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Disruption of *LACCASE4* and *17* Results in Tissue-Specific Alterations to Lignification of *Arabidopsis thaliana* Stems [W]

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Peroxidases have been shown to be involved in the polymerization of lignin precursors, but it remains unclear whether laccases (EC 1.10.3.2) participate in constitutive lignification. We addressed this issue by studying laccase T-DNA insertion mutants in *Arabidopsis thaliana*. We identified two genes, *LAC4* and *LAC17*, which are strongly expressed in stems. *LAC17* was mainly expressed in the interfascicular fibers, whereas *LAC4* was expressed in vascular bundles and interfascicular fibers. We produced two double mutants by crossing the *LAC17* (*lac17*) mutant with two *LAC4* mutants (*lac4-1* and *lac4-2*). The single and double mutants grew normally in greenhouse conditions. The single mutants had moderately low lignin levels, whereas the stems of *lac4-1 lac17* and *lac4-2 lac17* mutants had lignin contents that were 20 and 40% lower than those of the control, respectively. These lower lignin levels resulted in higher saccharification yields. Thioacidolysis revealed that disrupting *LAC17* principally affected the deposition of G lignin units in the interfascicular fibers and that complementation of *lac17* with *LAC17* restored a normal lignin profile. This study provides evidence that both *LAC4* and *LAC17* contribute to the constitutive lignification of *Arabidopsis* stems and that *LAC17* is involved in the deposition of G lignin units in fibers.

INTRODUCTION

Angiosperm lignins are complex phenolic polymers that consist mostly of guaiacyl (G) and syringyl (S) units, together with small or trace amounts of *p*-hydroxyphenyl (H) units. Monolignols are synthesized in the cytosol and transported to the cell wall, where their oxidation generates lignins (Vanholme et al., 2008). Laccases were first identified in the lacquer tree (*Rhus vernicifera*) secreted resin (Yoshida, 1883). It has been suggested repeatedly that these enzymes are involved in lignin biosynthesis, based on their capacity to oxidize lignin precursors in vitro (Higuchi and Ito, 1958; Sterjiades et al., 1992; Bao et al., 1993; Kärkönen et al., 2002; Liang et al., 2006) and their localization in zones of lignification in various plant species (Dean and Eriksson, 1994; Richardson and McDougall, 1997; Dean et al., 1998; Ranocha et al., 2002; Caparros-Ruiz et al., 2006). However, the use of an antisense strategy to decrease laccase expression in transgenic poplar (*Populus tremula* × *Populus alba*) lines affected the pool of soluble phenolic compounds but had no detectable impact on lignification (Ranocha et al., 2002). *Arabidopsis thaliana* transgenic lines overexpressing *LAC1*, which encodes a cotton (*Gossypium hirsutum*) root-secreted laccase, were found to be resistant to phenolic allelochemicals and 2,4,6-trichlorophenol (Wang et al., 2004). Expression of this laccase gene in transgenic poplars (*Populus deltoides*) increased the lignin content of stems (Wang et al., 2008). In the last decade, several studies have focused on *Arabidopsis* laccases (McCaig et al., 2005; Pourcel et al., 2005; Cai et al., 2006). Seventeen laccase genes have been identified in *Arabidopsis* and classified into six groups based on the alignment of their amino acid sequences with those of laccase-like multicopper oxidases (McCaig et al., 2005; Hoegger et al., 2006). Among T-DNA insertion mutants for 12 laccase genes, the mutants corresponding to *LAC2*, *LAC8*, and *LAC15* displayed changes in root elongation, earlier flowering, and changes in seed color, respectively (Cai et al., 2006). The first biological function assigned to an *Arabidopsis* laccase was revealed by the study of *tt10* mutants, which display changes in seed color (Pourcel et al., 2005). This work provided evidence for the involvement of *LAC15* in the oxidative polymerization of flavonoids in the *Arabidopsis* seed coat. Another study suggested that the extractable lignin content of the seed coat was lower in the *tt10* mutant (Liang et al., 2006).

In this study, we aimed to establish whether laccases were involved in the lignification of *Arabidopsis* stems. We addressed this issue by demonstrating that *LAC4* and *LAC17* were expressed in the lignified tissues of the inflorescence stems, making these genes good candidates for involvement in lignification. We

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produced two double mutants and studied the impact of single and double mutations on the lignification of inflorescence stems and on the corresponding lignified tissues recovered by laser capture microdissection. The various histological and chemical analyses, together with complementation of the *lac17* mutant with *LAC17* constructs, provided evidence that both the *LAC4* and *LAC17* genes are involved in the lignification of *Arabidopsis* stems.

RESULTS

Identification of *Arabidopsis* Laccases Strongly Expressed in Inflorescence Stems

According to the Web-based GeneCAT coexpression tool (http://genecat.mpg.de), eight of the 17 genes encoding laccases in *Arabidopsis* are expressed in the inflorescence stem to various extents (see Supplemental Figure 1 online) and might be involved in lignification (*LAC2*, *LAC4*, *LAC5*, *LAC6*, *LAC10*, *LAC11*, *LAC12*, and *LAC17*). We monitored the expression profiles of these genes in the stem during plant development by RT-PCR (Figure 1). The levels of most laccase transcripts increased from early developmental stages (stages 6.0 to 6.2; Boyes et al., 2001) to reach a plateau, decreasing thereafter when the plant reached maturity. This RT-PCR experiment revealed that *LAC4* and *LAC17* were strongly expressed in stems (Figure 1). In addition, the GeneCAT tool (http://genecat.mpg.de) indicated that *LAC4* and *LAC17* were coexpressed (see Supplemental Figure 1 online), suggesting functional redundancy between them. We therefore hypothesized that *LAC4* and *LAC17* were the candidate genes most likely to be involved in constitutive lignification.

