Running title: Laccases are required for grass stem lignification.

Corresponding author: Richard Sibout

1Institut Jean-Pierre Bourgin, UMR1318 INRA-AgroParisTech, INRA Centre de Versailles-Grignon, Route de St- Cyr (RD10), 78026 Versailles France.

Tel : +33 (0)1 30 83 37 51 or +33 (0)6 48 29 06 95

Email : richard.sibout@versailles.inra.fr

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Title: LACCASE 5 is required for lignification of the 
Brachypodium distachyon culm (†)

Yin Wang1,2, Oumaya Bouchabke-Coussa1,2, Philippe Lebris1,2, Sébastien Antelme1,2, Camille 
Soulhat1,2, Emilie Gineau1,2, Marion Dalmais3, Abdelafid Bendahmane3, Halima Morin1,2, 
Grégory Mouille1,2, Frédéric Legée1,2, Laurent Cézard1,2, Catherine Lapierre1,2, Richard 
Sibout1,2*

1 INRA, Institut Jean-Pierre Bourgin, UMR 1318, ERL CNRS 3559, Saclay Plant Sciences, 
RD10, F-78026 Versailles, France

2 AgroParisTech, Institut Jean-Pierre Bourgin, UMR 1318, ERL CNRS 3559, Saclay Plant 
Sciences, RD10, F-78026 Versailles, France

3 URGV, Unité de Recherche en Génomique Végétale, Université d'Evry Val d'Essonne, 
INRA, 2 rue Gaston Crémieux CP 5708, 91057 EVRY cedex

A mutation in a laccase gene alters lignin deposition and cell wall recalcitrance to 
saccharification in Brachypodium distachyon.
Footnotes:

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Address correspondence to richard.sibout@versailles.inra.fr
Abstract (176 words)

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 (LACCASE 5 and LACCASE 6) are present in lignifying tissues. A mutant with a mis-spliced LACCASE 5 mRNA was identified in a TILLING 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 to 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 Brachypodium lignification and are promising targets to alleviate the recalcitrance of grass lignocelluloses.
**Introduction**

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, 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 \( \text{H}_2\text{O}_2 \)-dependent peroxidases and/or \( \text{O}_2 \)-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, the genetic model plant. While the T-DNA mutants of \( \text{AtLAC4} \) and \( \text{AtLAC17} \) showed moderate alterations in lignification, the double mutant \( \text{lac4 lac17} \) 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, \( \text{AtLAC4} \) and \( \text{AtLAC17} \) 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, \( \text{SofLAC} \) shown to partially complement \( \text{Athal17} \) (Cesarino et al., 2013). To this aim, we used the Brachypodium mutant collection and the TILLING platform previously established (Dalmais et al., 2013) to isolate laccase mutants. By doing so, we demonstrated that the \( \text{BdLAC5} \) gene is involved in the lignification of Brachypodium culms and that laccases might be good candidates to target for cell wall engineering.

**Results and discussion**

**BdLAC5 and BdLAC6 proteins are closely related to lignin-specific AtLAC17 and AtLAC4 laccases**
In order to identify the laccases of Brachypodium \textit{in silico}, we blasted the amino acid sequences of Arabidopsis AtLAC4 or AtLAC17 (http://mips.helmholtzmuenchen.de/plant/brachypodium/) against proteins from the Brachypodium genome. This search allowed the identification of 29 non-redundant laccase genes named \textit{BdLAC}1 to \textit{BdLAC}29, according to their chromosome localization (Supplemental Table 1). A phylogenetic tree was constructed with the deduced 29 Brachypodium laccases proteins together with the 17 Arabidopsis laccases (Fig. 1). In addition, we incorporated in this tree some other laccases reported to catalyse the oxidative polymerization of various phenolics, such as SofLAC from sugarcane (Cesarino et al., 2013), ZmLAC3 from \textit{Zea mays} (Caparrós-Ruiz et al., 2006), PtLAC3 from \textit{Populus trichocarpa} (Ranocha et al., 2002), GaLAC1 from \textit{Gossypium arboretum} (Wang et al., 2008) and BnTT10-1 from \textit{Brassica napus} (Zhang et al., 2013) (Supplemental Table 2). The resulting phylogeny comprised four large clades (Fig. 1). Clade I and II gathered fourteen and three Brachypodium laccases respectively and some Arabidopsis proteins of unknown functions. Clade III comprised three Brachypodium 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 catalyse 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 sub-cluster with AtLAC4 (66/83% identity/similarity), AtLAC11 (Zhao et al., 2013) and PtLAC3 (Ranocha et al., 2002) also reported to be involved in lignification. BdLAC5 (Bradi1g66720), BdLAC8 (Bradi2g23370) and BdLAC12 (Bradi2g54740) gathered together in a sub-cluster 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 \textit{Atlac17} mutant (Cesarino et al., 2013). Based on this analysis, the \textit{BdLAC}5 and \textit{BdLAC}6 genes were chosen as the best lignin-specific candidates for further investigation.

