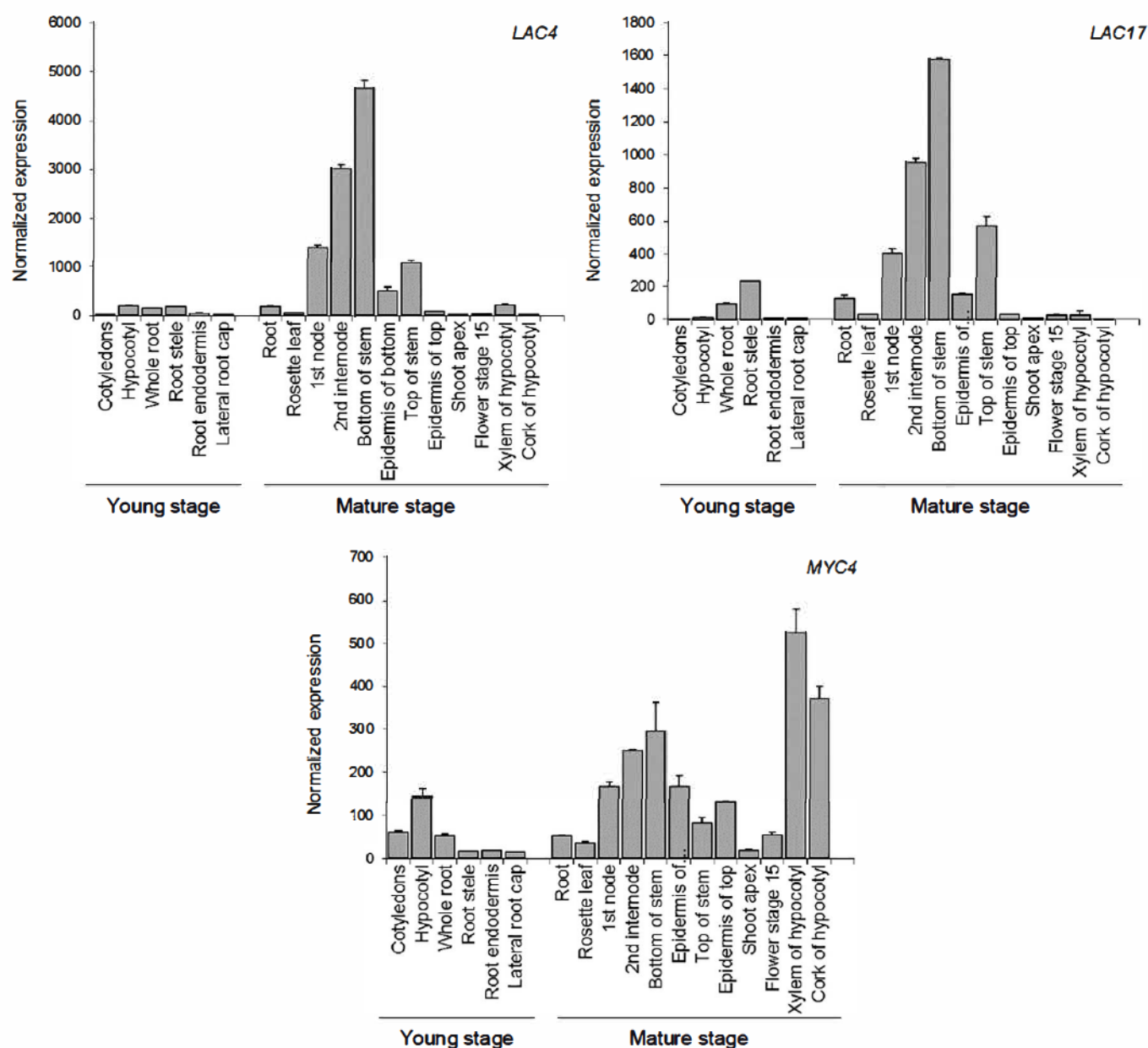
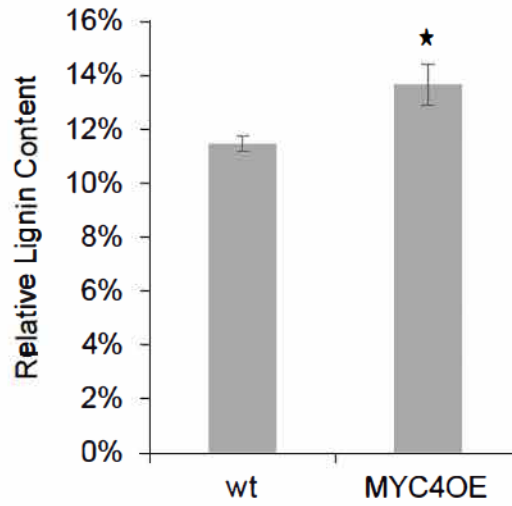


Figure 3.2-1. The expression patterns of the lignin specific laccases (*LAC4* and *LAC17*) and *MYC4*.

Data are extracted from eFP-Browser and normalized by GCOS method, TGT value of 100.

A



B



Figure 3.2-2. Lignin content and Phloroglucinol-HCl staining in the basal stem of *AtMYC4* overexpressors (*ws* background).

A: Relative lignin content was determined by the TGA method. Plants were grown under the long day condition (18h light/ 6h dark). Data were produced from three biological repeats of WT (*ws*) and three independent transformed lines of *MYC4* overexpressors (*MYC4OE*, *ws* background).

B: Tissues of mature bottom stems (6-weeks-old) stained with phloroglucinol-HCl reagent.

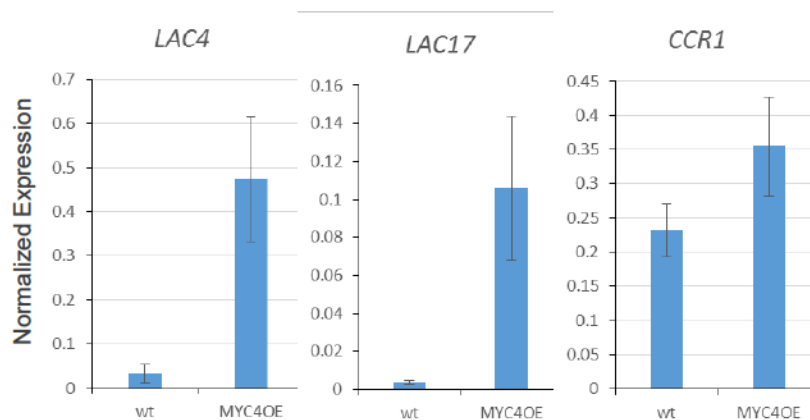


Figure 3.2-3 Expression levels of *LAC4*, *LAC17* and *CCR1* in *MYC4* overexpressors.

Data came from three biological repeats of wt and three transformed lines of *MYC4* overexpressors (MYC4OE). All data represent the mean with the standard deviation (error bars) from three biological replicates.

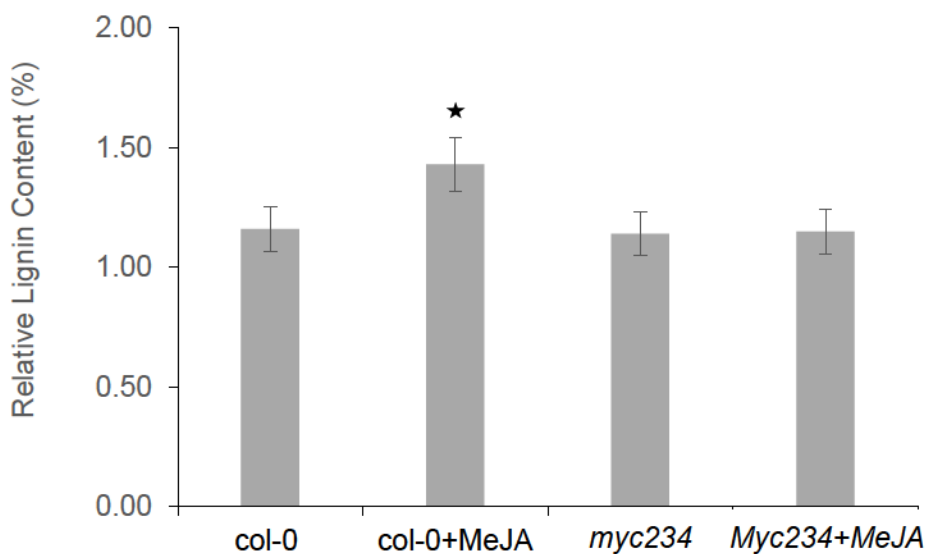
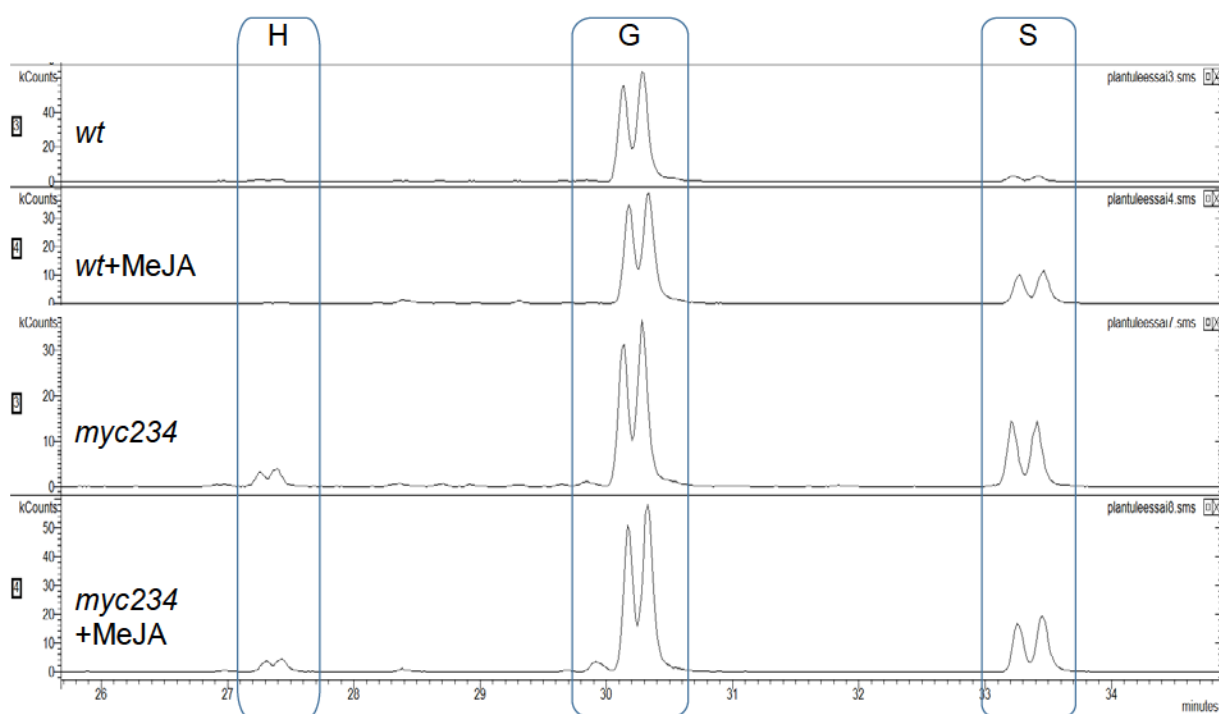


Figure 3.2-4. Lignin content in WT (*col-0*) and *myc234* seedlings treated with MeJA treatment.