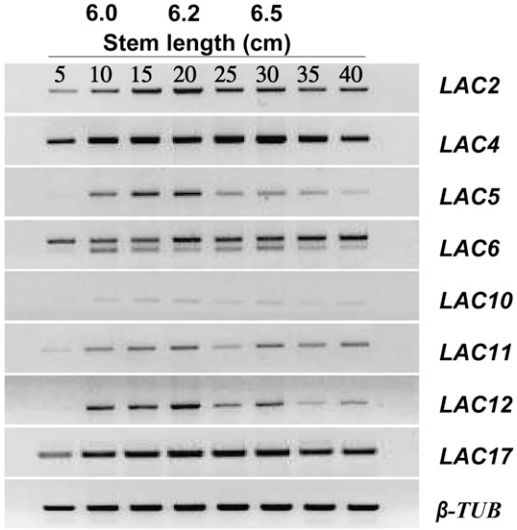

Figure 1. Laccase Gene Expression in *Arabidopsis* Floral Stems (Plants Grown under Long-Day Conditions).

RT-PCR expression profiles of eight laccase genes expressed in the inflorescence stem at eight different growth stages of *Arabidopsis*. Expression was normalized against the housekeeping β-TUBULIN (*β-TUB*) gene. Two signals were obtained for *LAC6*, corresponding to alternative splicing events. Growth stages, according to Boyes et al. (2001), are indicated at the top.

Molecular Characterization of *LAC4* and *LAC17* T-DNA Insertion Mutants and Production of the Corresponding Double Mutants

Two *LAC4* (S_051892, referred to as *lac4-1*, corresponding to *irx12* in Brown et al. [2005], and GabiKat-720G02, referred to as *lac4-2*) and one *LAC17* (S_016748, referred to as *lac17*) mutants were identified in the SALK and GABI collections (Table 1). According to Brown et al. (2005), the *irx12* mutant, identified on the basis of the coregulation of the corresponding gene, *LAC4*, with the *CesA* genes, has a weak irregular xylem (*irx*) phenotype. *LAC4* homozygous lines were selected by PCR, with primers binding before and after the T-DNA insertion site (see Supplemental Table 1 online). The flanking regions of the T-DNA insertions were amplified by PCR with specific primers (see Supplemental Table 1 online) and sequenced. This analysis revealed that *lac4-1* and *lac17* had T-DNAs inserted in their promoter sequences, 125 and 145 bp upstream from the ATG, respectively, whereas *lac4-2* had an insertion in the third exon of the coding sequence (Figure 2A). A single T-DNA insertion was found in the *LAC4* mutants, whereas two contiguous T-DNAs were found to be inserted in tandem in the *lac17* promoter. RT-PCR on total RNA extracted from the stem confirmed that these T-DNA insertion lines were null mutants for the corresponding genes (Figure 2B; see Supplemental Figure 2 online).

Homozygous *lac4-1* and *lac17* mutants were crossed to generate a double mutant. We checked the double mutant status of the offspring using primers specific for each mutation in the T2 progeny (see Supplemental Table 1 online). Surprisingly, *lac4-1 lac17* was found to display effective downregulation of *LAC17* but with the partial restoration of *LAC4* expression (Figure 2B). We therefore generated another double mutant by crossing *lac4-2* and *lac17* and established that both *LAC4* and *LAC17* were knocked out in this mutant (Figure 2B). Immunoblot analyses were performed with partially purified protein extracts from stem samples and an antibody specific for LAC17 (Figure 2C). Consistent with the findings of the transcriptomic studies, no LAC17 was detected in lines in which the *LAC17* gene was disrupted (Table 2).

We evaluated laccase expression levels in the single and double mutants by comparing laccase activity in protein extracts recovered from stems harvested at stage 6.4 (Boyes et al., 2001; Figure 2D). Consistent with the levels of expression of the genes for several stem-specific laccases, laccase activity was ~30% below control levels in *lac4-1*, *lac4-2*, *lac17*, and *lac4-1 lac17* stems. Moreover, in accordance with the simultaneous disruption of *LAC4* and *LAC17*, the double mutant *lac4-2 lac17* displayed a larger decrease (53%) in laccase activity (Figure 2D).

Phenotypes of the Lines Displaying *LAC4* and/or *LAC17* Downregulation

The *lac4-1*, *lac4-2*, *lac17*, and *lac4-1 lac17* mutants had growth and development characteristics similar to those of the wild-type

Table 1. List of Single Mutants Used in This Study

Gene Identification	Accession No.	Mutant Identification	Mutant Name	T-DNA Insertion Site	RT-PCR Confirmation
LAC4	At2g38080	Salk_051892	*lac4-1*	Promoter	Knockdown
		GabiKat-720G02	*lac4-2*	Exon	Knockout
LAC17	At5g60020	Salk_016748	*lac17*	Promoter	Knockdown

line grown under long-day or continuous light conditions (Figure 3). By contrast, the development of *lac4-2 lac17* was dependent on the growth conditions. We repeatedly observed that this double mutant had a normal size (Figure 3A) when grown in long-day conditions (either in the growth chamber or in the greenhouse). By contrast, it displayed a semidwarf phenotype (Figure 3B) when grown under continuous light in the growth chamber.

We further investigated the putative impact of the mutations at the tissue level by observing stem cross sections after Wiesner (Figures 3C to 3H) or Maüle (Figures 3I and 3J) staining. In the Wiesner test (phloroglucinol-HCl), lignins are stained a violet-red color, principally through reactions involving coniferaldehyde end groups (Nakano and Meshitsuka, 1992; Kim et al., 2002). Despite their low frequency in native lignins (<2% of the phenylpropane units) (Adler, 1977; Lapierre, 2010), these coniferaldehyde end groups efficiently detect lignins due to the high extinction coefficient of the coniferaldehyde-phloroglucinol adduct. Wiesner staining was weaker in the interfascicular fibers of *lac4-2 lac17* mutants than in other lines, regardless of the growth conditions (Figure 3H for plants grown under continuous light and in the growth chamber; see Supplemental Figure 3 online for plants grown in long-day conditions). This suggests that this mutant had a lower lignin level and/or lower coniferaldehyde end group content. Maüle reagent specifically stains S lignin units bright red. Maüle staining revealed no major staining differences between the control and *lac4-2 lac17* lines (Figures 3I and 3J). Accordingly, the deposition of S units did not seem affected in *lac4-2 lac17* fibers.