\textbf{\textit{BdLAC}5 and \textit{BdLAC}6 are mainly expressed in lignified tissues.}

The expression and co-expression of the selected \textit{BdLAC}5 and \textit{BdLAC}6 genes were studied in different organs and at different development stages using the PlaNet tools (http://aranet.mpimp-golm.mpg.de/) (Supplemental Fig. 1). Both genes were highly expressed in lignified organs (internode, node and peduncle) and poorly expressed in organs with low
lignin levels (leaf for instance), or in non-lignified tissue (endosperm). This co-expression study (Supplemental Table 3) revealed that both laccases are co-expressed with genes involved in monolignol biosynthesis such as O-methyltransferase (Bradi3g16530) (Bragg et al., 2012; Dalmais et al., 2013), cinnamoyl-CoA reductase (Bradi3g36887) or phenylalanine ammonia lyase (Bradi3g49250 and Bradi3g49260). They are also co-expressed with three cellulose synthase genes specific to the secondary cell wall, CESAs4 (Bradi2g49912), CESAs7 (Bradi3g28350) and CESAs9 (Bradi4g30540) (Handakumbura et al., 2013).

The expression levels of *BdLAC5* and *BdLAC6* were analyzed by quantitative RT-PCR on RNA isolated from different organs at three developmental stages (see Materials and Methods), namely 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 stage. 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 Brachypodium lignification was performed on stem and leaf samples at the same stages in addition to the senescence stage (SS). Lignin content and structure of extractive-free material was estimated using thioglycolic acid (TGA) and thioacidolysis methods (Fig. 3A and Fig. 3B). 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. 3A and Fig. 3B). Histochemical Wiesner and Maüle staining of Brachypodium stems (Fig. 3C) were consistent with these chemical analyses. At the TS stage, using Wiesner staining, lignins could be detected mainly in vessels (MX, PX), mestome (Me) and intrafascicular fibers (VF) of vascular bundles. It is only at the EFS and later stages that interfascicular fibers (IF) 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 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 tissue level, *in situ* hybridization experiments were performed with appropriate RNA probes applied to internode sections collected at the EFS stage. At this stage, the sclerenchyma (IF) tissue, a few parenchyma cells and vascular bundles (VB) are lignified (Fig. 3C). The intense signal of the *BdLAC5* antisense probe was detected in most of these tissues (Fig. 4A) when compared to the negative control performed with 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 stage (Bollhöner et al., 2012, Schuetz et al., 2013). The most intense signal of *BdLAC5* transcripts was observed in most sclerenchyma cells and some lignifying neighbouring parenchyma cells of the interfascicular area, whereas the non-lignified 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-aa polypeptide (theoretical molecular mass and isoelectric point (pI): 62.7 kDa and 8.96) while the *BdLAC6* gene (1,689 bp coding sequence) encodes a predicted 561-aa polypeptide (theoretical molecular mass and pI: 62.1 kDa 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. 2). Little experimental evidence is published regarding subcellular location of laccases (Berthet et al., 2011; Cesarino et al., 2013). It is worth noting that Pang and colleagues (2012) 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 immunolabelling 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 immunolabelling assay revealed anti-BdLAC5 labeled particles in or next to the walls of the interfascicular fibers (Figure 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 apoplasm 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 TILLING (Targeting Induced Local Lesion IN Genome) screen was carried out on the Versailles Brachypodium 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 (Durao 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 an RT-PCR analysis to amplify the *BdLAC5* mRNA in *Bd4442*. When compared to the wild-type (WT) sample, the *BdLAC5* mRNA from *Bd4442* displayed an insertion of thirteen nucleotides from the last intron, which is predicted to cause a truncation of the C-terminal domain of the BdLAC5 protein (Supplemental Fig. 3). The mutant *Bd5024* possesses a single G-to-T nucleotide substitution at position 1270 of the *BdLAC6* cDNA, which introduces a premature stop codon at glycine 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 to the WT 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 stem height and internode diameter of the *Bd4442* line were systematically smaller than those of the WT line (Supplemental Table S4). At the tissue level, the area of the vascular bundles from the homozygous *Bd4442* line was also found to be slightly reduced, as compared to WT.