Arabidopsis seedlings were grown in MS medium with or without MeJA (50 μ M) for 12 days. The relative lignin content was determined with the thioglycolic acid method. About 30 seedlings were pooled in one sample, standard deviations were calculated from two technical repeats. Stars indicate significant differences analyzed by Student's t-test ($P < 0.05$).

A



B

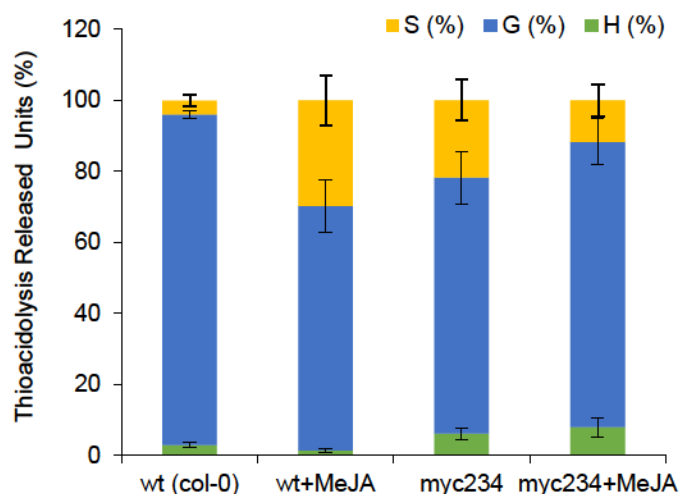
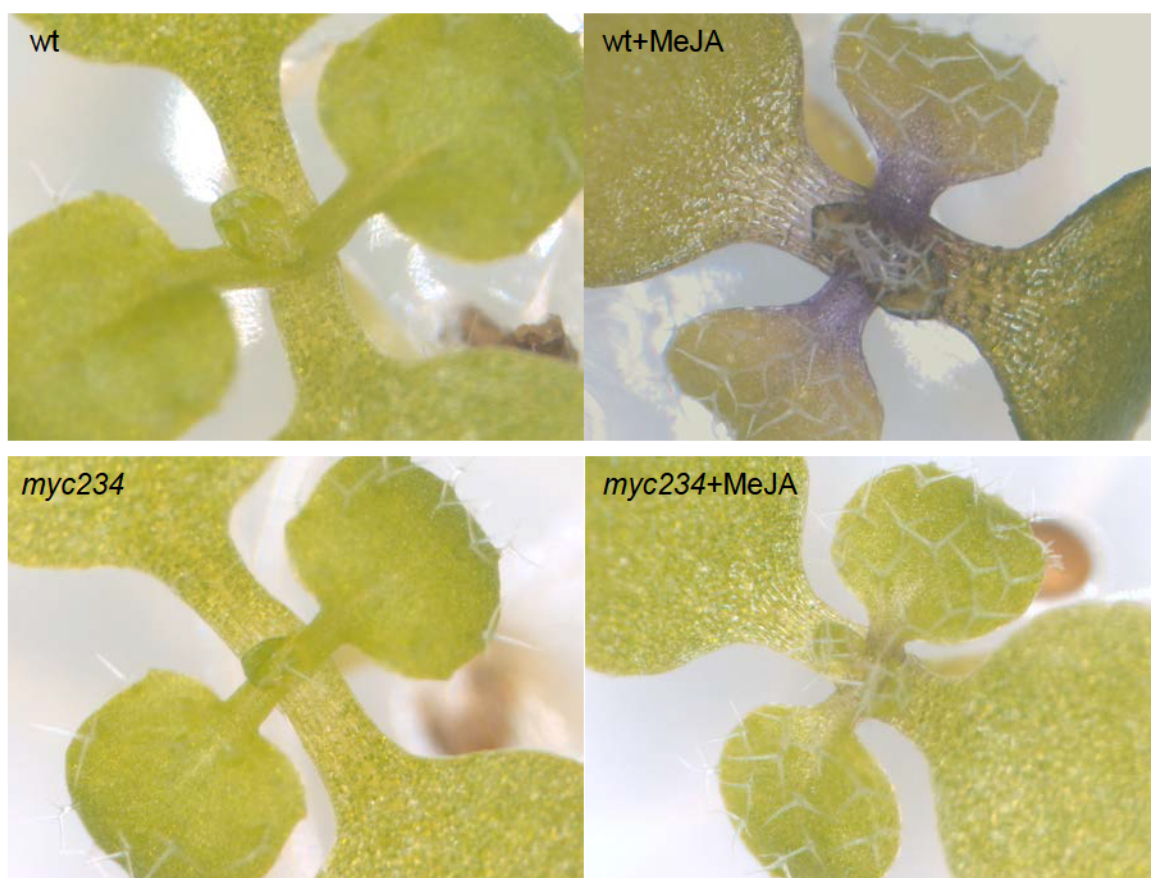


Figure 3.2-5 Lignin structure in wt and *myc234* treated with MeJA.

The β -O-4 substructures of lignins were analyzed by gas chromatography mass spectrometry (GC-MS) of the thioacidolysis products. The seedlings of *myc234* and wt were grown in the medium with MeJA (50 μ M) or mock (control) treatments. A: GC-MS spectra show the peaks of H, G and S units identified after by thioacidolysis; B: determination of β -O-4 linked H, G and S units in lignin polymers. All data represent the mean with the standard deviation (error bars) from three biological replicates.

A



B

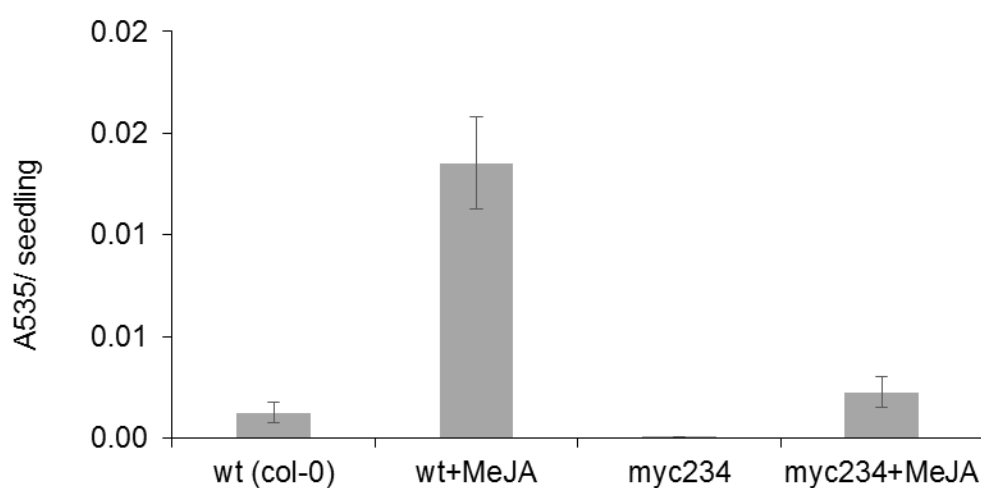


Figure 3.2-6. Anthocyanin content in *myc234* and WT seedlings treated with MeJA.

A: Phenotypes of plants treated with MeJA (50 μ M). B: relative anthocyanin content in seedlings. Units of Y axis represent absorbance at 535 nm of samples treated with or without MeJA (Ronchi et al., 1997). All data represent the mean with the standard deviation (error bars) from three biological replicates.

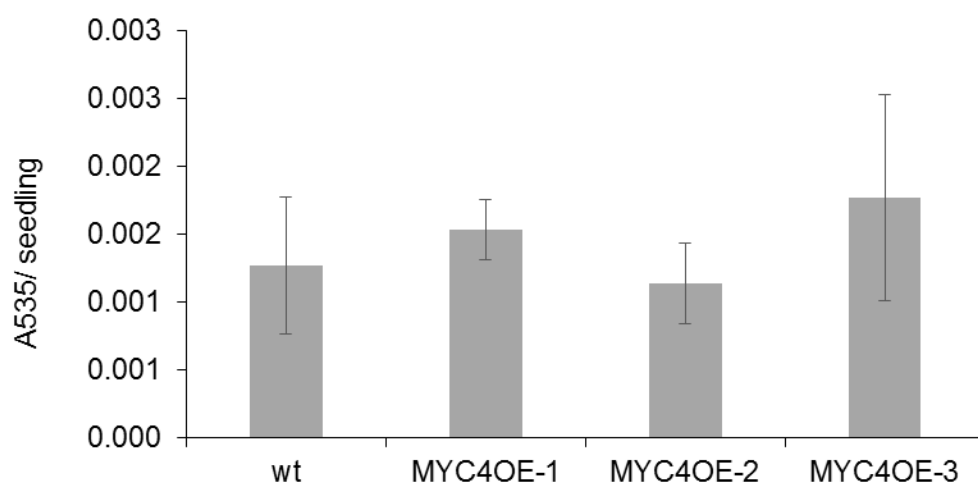


Figure 3.2-7. Anthocyanin content in *myc4* overexpressors and WT seedlings treated with MeJA.

Units of Y axis represent absorbance at 535 nm of samples treated with or without JA (Ronchi et al., 1997). All data represent the mean with the standard deviation (error bars) from three biological replicates.