The weak *irx* phenotype reported by Brown et al. (2005) for the *irx12* mutant could not be observed in the stem sections of *lac4-1*, *lac4-2*, *lac17*, and *lac4-1 lac17* when grown in long-day conditions (see Supplemental Figure 3 online). In conditions that generate the semidwarf phenotype for *lac4-2 lac17* (i.e., continuous light in the growth chamber), some collapsed vessels were observed in the stem cross sections of *lac4-1* and *lac4-2* (Figures 3D and 3E). When its growth was not affected, the double mutant *lac4-2 lac17* still had vessels with a slight *irx* phenotype (see Supplemental Figure 3 online). By contrast, the semidwarf stems

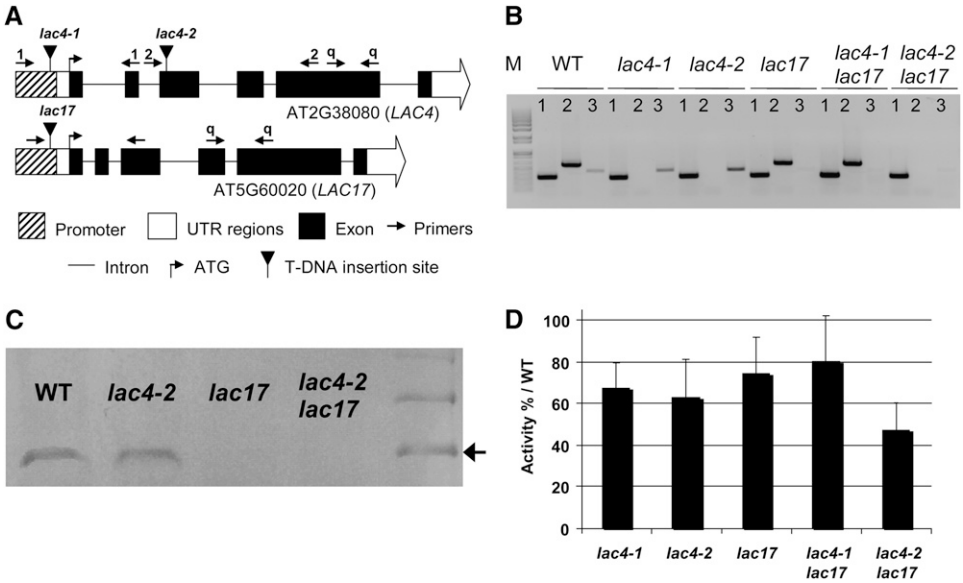

Figure 2. Characterization of Laccase T-DNA Insertion Mutants (Grown in Long-Day Conditions).

(A) Schematic diagram of the T-DNA insertion in *lac4-1*, *lac4-2*, and *lac17* mutants. The positions of primers used for genotyping *lac4-1* and *lac4-2* and for quantitative PCR are indicated by 1, 2, and q, respectively.

(B) Confirmation of the downregulation of laccase transcripts by RT-PCR. Lane 1, expression of the β-tubulin gene (housekeeping gene); lane 2, *LAC4* expression; lane 3, *LAC17* expression; lane M, 1 kb + ladder (Invitrogen).

(C) Immunoblot analysis with an antibody against *Arabidopsis* LAC17. The molecular mass of *Arabidopsis* LAC17 is indicated by an arrow at 75 kD.

(D) Quantification of laccase activity in partially purified protein extracts, with ABTS as the substrate. Data represent means ± SD (n = 3).

Table 2. A Selection of the Differentially Expressed Genes in the Five Laccase Mutants

Selected Gene Chip Hybridization Data						
Putative Gene Function	Accession No.	log2 Ratio *lac4-1*/WT	log2 Ratio *lac4-2*/WT	log2 Ratio *lac17*/WT	log2 Ratio *lac4-1 lac17*/WT	log2 Ratio *lac4-2 lac17*/WT
Monolignol Genes						
Phenylalanine ammonia-lyase 1 (*PAL1*)	At2g37040	−0.25	−0.57	−0.24	**−0.92**	**−1.09**
Phenylalanine ammonia-lyase 3 (*PAL3*)	At5g04230	−0.47	**−0.76**	−0.57	**−1.06**	**−1.59**
Phenylalanine ammonia-lyase 4 (*PAL4*)	At3g10340	0.01	−0.40	−0.01	**−1.43**	**−2.03**
Cinnamate 4-hydroxylase (*C4H*)	At2g30490	−0.28	−0.53	−0.32	**−1.25**	**−1.24**
4-Coumarate:CoA ligase 2 (*4CL2*)	At3g21240	−0.32	**−0.75**	−0.19	**−0.76**	**−0.78**
Coumarate 3-hydroxylase (*C3H*)	At2g40890	−0.07	−0.31	−0.14	**−0.76**	**−1.10**
Ferulate 5-hydroxylase (*F5H*)	At4g36220	0.04	−0.30	−0.07	−0.52	**−1.04**
O-methyltransferase 1 (*OMT1*)	At5g54160	**0.61**	0.55	0.31	−0.25	−0.22
Caffeoyl-CoA 3-*O*-methyltransferase (*CCoAOMT1*)	At4g34050	−0.16	−0.17	0.13	**−0.99**	**−0.63**
Cinnamyl alcohol dehydrogenase (*CAD-C*)	At3g19450	−0.11	−0.11	−0.13	−0.34	**−0.74**
Cinnamyl alcohol dehydrogenase (*CAD-D*)	At4g34230	−0.18	−0.45	−0.22	**−0.81**	−0.51
β-Glucosidase 46 (*βGLU46*)	At1g61820	−0.13	0.01	−0.10	0.03	**−0.62**
Laccase Genes						
Laccase 2 (*LAC2*)	At2g29130	−0.08	−0.27	−0.30	−0.38	−0.32
Laccase 4 (*LAC4*)	At2g38080	**−4.53**	**−4.21**	−0.05	**−1.03**	**−3.76**
Laccase 5 (*LAC5*)	At2g40370	0.28	0.15	−0.03	0.01	−0.10
Laccase 6 (*LAC6*)	At2g46570	0.01	−0.07	−0.04	0.15	0.13
Laccase 10 (*LAC10*)	At5g01190	−0.06	−0.11	0.03	−0.10	0.10
Laccase 11 (*LAC11*)	At5g03260	−0.12	0.02	−0.41	0.00	0.19
Laccase 12 (*LAC12*)	At5g05390	0.26	0.28	0.08	−0.42	−0.08
Laccase 17 (*LAC17*)	At5g60020	0.49	0.43	**−3.93**	**−4.36**	**−4.24**

The five mutant samples were analyzed with wild-type (WT) samples grown in the same conditions as references (in growth chamber and under long-day conditions). A negative ratio in bold is indicative of downregulation of the gene in mutants. A positive ratio in bold indicates that the gene is upregulated in mutants. Other ratios (not in bold) were not found to be statistically significant after Bonferroni correction ($P < 0.05$).

of *lac4-2 lac17* had more severely collapsed vessels (Figures 3H and 3J).