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 to the WT level) was systematically observed for independent co-cultures of *Bd4442* and WT 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. 4). Lignin structure of the WT and mutant lines was also investigated by thioacidolysis (Lapierre, 1993). Compared to the corresponding WT 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 to WT, Wiesner staining of *Bd4442* stems at MS stage was substantially weaker, particularly in the sclerenchyma (Fig. 7). By contrast, this test did not show obvious differences between the *Bd5024* and WT lines. To further investigate the topochemistry of lignification in *Bd4442* culms as compared to control plants, FTIR absorbance spectra of vascular bundle or interfascicular fiber areas were recorded with a spectra-microscope system as previously described (Mouille et al., 2003; Sibout et al., 2005). Relative to WT, the most striking impact of the *Bd4442* mutation on the FTIR spectra was a substantial reduction of the lignin-specific aromatic signal at 1508 cm$^{-1}$ and mainly in the interfascicular fiber area (Fig. 8, Supplemental Fig. 5). 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 *Brachypodium* culm was affected by the *Bd4442* mutation, more specifically in interfascicular fibers.

From these analyses, we may conclude that *BdLAC5* disruption results in 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 spatio-temporally-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 arabinose 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* line than from WT (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., 2004 a, b). The amount of FA ethers in the *Bd4442* and WT samples was 5.89 ± 0.36 and 6.93 ± 0.48 mg g⁻¹, respectively. According to model studies carried out with artificially-lignified maize cell walls, these changes could be linked to the reduced lignification of *Bd4442* cell walls (Grabber et al., 2000). In addition and together with peroxidases, *BdLAC5* laccase 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 *Brachypodium* culms was provided by complementation experiments. We produced transgenic lines overexpressing a *BdLAC5* WT gene copy driven by a maize ubiquitin promoter into *Bd4442*. We obtained few transgenic lines suggesting recalcitrance of this line to embryogenesis. However, we selected one transformed line with detectable levels of the transgene (Supplemental Fig. 6). 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. 7). Not unexpectedly, the saccharification yield of the extractive-free mature stems was found to be improved for the *Bd4442* mutant, and comparable to the WT sample (Table 4). The weight loss induced by a cellulase treatment without any pretreatment was found to be 30.5 ± 1.3% for *Bd4442* samples versus 23.5 ± 1.4% for WT samples and 20.6 ± 0.4% for the complemented line (mean and SD values for 2 independent experiments performed each on 3 different samples per line).

**Conclusion**

This study provides the first evidence that a laccase enzyme, BdLAC5, is involved in the lignification of *Brachypodium*, 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 protein 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 much lower level than *BdLAC5*, and redundancy might prevent any detectable impact on lignin content in the *Bd lac6* mutant. Only a double mutant would reveal a role for both genes in lignin formation. *Brachypodium* possess 29 laccases in its genome and nine of them belong to the same cluster of BdLAC5 and 6. More functional experiments are required to determine precisely which other enzymes participate in lignification, however the current study provides evidence that, like Arabidopsis, laccases are involved in lignification of *Brachypodium*, the model plant for grasses.
**Materials and Methods**

**Plant Material and Growth Conditions**

Mutants were identified in the collection of chemically-induced Brachypodium mutants at the Research Center of INRA-Versailles-Grignon (Dalmais et al., 2013). Brachypodium plants (accession *Bd21-3*) were grown in a greenhouse under long-day conditions (18 h light, 400 watt 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): Tillering Stage (TS), 17 days after germination (DAG) when the plants are tillering and the first true internode is elongating; Early Flowering Stage (EFS~ 35 DAG) when an inflorescence appears with immature flowers; Maturity Stage (MS~ 60 DAG), when seeds are filled and the oldest leaves are turning yellow; Senescence Stage (SS~120 DAG), 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 tillering stage where the first internode above the crown was selected. Samples were fixed in FAA (50% ethanol, 10% formalin, 5% Acetic Acid) solution overnight, and then embedded in 7% agarose before being transversely sectioned at a thickness of 100 um using a vibratome (Leica VT1000S, Leica, Germany). Lignin deposition and composition were investigated histochemically by Maüle and Wiesner staining as previously published (Bouvier d’Yvoire et al., 2013). All sections were observed under a Zeiss AxioPlan 2 microscope system with automatic exposure times.