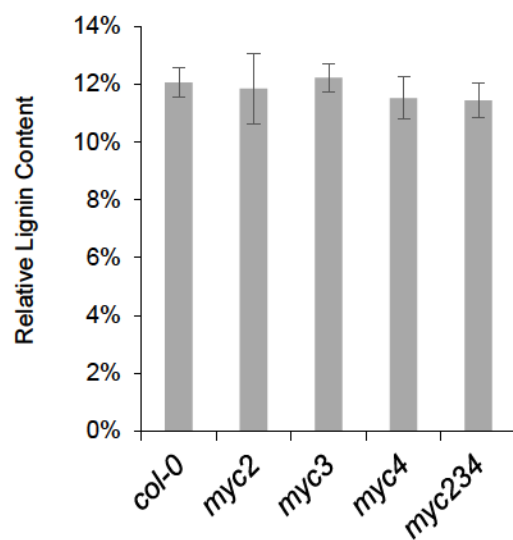


Figure 3.2-8. Relative lignin content in mature stems of *myc2*, *myc3*, *myc4*, *myc234* and WT.

Relative lignin content was determined by TGA method. Data included three biological repeats.

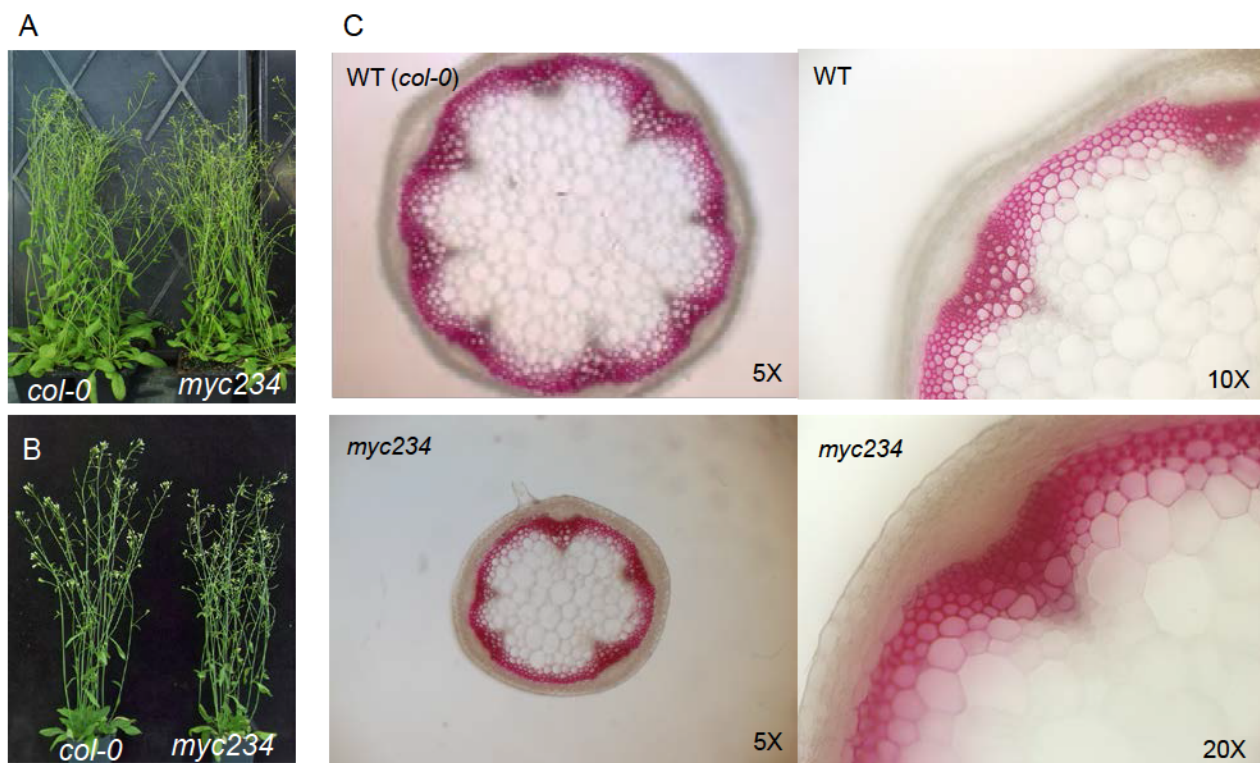


Figure 3.2-9. Phenotypes and lignin patterning in *myc234* and *wt*.

A: phenotype of mature plants grown under long day condition (16h light/ 8h dark).

B: phenotype of mature plants grown under longer day condition (18h light/ 6h dark).

C: Wiesner staining of transverse sections at the bottom of mature stems in *myc234* and WT (*col-0*).

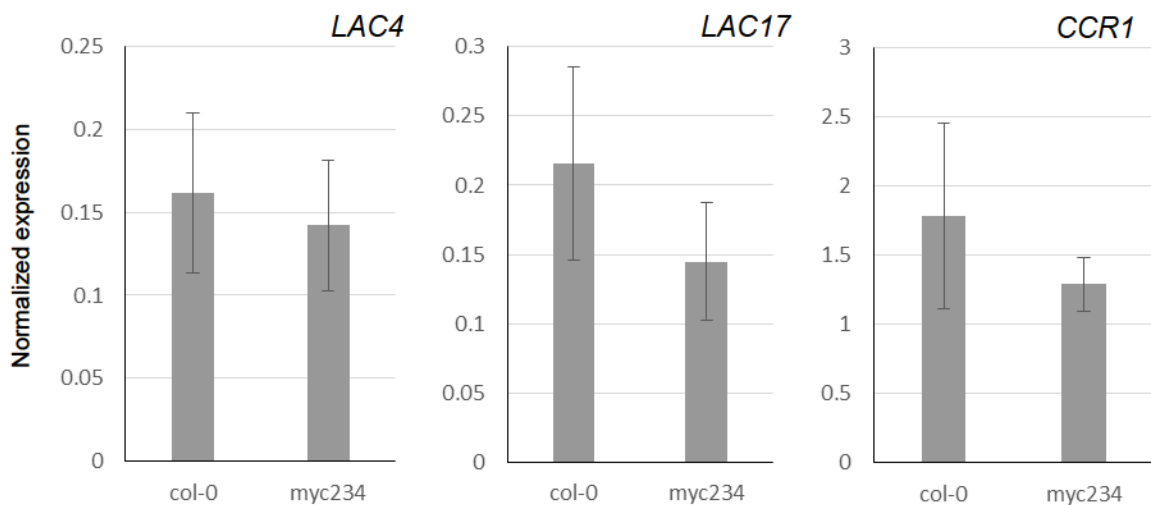


Figure 3.2-10. The transcription levels of *LAC4*, *LAC17* and *CCR1* in mature stems of *myc234* and WT.

Bottom stems (6 cm) of mature plants were used for qRT-PCR. Plants were grown under long day condition (16h light/ 8h dark) during 6 weeks. All data represent the mean with the standard deviation (error bars) from three biological replicates.

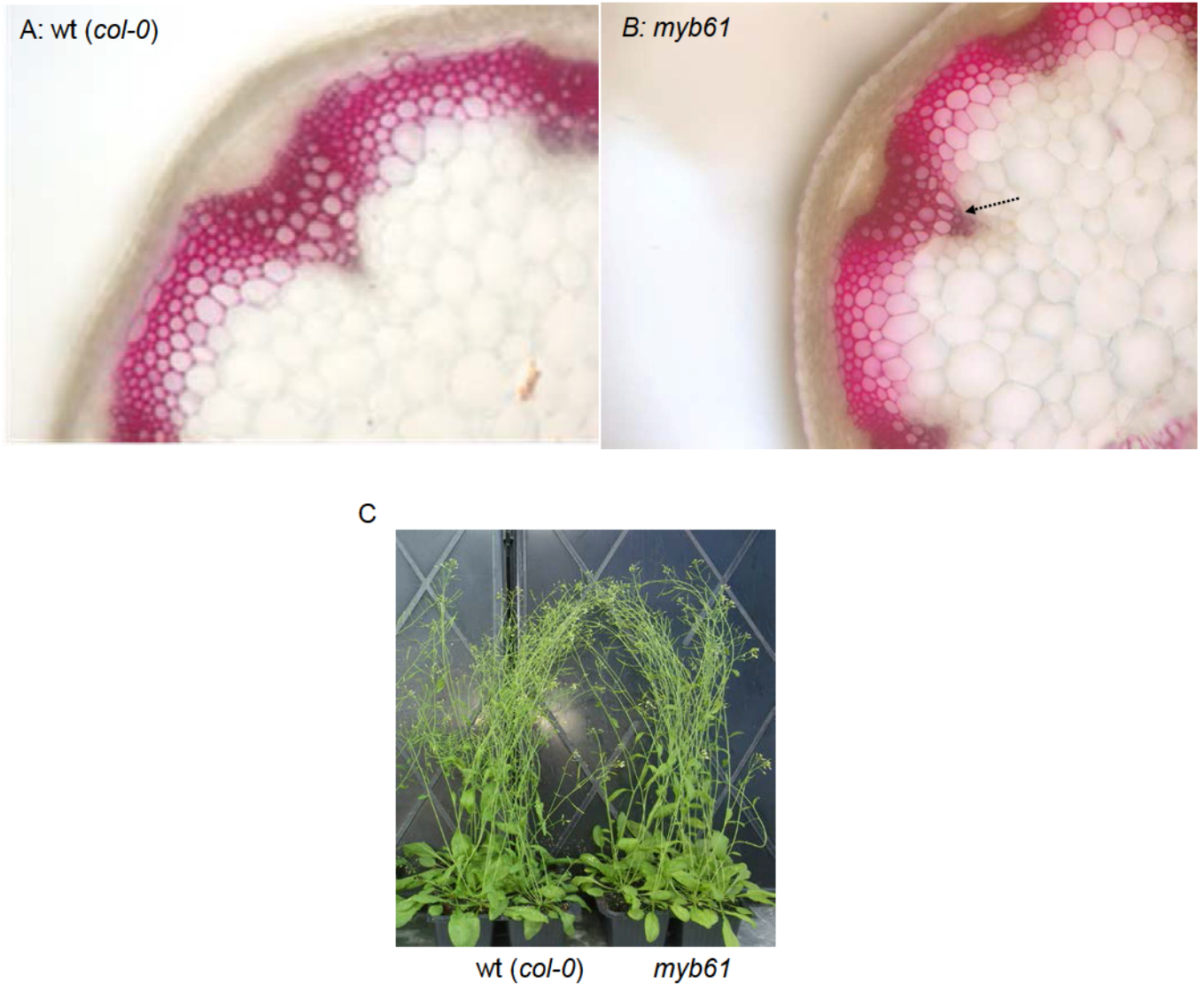


Figure 3.2-11. Phenotypes of *myb61*.