Effect of the *LAC4* and/or *LAC17* Mutations on the Expression of Other Genes

Complete *Arabidopsis* transcriptome microarray experiments were performed on RNA extracted from stems grown in a growth chamber under continuous light and collected at stage 6.2 (Boyes et al., 2001). *LAC4* and/or *LAC17* repression had no significant impact on the expression of other stem-specific laccases (Table 2). We also investigated the expression of some of the genes involved in the monolignol biosynthesis pathway. The *lac4-1*, *lac4-2*, and *lac17* single mutants displayed no significant modification of the level of expression of these genes (Table 2). In addition, the two double mutants, *lac4-1 lac17* and *lac4-2 lac17*, displayed decreases in the expression of 8 and 10 genes involved in lignin biosynthesis, respectively. Thus, disruption of both the *LAC4* and *LAC17* genes induced some feedback control on the genes of the phenylpropanoid (*C4H*, *PAL1*, *PAL3*, and *PAL4*) or lignin-specific (*CAD-C*, *CAD-D*, *CCoAOMT1*, *OMT1*, *F5H*, *C3H*, *4CL2*, and *β-GLU46*) pathways. Only a few other genes were affected in the single and/or double mutants. The functions of these genes were not known, with the exception of a small number of genes involved in tolerance to water stress or in copper metabolism (see Supplemental Data Set 1 online).

Impact of *LAC4* and/or *LAC17* Silencing on Lignification, Enzymatic Hydrolysis, and Soluble Phenolic Compounds in Mature *Arabidopsis* Stems

Lignin content was determined by measuring both the Klason lignin and acid-soluble lignin concentrations of the extract-free stems. Regardless of the growth conditions (long-day or continuous light), the single mutants *lac4-1*, *lac4-2*, and *lac17* consistently had lower Klason lignin levels (lignin levels 8 to 14% lower; Table 3) than the corresponding control. These levels were even lower in the double mutant *lac4-1 lac17* (~20% lower than the wild-type level). The *lac4-2 lac17* double mutant had much lower lignin levels (40% less than the control sample) regardless of its phenotype (normal or semidwarf size). Acid-soluble lignin contents were similarly low, whatever the sample (in the 1 to 2% range; Table 3) and did not compensate for the lower Klason lignin levels of the single and double mutants.

Lignin content is known to be a factor in decreasing cell wall susceptibility to enzymatic hydrolysis. We therefore subjected the various extract-free samples to treatment with a commercial cellulase preparation to evaluate the saccharification potential of the various single and double mutants. Consistent with their low

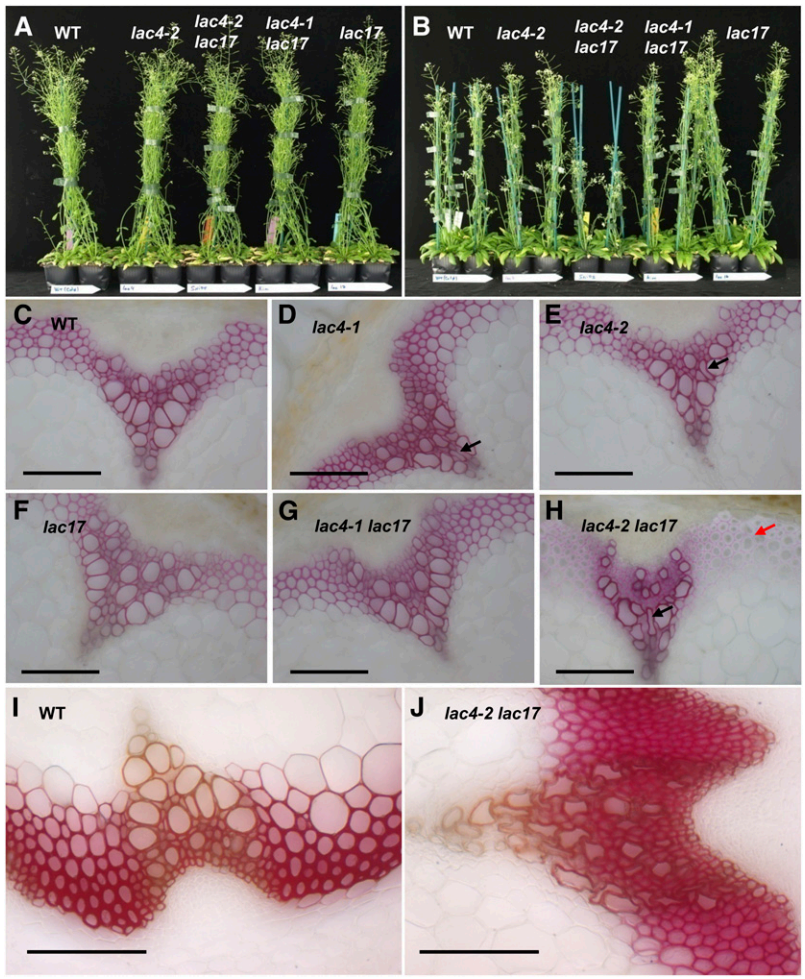

Figure 3. Phenotype of Laccase Mutants.