**Phenotype measurements and image analysis**

All the measurements were performed on the tallest stems. The 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 the tillering stage. ImageJ software was used for quantification of cell wall thickness and vascular bundles. Significant differences were analyzed by one-way ANOVA (Tukey's HSD, 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 thioglycolic acid lignin assay was optimized from (Hatfield and Fukushima, 2005; Suzuki et al., 2009). Extractive-free cell wall residues of stem were treated with thioglycolic acid (TGA). Briefly, 10–15 mg of plant cell wall residues were treated with thioglycolic acid at 80 C° for 8 hours. 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 NaOH (1 M). After six-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 Brachypodium. 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 previously described (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 HSD, 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 in Harholt et al., (2006). To determine the cellulose content, the residual pellet obtained after the monosaccharide analysis was rinsed twice with ten volumes of water and then hydrolysed with H₂SO₄ as described by Updegraff (1969). The released glucose was diluted 500 fold and then quantified using a HPAEC-PAD chromatography as described in Harholt et al., (2006).
RNA extraction and quantitative RT-PCR.

Total RNA was extracted using the Qiagen Plant RNAeasy kit (Qiagen, Courtaboeuf, France) with an additional DNase step. RNA samples were quantified using a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). 1 µg of total RNA was used for reverse transcription using the Superscript Reverse Transcriptase II kit (Invitrogen, Illkirch, France). The absence of genomic contamination was confirmed by PCR with primers target introns. The quantitative RT-PCR (qRT-PCR) were performed on the Eppendorff Realplex2 Mastercycler using the SYBR Green kit (Bio-Rad, Marnes-la-Coquette, France) and the 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 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 with amplification efficiency of PCR reaction of Muller et al., (2002). The Brachypodium 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

Primers sequences used for the DIG-labeled probe amplification are shown in Table S1. Anti-sense probes were synthesized by *in vitro* transcription with T7 RNA polymerase using a digoxygenin (DIG) RNA labelling kit (Roche Applied Science, Boulogne-Billancourt, France), 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* hybridizations were carried out as described by Chapelle et al., (2012) with minor changes: after washing and dehydration of the tissues, sections were pre-hybridized for 1 h at 60°C with hybridization solution (50% formamide, 5x SSC, 100 µg ml⁻¹ tRNA, 50 µg ml⁻¹ heparin and 0.1% tween). Sections were then hybridized with DIG-labeled anti-sense or sense probes (1:1000) overnight at 60°C and then 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, Boulogne-Billancourt, France). All sections were observed under a Zeiss AxioPlan 2 microscope system with automatic exposure time.

Phylogeny tree and 3D-structure prediction

An unrooted phylogenetic tree was constructed with PhyML in the Phylogeny.fr platform (http://www.phylogeny.fr) with Blosum62 as a substitution model, 1000 bootstraps after loading the protein ClustalW alignment (Dereeper et al., 2008). 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 BdLAC5 splicing mutants Bd4442

The full-length cDNA including the 5'-UTR and 3'-UTR of *BdLAC5* was cloned from Brachypodium 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 Brachypodium transformation by the LR Gateway® (Invitrogen) reaction. The pIPKb2-BdLAC5 was electroporated into *Agrobacterium tumefaciens* (AGL1 strains). 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 hygromycin.

Fourier transform infrared (FTIR) spectroscopy

Fixed internodes were cut into 50 µm thick sections with a vibratome, and then were rinsed abundantly with distilled water for 2 min and dried at 37 ºC for 20 min. FTIR spectra were collected from a 60 µm x 60 µm window targeting vascular bundles or interfascicular fibers. For each genotype, 3 sections from 3 different plants were analyzed. Normalization of the spectral data and statistical analyses were performed as described by Mouille et al., (2003). A Student’s t-test was performed to estimate statistical differences between mutants and wild type.

Cell wall saccharification (Cellulolysis)
Saccharification assays were performed as described by Berthet et al., (2011). For each sample, 30 mg extractive-free cell wall residues were incubated with 4 ml of acetate buffer pH 4.5, containing 4 mg/ml commercial cellulase (cellulase Onozuka-R10; Serva) and 0.5 mg/ml NaN$_3$ for 3 days at 45°C on a carousel. After centrifugation, the recovery of the supernatant for glucose was determined via enzymatic assay with the bioMérieux Kit (bioMérieux, Crapone, France). The pellet was washed twice with water, then freeze-dried and weighed to evaluate the weight loss.