Shape of xylem vessels is irregular (arrows) in *myb61* basal stem (Wiesner staining). Plants grew under long day condition (16 h light/ 8 h dark). A: wt (*col-0*, 42-days-old); B: *myb61* knock-out mutant (42-days-old). C: The *myb61* mutants have normal phenotypes indistinguishable from the wt.

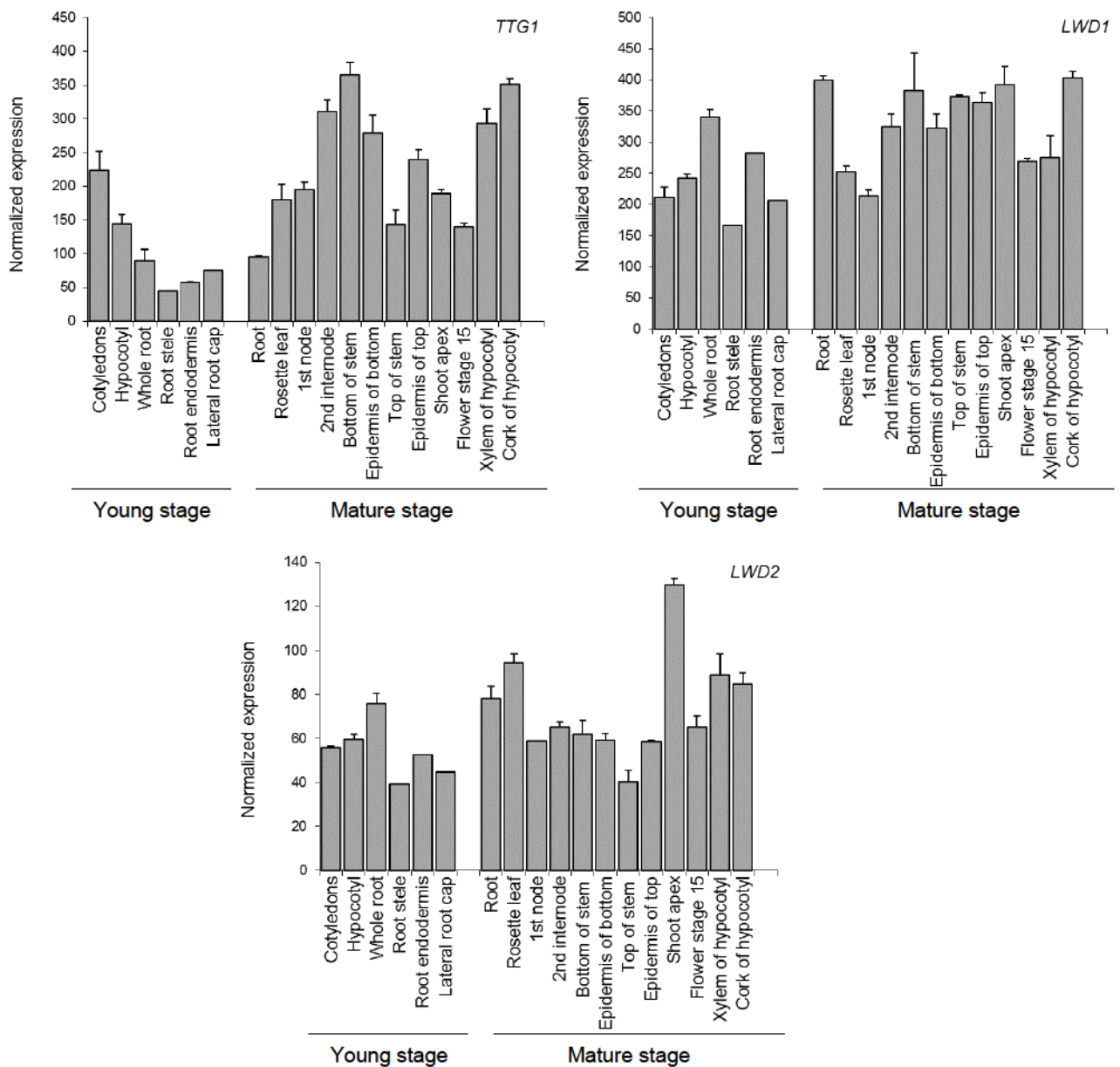


Figure 3.2-12. Expression of *TTG1*, *LWD1* and *LWD2* genes in various organs at different development stages.

Data are extracted from eFP-Browser, and normalized by GCOS method, TGT value of 100.

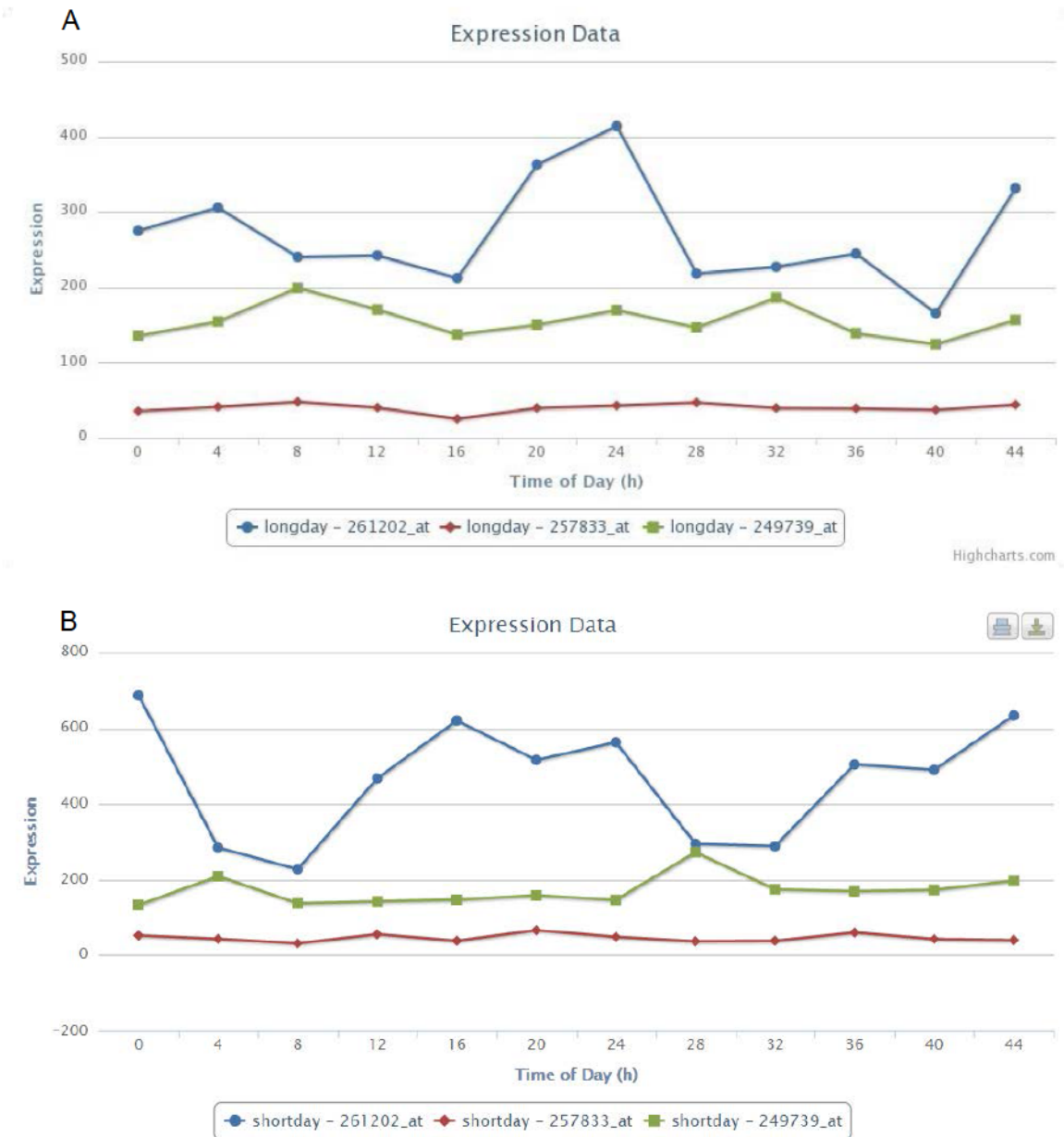
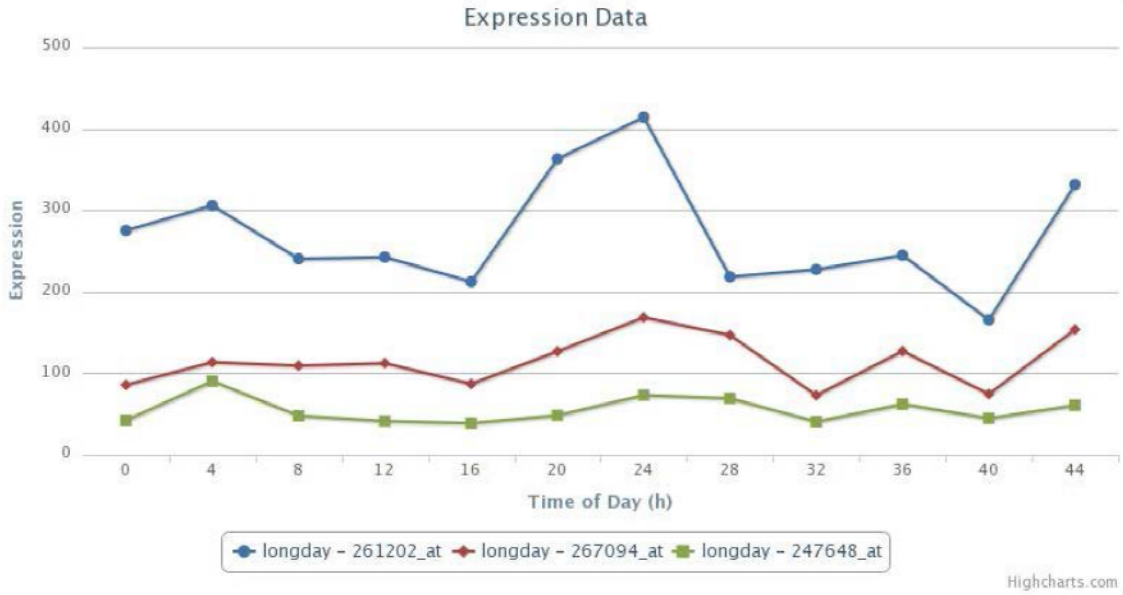


Figure 3.2-13. Circadian expression patterns of *WDR* genes

A: short day condition (8h light/ 16h dark) ; B: long day condition (16h light/ 8h dark).