(A) Plants grown in the growth chamber (long-day conditions: no phenotype was observed).
(B) Plants grown in the growth chamber (continuous light: *lac4-2 lac17* displayed a semidwarf phenotype).
(C) to **(H)** Wiesner staining of stem cross sections from plants grown under continuous light. The black arrows show collapsed xylem, and the red arrow shows hypolignified fibers. Bars = 100 μm.
(I) and **(J)** Maüle staining of stem cross sections from wild-type and *lac4-2 lac17* plants grown under continuous light. Bars = 100 μm.

Klason lignin levels, commercial cellulase treatment (cellulase and hemicellulase activities) resulted in weight losses of 53 to 62% for *lac4-2 lac17* samples, much greater than those observed for the control samples (Table 3). About half of this loss could be accounted for by Glc (Table 3). Both the *lac4-1* and *lac4-2* stems displayed moderately higher levels of saccharification than the wild type, as shown by weight loss after cellulase treatment. By contrast, *lac17* was no more easily hydrolyzed than control samples despite having 11 to 14% lower lignin levels.

We evaluated the impact of laccase deficiency on lignin structure by thioacidolysis (Table 4). This analytical degradation method generates H, G, and S monomers from H, G, and S lignin units involved in β-O-4 bonds only (Lapierre et al., 1995). Thus, total monomer yield closely reflects the amounts of these lignin structures in the sample. In addition, when calculated on the basis of Klason lignin content, this yield is inversely correlated with the frequency of lignin units involved in resistant interunit linkages (condensed linkages). Whatever the sample, only small amounts of H thioacidolysis monomers were recovered (0.6 to 2 μmol per gram of extract-free sample); therefore, these monomers will not be discussed further below. Thioacidolysis yields calculated on the basis of lignin content did not significantly differ from control levels (arbitrarily set at 100) other than for the semidwarf *lac4-2 lac17* sample (obtained under continuous light). Thus, laccase deficiency did not affect the frequency of condensed bonds in the lignins of the single and double mutants, except for the semidwarf *lac4-2 lac17* sample, which contained lignins enriched in condensed bonds.

When calculated on the basis of extract-free stems, the total yields of monomers released by the laccase mutants were lower than the control value, due directly to the lower lignin levels of these mutants. The extract-free stems of the mutant lines with a

Table 3. Lignin Content and Enzymatic Hydrolysis of Mature Stems from Laccase Mutants

Growth Conditions	Line	Lignin Content		Enzymatic Hydrolysis	
		KL%	ASL%	Weight Loss%	Released Glc%
Long-day	Wild type	20.05 ± 0.18 (100)	1.37 ± 0.07	28.2 ± 1.0 (100)	9.0 ± 0.3
(no phenotype)	*lac4-2*	17.35 ± 0.13 (87)	1.70 ± 0.01	31.4 ± 0.1 (111)	9.3 ± 0.9
	lac17	17.26 ± 0.12 (86)	1.63 ± 0.05	28.2 ± 0.5 (100)	9.7 ± 0.4
	lac4-1 lac17	15.37 ± 0.02 (77)	1.95 ± 0.02	36.9 ± 0.1 (131)	11.0 ± 0.0
	lac4-2 lac17	12.66 ± 0.16 (63)	1.70 ± 0.01	52.6 ± 0.4 (187)	23.4 ± 0.4
Continuous light	Wild type	20.13 ± 0.09 (100)	1.04 ± 0.02	29.4 ± 0.4 (100)	9.9 ± 0.5
(semidwarf phenotype for *lac4-2 lac17)*	*lac4-1*	18.42 ± 0.01 (92)	1.10 ± 0.03	35.8 ± 0.6 (122)	13.6 ± 0.5
	lac4-2	18.27 ± 0.10 (91)	0.96 ± 0.01	35.9 ± 1.1 (122)	13.1 ± 0.7
	lac17	17.97 ± 0.13 (89)	1.20 ± 0.01	29.0 ± 0.4 (99)	9.4 ± 0.3
	lac4-1 lac17	16.22 ± 0.00 (81)	1.10 ± 0.01	30.6 ± 0.5 (104)	11.0 ± 0.2
	lac4-2 lac17	12.24 ± 0.01 (61)	1.56 ± 0.01	61.9 ± 0.5 (210)	30.5 ± 0.6

The Klason lignin (KL) and the acid-soluble lignin (ASL) contents are expressed as a percentage of the extract-free samples, in terms of weight. Data are mean values and standard errors from duplicate experiments. The values in parentheses are the percentages relative to the corresponding control.

LAC17 gene knockout released moderately (*lac17* and *lac4-1 lac17*) or substantially (*lac4-2 lac17*) smaller amounts of G thioacidolysis monomers than the other lines (Table 4). By contrast, they released S monomers in similar (*lac17* and *lac4-1 lac17*) or slightly smaller amounts (*lac4-2 lac17*, only when semidwarf) than the control. These variations made the S/G ratios of these mutants higher than those of the wild type or *LAC4* single mutants.

Several recent studies have shown soluble phenolic content to be higher in the hypolignified stems of various *Arabidopsis* mutants with impaired lignin biosynthesis than in the wild type (Abdulrazzak et al., 2006; Besseau et al., 2007; Mir Derikvand et al., 2008). We further evaluated the impact of laccase mutations on phenylpropanoid metabolism by studying the soluble phenolic compounds extracted from the basal and the upper parts of stems (Figure 4). The *lac17*, *lac4-1 lac17*, and *lac4-2 lac17* mutants released substantially more sinapoyl malate (162, 153, and 270 in the basal part of stems; 140, 139, and 204 in the upper part of stems, respectively) than the wild type (level arbitrarily set at 100). We also investigated the flavonol glycoside pool of the samples, considering three kaempferol glycosides as the major representatives of this pool (Mir Derikvand et al., 2008). These flavonol glycosides were recovered in larger amounts from the basal and upper parts of *lac4-2 lac17* stems (172 and 129, respectively) than from the wild type (level arbitrarily set at 100). By contrast, the amount of soluble phenolic compounds released from *lac4-1* or *lac4-2* samples was similar to that from the wild type (Figure 4). Thus, disruption of the *LAC17* gene induced redirection of the phenylpropanoid pathway. Greater redirection was observed in *lac4-2 lac17* stems, which had the lowest lignin content, than in other mutant stems.