Immunolabelling

Anti-BdLAC5 and Anti-BdLAC6 Based on their amino acid sequences, the C-terminal domains were used to design the potentially antigenic peptide sequence. The peptide sequence (AGGWVAIRFYADNPGVWFMH) was used to produce Anti-BdLAC5 and peptide sequence (KMVFVVENGKRPSETLI) was used to produce Anti-BdLAC6. Antibodies were produced by (Genescript, Hong Kong).

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 immunolabelling was carried out as described in Verhertbruggen et al., (2009). Slides were incubated in Evans Blue solution (0.001% in PBS: 10 min) to decrease the autofluorescence of lignified cells. Sections were labeled with the primary antibody anti-BdLAC5 or anti-BdLAC6 from rabbit (1:1000), and the secondary antibody was an anti-rabbit conjugated to Alexa Fluor® 594 (Invitrogen, Illkirch, France) (1:500). This dye was found to be the best marker because of its low interference with autofluorescence of Brachypodium cell walls (data not shown). The labeled sections were viewed with a confocal laser-scanning microscope Leica TCS SP5 Confoca (Leica, Nanterre, France).

Full text: 4630 words

Acknowledgements

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References


FIGURE LEGENDS

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Figure 4. Tissue localization of BdLAC5 and BdLAC6 transcripts. Transcripts of BdLAC5 and BdLAC6 were detected by in situ hybridization at heading and flowering stages (ETS). A, cross-sections of the second internode were labeled with BdLAC5 anti-sense probes. C, BdLAC5 sense probes. B, BdLAC6 anti-sense probes. D, BdLAC6 sense probes. E, cross-section of internodes without labeled probes is shown. F, whole mount leaves labeled by BdLAC5 anti-sense probes are shown. Black arrows show immunolabeling signal. White arrows show veins. Scale bar = 100 μm.

Figure 5. Subcellular localization of BdLAC5 and BdLAC6 laccases in lignifying interfascicular fibers. Immunolocalizations are imaged with confocal microscopy. A, immunolocalization of Alexa 594 after detection of BdLAC5 primary antibody; B, immunolocalization of Alexa 594 after detection of BdLAC6 primary antibody. C, control

**Figure 6. Multiple alignment of deduced amino acid sequences from Brachypodium and other plant laccases.** Amino acid sequences of both Brachypodium laccases, *Zea mays* (ZmLAC3), *Oryza sativa* (OsLAC22) and *Arabidopsis thaliana* (AtLAC4 and AtLAC17) were aligned with ClustalW software. The copper binding domain motifs are underlined. Triangles represent location of amino acid changes in BdLAC5 of *Bd4442* (blue), and 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 wild type. B-D, transverse sections of second internodes stained with Wiesner reagent. Scale bar = 100 μm. For B-D, pictures were taken at same magnification.

**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 mature stage (MS). Black line, wild-type plants; red line, *Bd4442*. Arrow shows absorbance at 1508 cm⁻¹. Each spectrum corresponds to the normalized means of biological replicates (n=6).

**SUPPLEMENTAL FIGURE LEGENDS**

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

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

**Figure S3.** The aberrant transcript of *BdLAC5* and its deduced amino acid sequence in *Bd4442*. A, a substitution (G to A) modifies the splicing site of the last intron of *BdLAC5* 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 BdLAC5 protein is abnormal and truncated in Bd4442 (red) when compared to wild-type plant (black)

Figure S4. Hemicellulose and cellulose contents in extractive-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 ± 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 plants (WT) and Bd4442.

Figure S6. Genomic characterisation of the transgenic line. A genomic PCR was used to verify the presence of the BdLAC5 wild type allele in CP3 (upper row). Transcript of hygromycin phosphotransferase gene was also amplified in the transgenic line CP3 (middle row). BdUB14 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 I. Lignin analyses for wild-type (WT), BdLAC5 (*Bd4442*) and BdLAC6 (*Bd5024*) misregulated mutants: lignin content and structure for extractive-free mature stems as measured by Klason and thioacidolysis methods