Blue line is *LWD1*, red line is *LWD2* and green line is *TTG1*.

A



B



Figure 3.2-14. Circadian expression of *LAC4*, *LAC17* and *LWD1* under long day and short day conditions.

A: short day condition (8h light/ 16h dark); B: long day condition (16h light/ 8h dark). Blue line is *LWD1*; red line is *LAC4* and green line is *LAC17*.

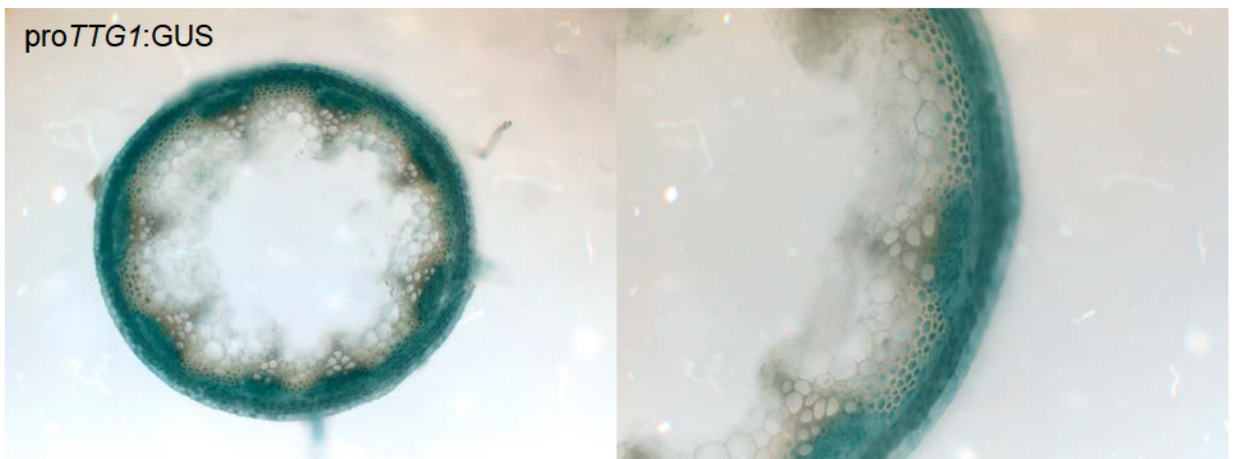


Figure 3.2-15. GUS staining of transformed lines with promoter of *TTG1* fused with GUS.

TTG1 promoter:GUS lines (Baudry et al., 2004) were grown under long day condition for 5 weeks, the transverse sections from the bottom of primary shoot were used for GUS staining. The staining is found in the epidermis, cortex, starch sheath, sclerenchyma of interfascicular fibers, cambium and phloem in vascular bundles but not in xylem and parenchyma cells. Left: the picture was taken under 5x objective. Right: the pictures was taken under 10x objective.

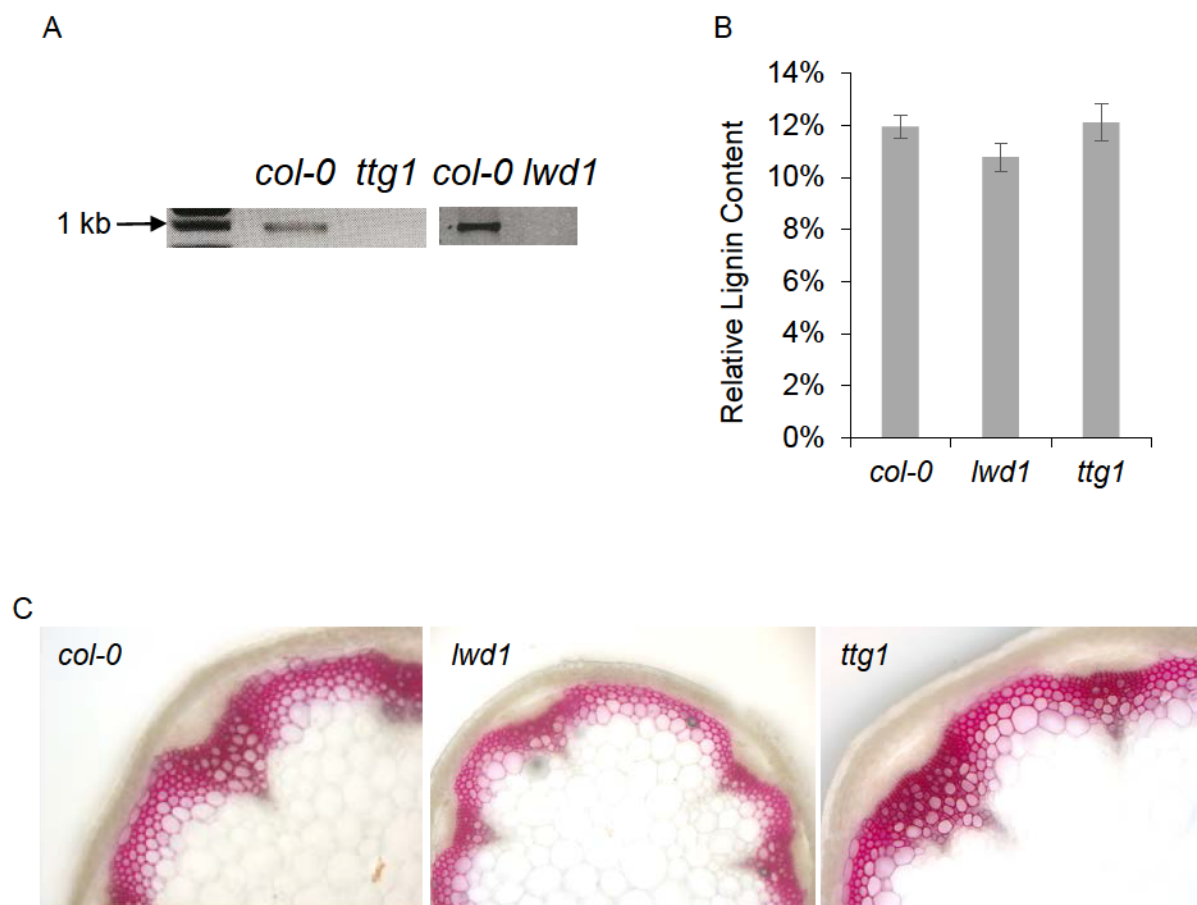


Figure 3.2-16. Lignification patterning in *ttg1* and *lwd1* single mutants

A: The homozygous T-DNA insertion mutants of *TTG1* (GK-580A05-021290) and *LWD1* (SALK_006874C) were identified and confirmed by RT-PCR with specific primers.

B: TGA lignin content in *ttg1* and *lwd1* single mutants.

C: Lignin patterning in the bottom of mature stems (6-weeks-old) was characterized by Wiesner staining.