Evaluation of the Tissue Specificity of *LAC4* and *LAC17*

According to in silico expression data, *LAC4* and *LAC17* are strongly expressed in the developing stems of *Arabidopsis*. We tried to localize the expression of these genes more precisely at the tissue level by inserting a 2.0-kb fragment corresponding to

Table 4. Determination of the Main H, G, and S Thioacidolysis Monomers Released by the Lignins of Extract-Free Mature Stems of Laccase Mutants

Growth Conditions	Line	Thioacidolysis Yield in μmole per Gram of Extract-Free Sample				S/G Molar Ratio	Relative Yield Based on Lignin Content[a]
		H	G	S	Total		
Long-day	Wild type	2.0 ± 0.3	179 ± 7	74 ± 4	254 ± 11	0.41 ± 0.02	100
(no phenotype)	*lac4-2*	1.4 ± 0.3	153 ± 17	62 ± 7	216 ± 24	0.41 ± 0.01	91 ± 10
	lac17	1.0 ± 0.2	143 ± 10	72 ± 2	215 ± 13	0.50 ± 0.02	93 ± 6
	lac4-1 lac17	1.1 ± 0.1	146 ± 9	87 ± 7	234 ± 16	0.59 ± 0.01	107 ± 7
	lac4-2 lac17	0.6 ± 0.2	114 ± 2	83 ± 6	198 ± 8	0.71 ± 0.04	107 ± 5
Continuous light	Wild type	1.4 ± 0.8	213 ± 1	93 ± 2	308 ± 4	0.44 ± 0.01	100
(semidwarf phenotype	*lac4-1*	1.0 ± 0.1	177 ± 1	83 ± 2	261 ± 3	0.47 ± 0.01	93 ± 1
for *lac4-2 lac17)*	*lac4-2*	1.1 ± 0.0	178 ± 3	79 ± 1	258 ± 2	0.45 ± 0.01	93 ± 2
	lac17	1.2 ± 0.1	171 ± 9	91 ± 4	263 ± 13	0.53 ± 0.01	96 ± 5
	lac4-1 lac17	1.1 ± 0.0	161 ± 1	101 ± 2	263 ± 3	0.63 ± 0.01	106 ± 3
	lac4-2 lac17	1.4 ± 0.0	78 ± 3	64 ± 1	143 ± 4	0.82 ± 0.02	76 ± 1

Data are mean values and standard errors from duplicate analyses.

[a]The relative thioacidolysis yield corresponds to the total yield of (H+G+S) monomers calculated per gram of Klason lignin content and compared to the corresponding control value set at 100.

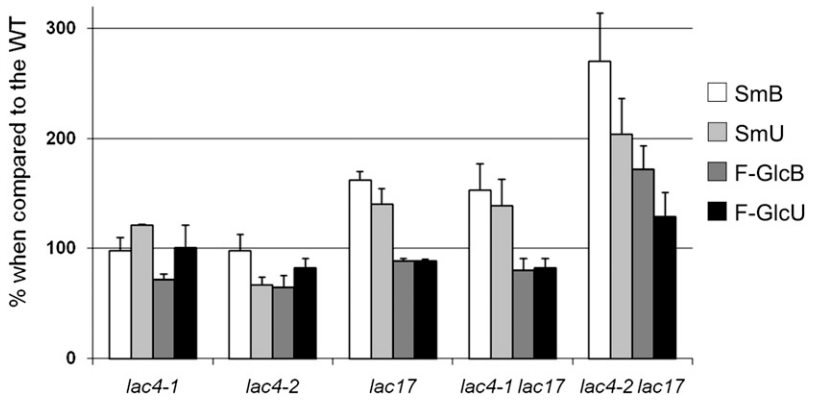

Figure 4. Analyses of Soluble Phenolic Compounds Extracted from the Stems of *lac4-1*, *lac4-2*, *lac17*, *lac4-1 lac17*, and *lac4-2 lac17* Mutants (Grown in Long-Day Conditions).

Data are mean values from three to nine biological replicates. Error bars indicate SD (n = 3 to 9). All results are expressed as percentages of wild-type levels (arbitrarily set at 100). SmB, sinapoyl malate in the basal part of stems; SmU, sinapoyl malate in the upper part of stems; F-Glc B, flavonol glycosides in the basal part of stems; F-Glc U, flavonol glycosides in the upper part of stems.

the promoters of *LAC4* and *LAC17* into p*BI101GUS-GTW*, a vector containing the β-glucuronidase (*GUS*) reporter gene (Baudry et al., 2006; Dubos et al., 2008). The resulting constructs were introduced into *Arabidopsis* via *Agrobacterium tumefaciens* transformation, and several transgenic lines were obtained for each construct. *GUS* expression was monitored in T1 lines and evaluated more precisely in selected T2 plants. *LAC4* was strongly expressed in the differentiating xylem and stem fibers, both highly lignified tissues (Figure 5A). By contrast, *LAC17* seemed to be more specifically expressed in the interfascicular fibers (Figure 5B). The localization of the LAC4 and LAC17 proteins was further studied by confocal microscopy with fluorophore-conjugated specific antibodies (see Supplemental Figure 4 online). LAC4 could be detected in the cambium of vascular bundles (see Supplemental Figure 5 online). It was also observed in the secondary wall and in the middle lamella of interfascicular fibers (see Supplemental Figure 6 online). By contrast, LAC17 could be detected only in fibers and not in vascular bundles (see Supplemental Figure 5 online).

As these experiments revealed the tissue specificities of *LAC4* (vascular bundles and fibers) and *LAC17* (fibers), we then investigated the impact of the downregulation of these genes on the lignification of specific tissues. We performed thioacidolysis on stem tissues recovered by laser capture microdissection. Vascular bundles and interfascicular fibers were recovered from stems collected at stage 6.4 (Boyes et al., 2001) for the wild-type, *lac4-2*, and *lac17* lines. The reproducibility of thioacidolysis yields between biological duplicates was poor. By contrast, measurements of S/G ratio were reproducible (standard errors in the 2 to 10% range). Consistent with previous findings (Ruel et al., 2009), analyses of microdissected samples confirmed that interfascicular fibers were richer in S units than vascular bundles (Table 5). The S/G ratios of vascular bundles did not clearly distinguish the mutant lines from the wild type. By contrast, the S/G ratio was higher in *lac4-2* fibers (S/G = 0.75) and much higher in *lac17* fibers (up to 0.91) than in wild-type fibers (S/G = 0.57). This finding supports the hypothesis that a LAC17 deficiency affects the deposition of G lignin units in interfascicular fibers.