<table>
<thead>
<tr>
<th>Culture and genotype</th>
<th>Lignin content (%)</th>
<th>Relative frequency of lignin-derived thioacidolysis monomers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%H</td>
</tr>
<tr>
<td>1st culture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>17.63 ± 0.01</td>
<td>2.8 ± 0.1</td>
</tr>
<tr>
<td><em>Bd4442</em></td>
<td>15.71 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td><em>Bd5024</em></td>
<td>17.53 ± 0.14</td>
<td>2.8 ± 0.1</td>
</tr>
<tr>
<td>2nd culture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>16.93 ± 0.57</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td><em>Bd4442</em></td>
<td>15.60 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.6 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3rd culture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>17.59 ± 0.49</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td><em>Bd4442</em></td>
<td>16.19 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.7 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± standard deviations (SD) from individually analyzed plants (n=3).<sup>a</sup>, significantly different from the corresponding WT sample (one way ANOVA, Tukey’s HSD) at p < 0.05.
Table II. Amount of *p*-coumaric acid (CA) and ferulic acid (FA) released by mild alkaline hydrolysis of mature stems from wild-type (WT), BdLAC5 (*Bd4442*) and BdLAC6 (*Bd5024*) misregulated mutants

<table>
<thead>
<tr>
<th>Culture and genotype</th>
<th>Compound (mg g(^{-1}))</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CA</td>
<td>FA</td>
<td></td>
</tr>
<tr>
<td>1(^{st}) culture</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>7.17 ± 0.04</td>
<td>4.69 ± 0.03</td>
<td></td>
</tr>
<tr>
<td><em>Bd4442</em></td>
<td>7.50 ± 0.43</td>
<td>6.48 ± 0.41 (^a)</td>
<td></td>
</tr>
<tr>
<td><em>Bd5024</em></td>
<td>8.20 ± 0.18 (^a)</td>
<td>4.42 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>2(^{nd}) culture</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>6.83 ± 0.52</td>
<td>5.31 ± 0.44</td>
<td></td>
</tr>
<tr>
<td><em>Bd4442</em></td>
<td>6.52 ± 0.31</td>
<td>7.69 ± 0.15 (^a)</td>
<td></td>
</tr>
<tr>
<td>3(^{rd}) culture</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>WT</td>
<td>9.42 ± 0.21</td>
<td>5.05 ± 0.07</td>
<td></td>
</tr>
<tr>
<td><em>Bd4442</em></td>
<td>9.48 ± 0.23</td>
<td>7.42 ± 0.11 (^a)</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± standard deviations (SD) from individually analyzed plants (n=3).

\(^a\), significantly different from the corresponding WT sample (one way ANOVA, Tukey’s HSD) at p<0.05.
Table III. Lignin and ferulic acid (FA) analyses for the wild-type (WT) and BdLAC5 (*Bd4442*) mutant lines as compared to the *Bd4442 CP3* mutant line complemented with the *BdLAC5* WT allele (T2 transformants): lignin content and structure for extractive-free mature stems as measured by Klason and thioacidolysis methods; FA level ester-linked to the cell walls as measured by mild alkaline hydrolysis.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Lignin content (%)</th>
<th>Relative frequency of lignin-derived thioacidolysis monomers</th>
<th>FA amount</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%H</td>
<td>%G</td>
</tr>
<tr>
<td>WT</td>
<td>18.40 ± 0.33</td>
<td>3.0 ± 0.1</td>
<td>27.1 ± 1.1</td>
</tr>
<tr>
<td><em>Bd4442</em></td>
<td>16.43 ± 0.19ᵇ</td>
<td>3.1 ± 0.1</td>
<td>23.7 ± 0.5ᵇ</td>
</tr>
<tr>
<td><em>Bd4442 CP3</em></td>
<td>18.15 ± 0.23</td>
<td>3.1 ± 0.1</td>
<td>32.6 ± 0.5</td>
</tr>
</tbody>
</table>

Values are means ± standard deviations (SD) from individually analyzed plants (n=3). ᵐ All the plants were obtained from the same culture experiment in the greenhouse. ᵇ Significantly different from the corresponding WT sample (one way ANOVA, Tukey’s HSD) at p<0.05.
Table IV. Saccharification assays of extractive-free mature stem from the wild-type (WT) and BdLAC5 (*Bd4442*) mutant lines as compared to the *Bd4442 CP3* mutant line complemented with the *BdLAC5* WT allele (T2 transformant). Saccharification is evaluated both by the weight loss percentage (%WL) and by the amount of glucose released from the samples.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>%WL</th>
<th>glucose (mg·g⁻¹)</th>
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<tbody>
<tr>
<td>Bd21-3 WT</td>
<td>23.5±1.4 a</td>
<td>68.5±3.9 a</td>
</tr>
<tr>
<td>Bd4442 HO</td>
<td>30.5±1.3 b</td>
<td>113.6±4.4 d</td>
</tr>
<tr>
<td>Bd4442 CP3</td>
<td>20.6±0.4 c</td>
<td>75.3±3.3 ab</td>
</tr>
</tbody>
</table>

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).
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