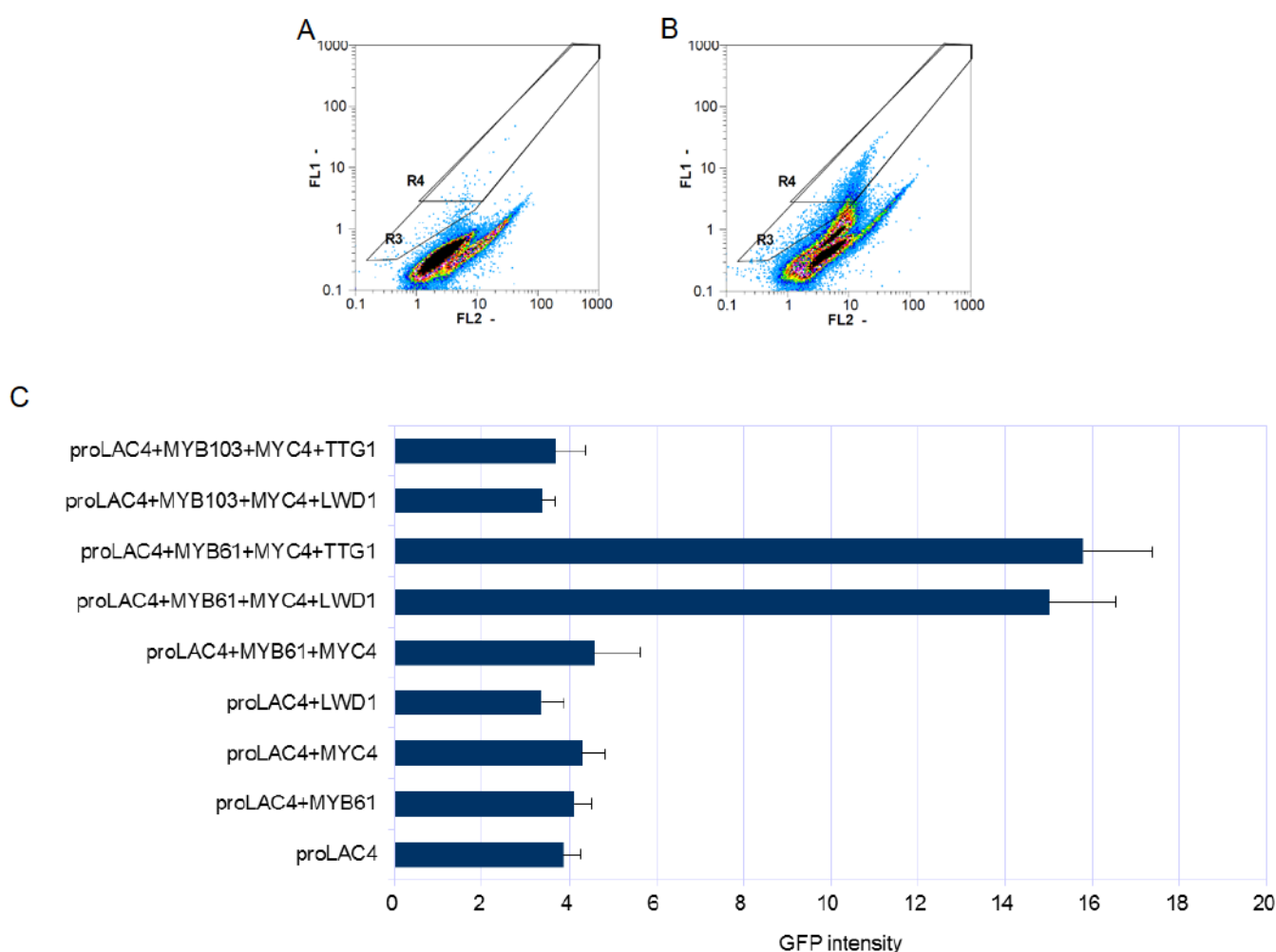
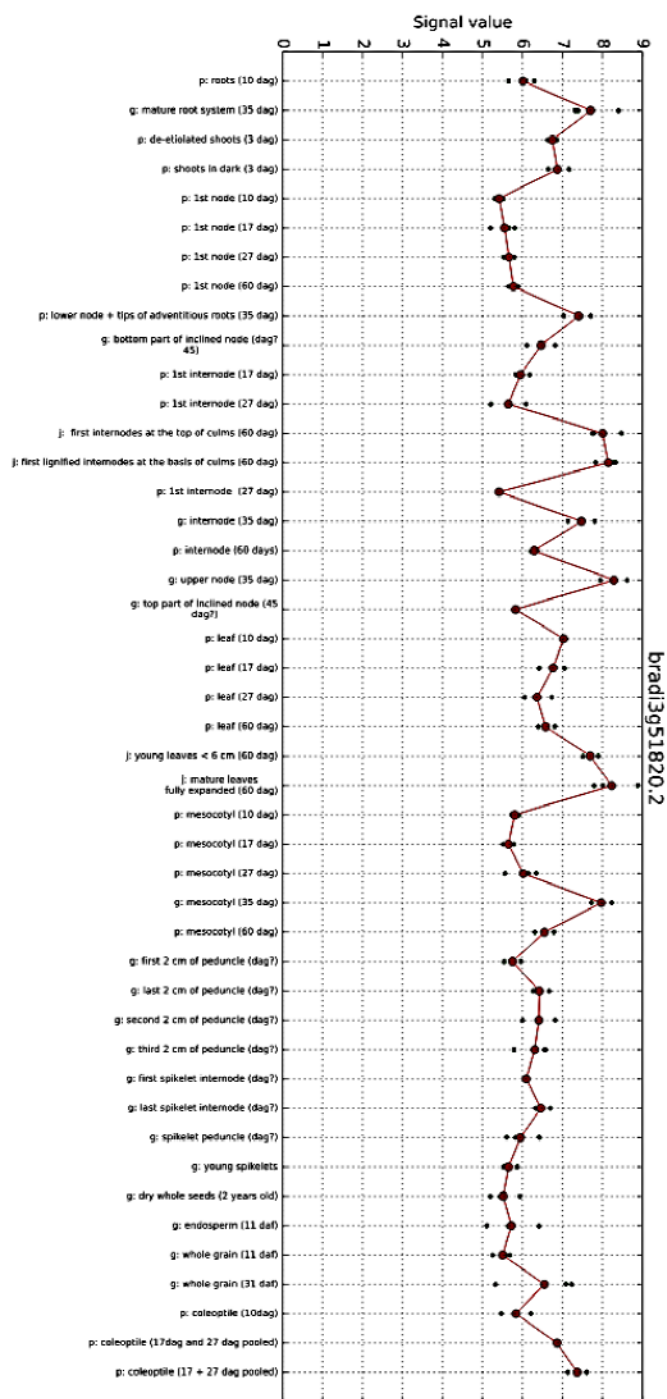
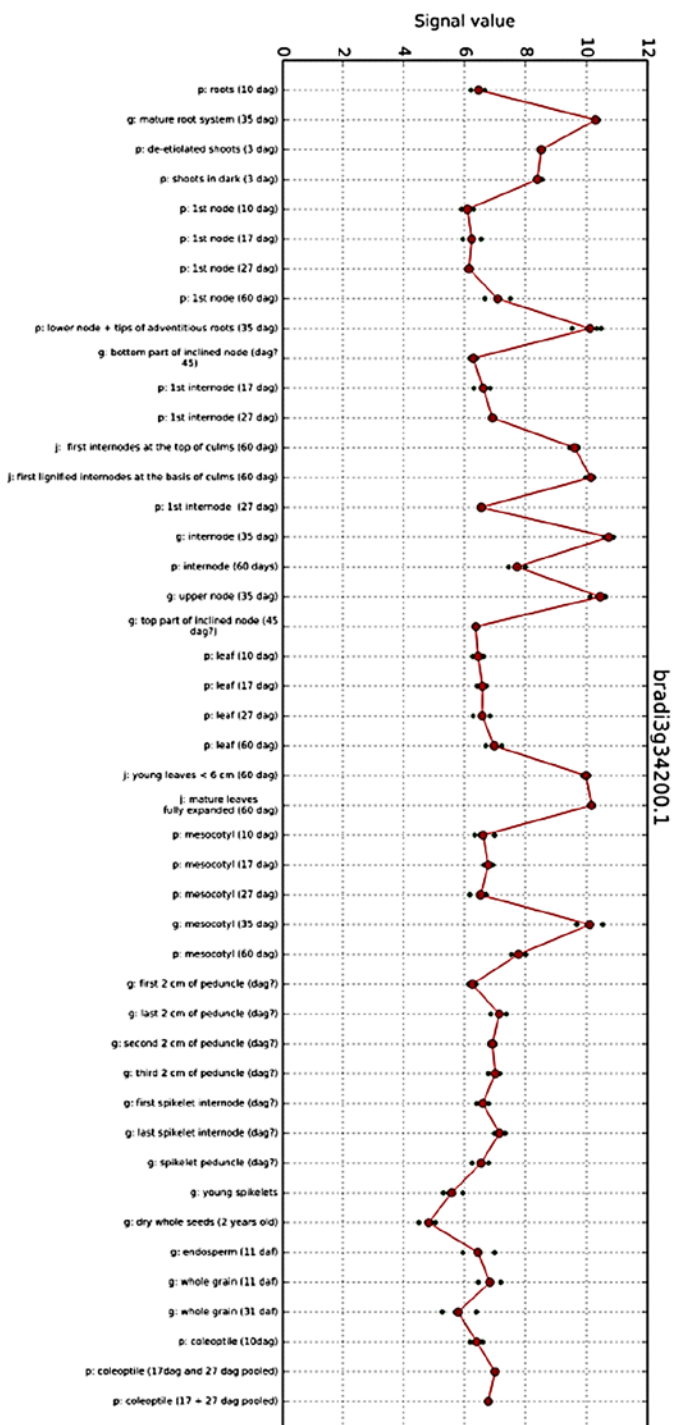
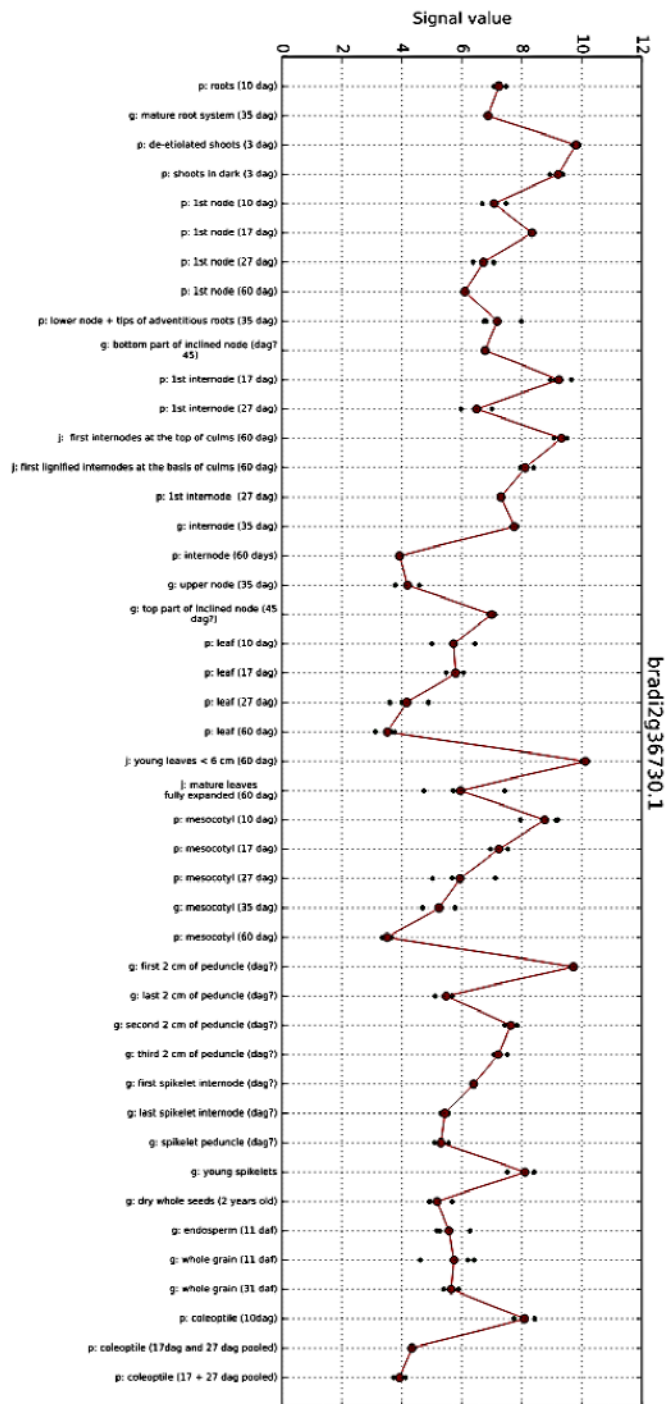
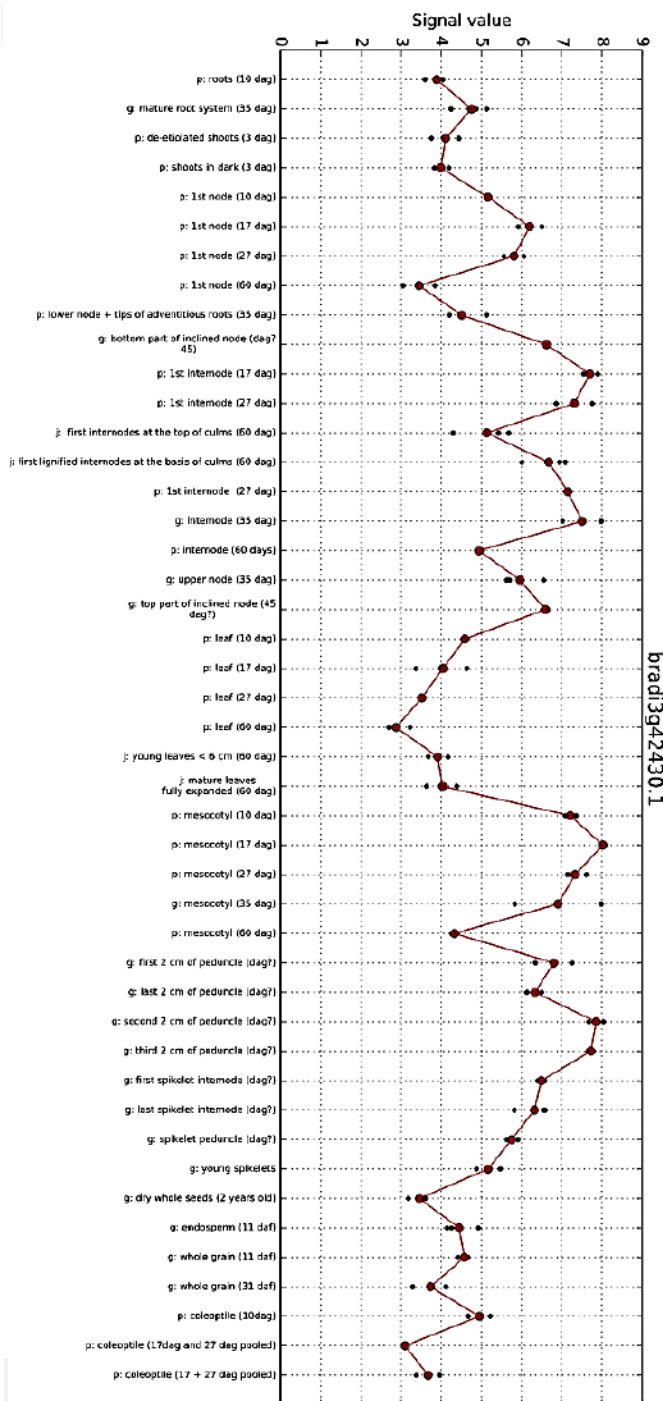