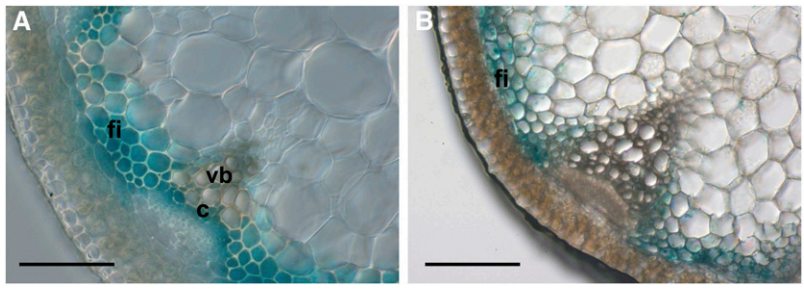

Figure 5. Localization of Laccase Transcripts in Inflorescence Stem Cross Sections of *GUS* Promoter Fusion Lines (from Plants Grown under Long-Day Conditions).

(A) Expression profile of the 2-kb Pro*LAC4*:*GUS*.
(B) Expression profile of the 2-kb Pro*LAC17*:*GUS*.
fi, fibers; vb, vascular bundle; c, cambium. Bars = 100 μm.

Complementation of *lac17* with Various Constructs

For confirmation of the role of *LAC17* in lignification, *lac17* was complemented with various constructs (Figure 6A). As a higher thioacidolysis S/G ratio was systematically obtained in *LAC17*-silenced mutants, the effectiveness of complementation was checked by subjecting the stems of the various transformants to thioacidolysis based on the rationale that effective complementation should restore the S/G ratio to control levels. Complementation was first assayed with the coding sequences of *LAC17* (from the ATG to the stop codon) under the control of the cauliflower mosaic virus 35S promoter. This complementation was unsuccessful because the S/G ratio of the mutant was not restored to control levels (see Supplemental Figure 7 online).

Another assay was then performed with the genomic sequence of *LAC17* under the control of its endogenous promoter with and without the 3′ untranslated region (UTR). The degree of restoration of the S/G ratio indicated that complementation was partially effective in four transformants obtained without the 3′ UTR (Figure 6B), whereas it was complete in two transformants complemented with the construct containing the 3′ UTR (Figure 6C). The effectiveness of complementation with the construct containing the 3′ UTR was further confirmed by Klason analyses of the extract-free stems (Table 6).

DISCUSSION

The peroxidases and laccases potentially involved in the oxidation of lignin precursors in plant cell walls are encoded by multigene families. In *Arabidopsis*, 73 peroxidases and 17 laccases have been identified (Tognolli et al., 2002; Pourcel et al., 2005). Despite the high degree of redundancy of peroxidase genes, making it difficult to identify lignin-specific enzymes, peroxidase activity has clearly been implicated in the polymerization of lignins. The most compelling evidence of lignin-specific peroxidases has been provided by a small number of studies of transgenic or mutant plants in which the down- or upregulation of genes encoding particular peroxidases affected lignification (reviewed in Fagerstedt et al., 2010). By contrast, despite the smaller number of laccase genes, the role of laccases in lignification remains less clear. No one has yet demonstrated conclusively that laccases are involved in the lignification of stems. Only two studies have reported alterations to lignification in mutants or transgenic plants with impaired laccase gene expression. Levels of lignin 30% lower than those of the wild type have been found in the seed coat of the LAC15-deficient *tt10 Arabidopsis* mutant, as determined in thioglycolic acid assays (Liang et al., 2006). Using the same method, Wang et al. (2008) reported 19.6% higher UV absorbance by the thioglycolic acid extract recovered from a poplar transgenic line overexpressing a cotton laccase gene than by the control.

Table 5. Lignin Composition in the Vascular Bundles and in the Interfascicular Fibers of Wild-Type, *lac4-2*, and *lac17* Inflorescence Stems (of Plants Grown under Long-Day Conditions)

	S/G Molar Ratio	
Lines	Vascular Bundles	Fibers
Wild type	0.36 ± 0.05	0.57 ± 0.04
lac4-2	0.30 ± 0.01	0.75 ± 0.01
lac17	0.28 ± 0.02	0.91 ± 0.04

The S/G ratio was measured by thioacidolysis of selected tissues collected by laser capture microdissection. Data are mean values and standard errors from two samples (50 microdissections for each one) per tissue type.

Laccase Gene Expression Profiles in *Arabidopsis* Stems and the Generation of Two *LAC4 LAC17* Double Mutants

Consistent with previous transcript profiling in *Arabidopsis* primary stems (Ehlting et al., 2005), we found that *LAC4* and *LAC17* were strongly expressed in stems. Other studies have confirmed that *LAC4* and *LAC17* are coexpressed with *CesA* genes (Brown et al., 2005) and with the cinnamyl alcohol dehydrogenase (*CAD*) genes *CAD-C* and *CAD-D*, which are specifically involved in monolignol biosynthesis (Sibout et al., 2005). We therefore selected two different *LAC4* mutants, *lac4-1* and *lac4-2*, from the SALK and GABI collections, respectively, and a *lac17* mutant from the SALK collection. This choice was further supported by expression profiles from plants harboring the *LAC4* or *LAC17* promoter regions fused to the *GUS* reporter gene. *LAC4* expression was observed in both vascular bundles and interfascicular fibers, whereas *LAC17* expression seemed to be more specific to fibers. These results are consistent with the expression profile determined in another study using the same reporter gene (Koizumi et al., 2009).