Figure 3.2-17. Transient expression of TFs and *LAC4* promoter fused to the GFP in *P. patens* protoplasts.

Flow cytometry demonstrated the dot plots of the GFP fluorescence using 527-nm/30-nm band-pass filter (FL1, Y-axis) and autofluorescence from living protoplasts using 610-nm/30-nm band-pass filter (FL2 X-axis). A: a transfected protoplast suspensions with the plasmid of LAC4 promoter-GFP (proLAC4) alone. B: A protoplast suspension transfected with plasmids of MYB61, MYC4, LWD1 and proLAC4:GFP together.

C: the GFP intensity was detected by flow cytometry to quantify the promoter activity.





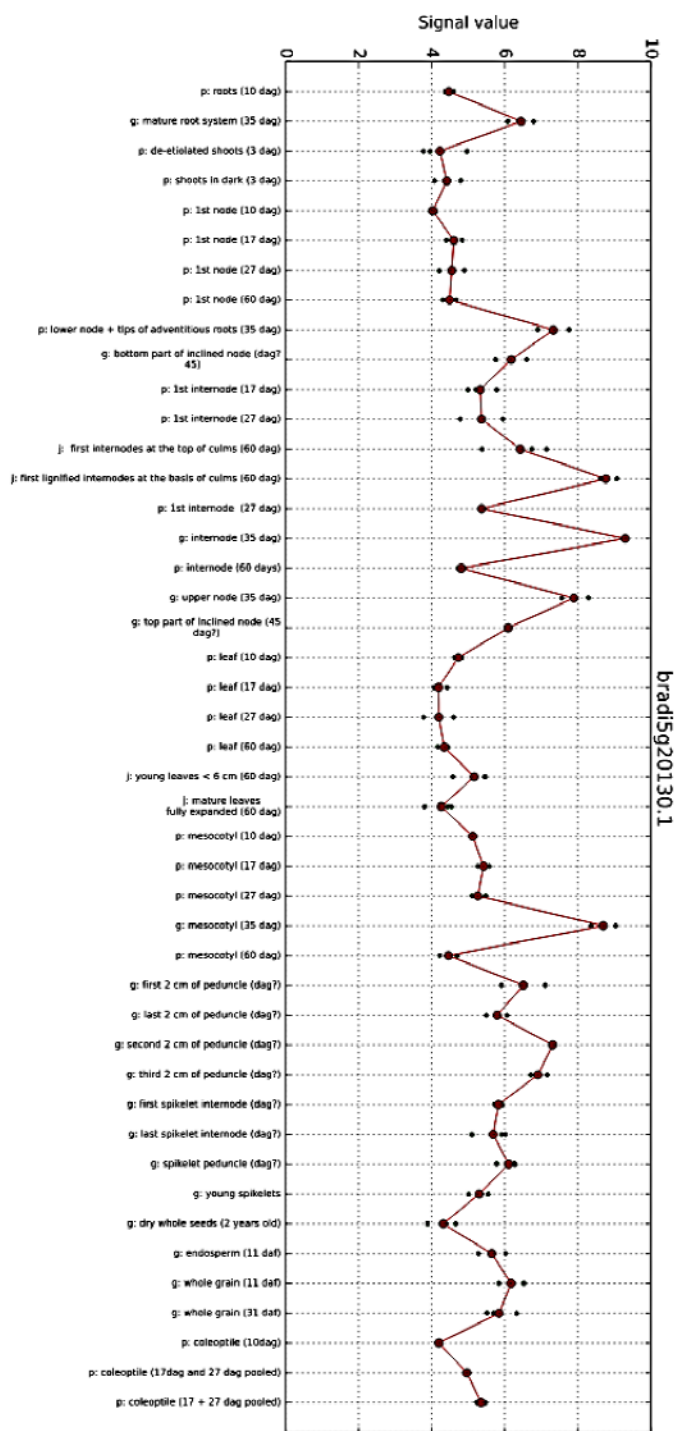
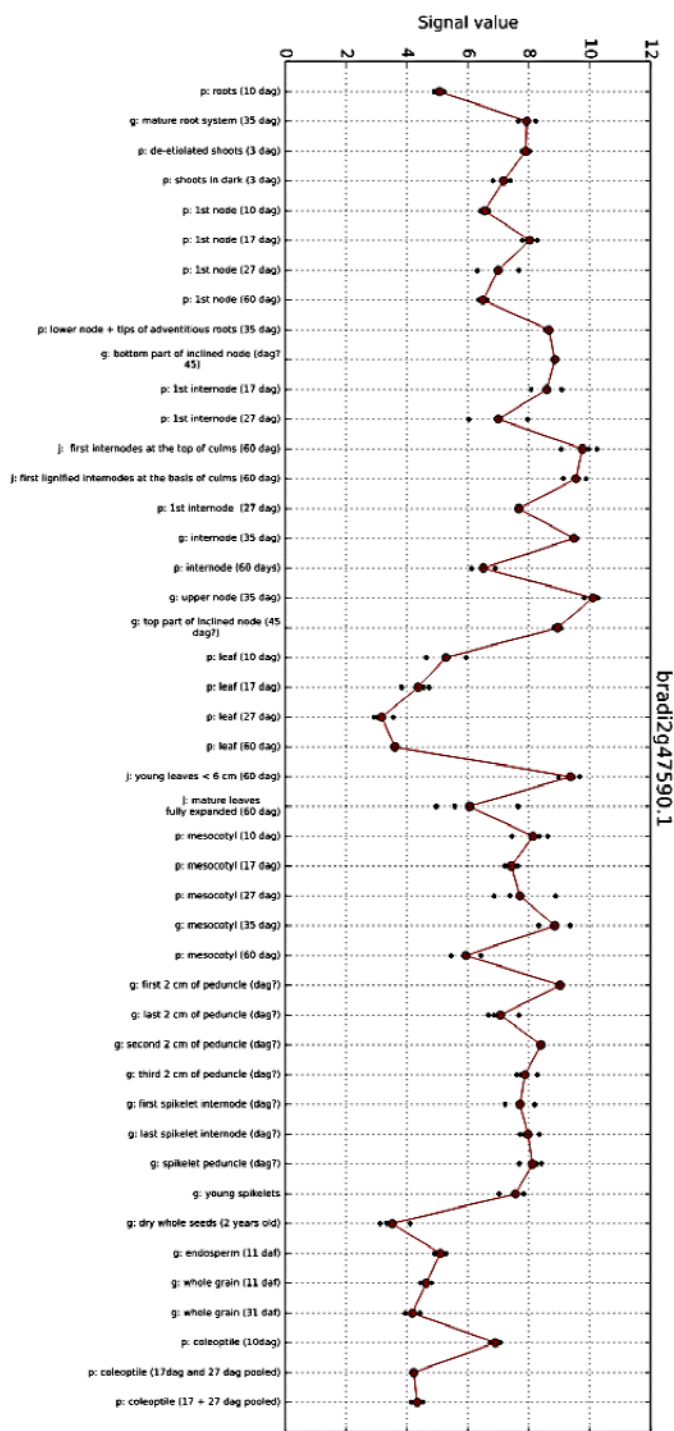


Figure 3.2-18. Expression profiles of candidate TFs in Brachypodium.

Expression data were adapted from PlaNet (<http://aranet.mpimp-golm.mpg.de/>). Y-axis: expression values; x-axis: tissues/treatments; red dots: average expression, black dots: expression from individual microarray/RNAseq experiments.

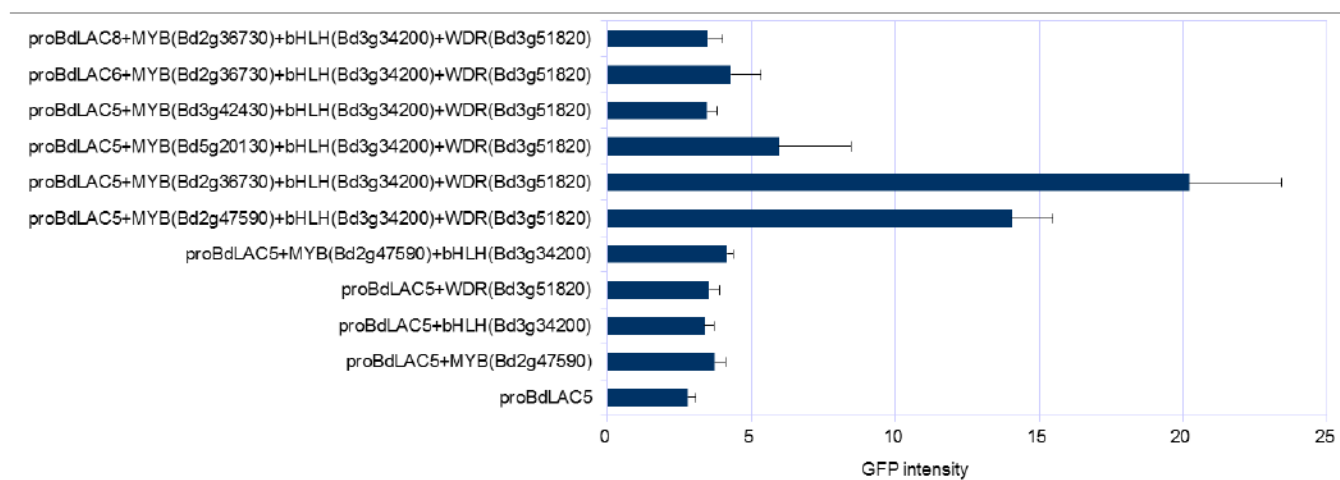


Figure 3.2-19. Transient expression of TFs and *BdLAC5/6/8* promoter-GFP in *P. patens* protoplasts.

The GFP intensity was detected by flow cytometry to quantify the promoter activity.

Chapter 4 Conclusions

Lignins are cell wall phenolic heteropolymers, mainly made from *p*-coumaryl, coniferyl, and sinapyl alcohols. These monolignols are synthesized in the cytoplasm from the phenylpropanoid pathway, then may be transported to the cell walls where they are oxidatively-polymerized into *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin units. This oxidative polymerization step is driven by H₂O₂-dependent peroxidases and/or O₂-dependent laccases (Vanholme et al., 2010 and Van Parijs et al., 2010). To our knowledge, no mutant or transgenics affected in laccase activity with a convincing impact on lignification have been reported in grasses. In this study we reported for the first time that laccases are also involved in lignification in grasses. Indeed, we identified a specific laccase gene (*BdLAC5*) among 29 non-redundant laccase genes in *Brachypodium distachyon* genome, which is responsible for the lignification in stems of *Brachypodium distachyon*, a model plant for grasses. *BdLAC5* gene was found highly expressed in lignified organs (internode, node and peduncle) and poorly expressed in organs with low lignin level (young leaf and spikelet) nor in non-lignified tissue (endosperm). The coexpression analysis reveals that *BdLAC5* is coexpressed with genes encoding enzymes involved in monolignol biosynthesis such as *O*-methyltransferase, cinnamoyl-CoA reductase, phenylalanine ammonia lyase and cellulose synthase which are involved in secondary wall formation. Two other laccases *BdLAC6* and *BdLAC8* are also found coexpressed with *BdLAC5* and interestingly enough they belong to the same phylogenetic clade. *BdLAC6* and *BdLAC8* are close orthologues of *Arabidopsis LAC4* and *LAC2* respectively. To more accurately localize *BdLAC5*, *BdLAC6* and *BdLAC8* transcripts at tissue level, *in situ* hybridization experiments demonstrated the most intense signal in the interfascicular fibers of the internode was detected with

BdLAC5 probes and then for *BdLAC8* and *BdLAC6* probes. Furthermore, immunolabelling assays revealed that BdLAC5 proteins might be secreted into the cell wall matrix because we detected some fluorescent particles close to or in the cell wall. It would be interesting to investigate further the localisation of BdLAC5 protein and check if it is localized only in secondary cell wall and not in middle lamella as suggested by Mathias Schtulz et al. (2014) for laccase 4 and 17 in the protoxylem cells of *Arabidopsis*. The double laccase mutant affected in *BdLAC5* and *BdLAC8* (*5ho8ho*) clearly showed that the lignification in interfascicular fibers involved different genes/proteins than the lignification in metaxylem cells of *Brachypodium*. Metaxylem cells were only poorly affected in the double mutant when interfascicular fibers showed dramatic decrease of Wiesner staining. The different mechanisms of lignification between xylem and fibers is discussed in chapter 1 (Voxeur et al. 2015). At last, the role of *BdLAC6* remains unknown. It would be worth crossing *BdLAC5* and *BdLAC6* mutants to determine whether functional redundancy between these two genes is hiding the genuine role of *BdLAC6*.

The regulation of the laccase transcription is almost completely unknown in plants. During this PhD, I tried to investigate what would be the protagonists involved in this mechanism. Because of the limited time of the PhD, I chose to begin this research project in *Arabidopsis* first and then work on *Brachypodium*.

MYBs are the most famous transcription factors involved in the regulation of lignin biosynthesis (Zhong et al., 2014). However, it is still necessary to figure out what are the interactors with MYBs and how they exactly regulate lignin biosynthesis. The physical interaction and regulatory synergy between specific R2R3-MYB, bHLH and WD repeat protein is well studied in the biosynthesis of flavonoids, root hair, trichome and seed mucilage development in different plant species (Xu et al., 2014). Several bHLHs were thus selected as putative

candidates from the whole bHLH family according to their expression patterns. In this study, we focused on *MYC4* (At4g17880) which is specifically expressed in lignified tissues, mainly the vasculature. The overexpressors of *MYC4* had upregulated levels of *LAC4* and *LAC17* transcription and displayed an enhanced lignification phenotype, with higher lignin content and ectopic lignin distribution when plants were grown under the longer day condition (18h light/ 6h darkness). Despite, we did not detect any significant difference in the lignification of *myc234* compared with WT our data suggest *MYC4* is a positive TF for lignin biosynthesis in *Arabidopsis* at least in stress conditions. Indeed, because *MYC2* and *MYC3* are close paralogs of *MYC4* and *MYC2* has been clearly described as involved JA signaling, we hypothesized that *MYC2/3/4* may participate to the JA-induced lignification *in planta*. With exogenous MeJA treatment, the lignin content in wt plants was increased, but there was no significant increase of lignin content in *myc234*. Interestingly, the β -O-4 substructures of lignin in wt plantlets is dramatically changed by MeJA treatment, a huge increase of S unit frequency was detected. Lignification in *myc234* plantlets was unchanged after JA treatment, suggesting disability of this mutant to perceive JA. Interestingly there is already high proportion of S units in non-treated *myc234* seedlings and H units are abnormally high. H units are often considered as marker of stress conditions. In conclusion, these results suggest that at least one gene among *MYC2*, *MYC3*, *MYC4* is involved in stress induced lignin biosynthesis.

Typical bHLH binding motifs such as E-box (CANNTG) and G-box (C/G)ACGT(A/G) and R2R3-MYB binding motifs, such as MYB-core (CAACTG) and AC-elements [ACC(T/A)(A/C)(A/C)C] were found in promoters of *Arabidopsis* *LAC4* and *LAC17* via promoter *in silico* analysis. Furthermore, a high throughput and quantitative analysis of transient expression in *Physcomitrella patens* protoplasts was adapted to demonstrate the MYB–bHLH–WDR can directly activate the promoter (2 kb) of *LAC4*. When the *MYB61*, *MYC4* and *TTG1* or *LWD1* genes were transfected together with the

LAC4 promoter:GFP into the protoplasts, a significantly increase of GFP signals were detected by flow cytometry. An interesting result reside in the disability of MYB 103 to replace MYB61 in this context, suggesting that MYBs specificities are important for the activity of MBW complex on *LAC4* promoter. To find the corresponding MBW complex in *Brachypodium*, the homology of protein sequences and gene expression patterns were both employed for screening candidates. Finally, four MYBs (Bradi2g47590, Bradi5g20130, Bradi3g42430 and Bradi2g36730), one bHLH (Bradi3g34200) and one WDR (Bradi3g51820) were chosen as candidates for further investigation. Again, when the MBW complex of BdMYC4 and BdWD1 and BdMYB (Bradi2g47590 or Bradi2g36730) were transfected together, the promoter of *BdLAC5* was activated in *P. patens* protoplasts, however BdMYBs (Bradi5g20130 and Bradi3g42430) did not activate the MBW complex. So far, we did not find the complex that can activate the promoters of *BdLAC6* and 8.

Identifying the protein complex involved in laccase transcription is a huge work that need further investigations. I did not identify a *bhlh* or a *wdr* mutant with a convincing decrease in lignification, but however, my work showed that the MBW complex might be involved in regulation of laccases both in *Arabidopsis* and *Brachypodium*. Bypass the functional redundancy between bHLH or between WDR proteins would probably answer definitely whether the mechanism I showed in *Physcomitrella patens* is a genuine phenomenon in *Arabidopsis* or *Brachypodium*.

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Wang Y, Bouchabké-Coussa O, Le Bris P, Antelme S, Soulhat C, Gineau E, Dalmais M, Bendahmane A, Morin H, Mouille GG, et al (2015) LACCASE5 Is Required for Lignification of the *Brachypodium distachyon* Culm. *Plant Physiol* 168: 192–204

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The contribution

The contribution of Yin WANG to this thesis was as follows:

I, Yin WANG, participated in the planning and designing of all the experiments and also wrote most of paragraphs in the publications that with my name as the first author.

I also performed experiments, with my supervisor and colleagues guide, many experiments are done by myself. I isolated and performed molecular and phenotypic characterization of mutants, gene expression analysis. For the Klason lignin content, thioacidolysis and mild alkaline hydrolysis, I collaborated with Dr. Catherine Lapierre. I prepared extracted cell wall residues and then she performed the experiments and analyzed the results. With my supervisor's help, I also performed data analysis and writing, formatting and proofreading this manuscript.

I had a main responsibility in assuring the accuracy and reliability of all the experimental results.

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