We then generated two double mutants by crossing *lac4-1* and *lac17* and by crossing *lac4-2* and *lac17*. Surprisingly, *lac4-1 lac17* displayed a partial restoration of *LAC4* transcript levels but efficient downregulation for *LAC17*. This restoration may be accounted for by the insertion of the *lac4-1* T-DNA into the promoter of the *LAC4* gene rather than its coding sequence. For unknown reasons, the promoter was not operational in the *lac4-1* single mutant, but its function was partially restored in the *lac4-1 lac17* double mutant. By contrast, the other double mutant, *lac4-2 lac17*, displayed effective silencing for both the *LAC4* and *LAC17* genes. In this double mutant *lac4-2 lac17*, laccase activity was reduced (to ~50% of control levels), further confirming that LAC4 and LAC17 make a major contribution to total laccase activity in stems.

Disruption of *LAC4* and/or *LAC17* Affects Plant Development, Vascular Tissues, Lignification, and Saccharification Efficiency to Various Extents

The finding that *lac4-2 lac17* displayed either normal development or a semidwarf phenotype, depending on the growth conditions, suggested an effect of stress-inducing conditions. The *LAC2*-deficient mutant has also been reported to display developmental defects (altered root elongation) only when subjected to dehydration stress (Cai et al., 2006). The precise conditions inducing the semidwarf phenotype in *lac4-2 lac17* should be investigated in a more systematic and comprehensive study.

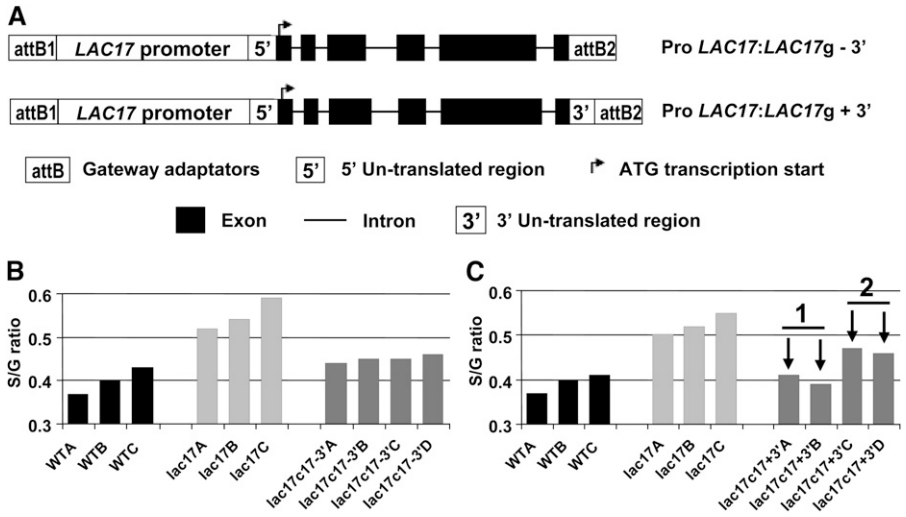

Figure 6. Complementation of *lac17* with Various Constructs.

The effectiveness of the complementation was tested by thioacidolysis of mature stems (from plants grown under long-day conditions), assuming that *lac17* mutants had stem lignins with a higher S/G thioacidolysis ratio.

(A) Schematic diagram of the Gateway constructs used for *lac17* complementation.

(B) Impact of *lac17* complementation by the endogenous promoter and genomic sequence of *LAC17* without the 3′ UTR (Pro*LAC17*:*LAC17*g - 3′) on the S/G ratio.

(C) Impact of *lac17* complementation by the endogenous promoter and genomic sequence of *LAC17* with the 3′ UTR (Pro*LAC17*:*LAC17*g + 3′) on the S/G ratio. The arrows indicate two lines selected for further lignin content determination on their pooled extract-free stems (*lac17*c17 +3′ 1; Table 6); the stems of the other two lines were pooled as *lac17*c17 +3′ 2 (*lac17*c17 +3′ 2; Table 6).

The *irx12* mutant (*lac4-1* in this work) has been reported to display a weak irregular xylem phenotype that varies in severity between plants and even between the vascular bundles of the same plant (Brown et al., 2005). In our growth conditions, only *lac4-2 lac17* consistently displayed a moderate to severe *irx* phenotype. In the other *LAC4*-deficient mutants (*lac4-1*, *lac4-2*, and *lac4-1 lac17*), some collapsed vessels were sporadically observed. These results suggest that xylem morphology is not greatly affected when mutants in which *LAC4* is silenced are grown in optimum conditions. The *lac17* mutant did not display any *irx* phenotype, consistent with the expression profile of *LAC17*, which tended to be specifically expressed in fibers.

The extract-free stems of the single mutants had consistently low lignin levels. Surprisingly, these lower lignin levels are reported here for the first time for the *lac4-1* mutant, which has been studied before (Brown et al., 2005). Up to eight laccase genes are expressed in *Arabidopsis* stems. However, the silencing of a single *laccase* gene (*LAC4* or *LAC17*) is sufficient to decrease lignin content slightly, but noticeably, as shown for two different *LAC4* insertion mutants and one *LAC17* insertion mutant. The larger decrease (by 40%) observed in the lignin level of the double *lac4-2 lac17* knockout mutant was not necessarily associated with a smaller size. Indeed, the semidwarf phenotype of *lac4-2 lac17* was obtained in continuous light, which could be related to growth in stress-inducing conditions. When *lac4-2 lac17* was not subjected to stress during its growth, its size was similar to that of wild-type plants, despite its much lower lignin content. Several studies have reported the absence of a strict correlation between low lignin levels (30 to 40% lower than the wild type) and dwarfism. For instance, the *cad c cad d* double mutant has 40% lower Klason lignin levels than the wild type but displays no dwarfism, only a bending stem phenotype (Sibout et al., 2005). The stems of the *CCoAOMT1* mutant are also normal in size, despite having lignin levels ~30% lower than those of the wild type (Do et al., 2007). By contrast, two *CCR1*-deficient mutants display a 40% decrease in lignin levels and a dwarf phenotype, whatever the growth conditions (Mir Derikvand et al., 2008). In this study, the hypolignified *lac4-2 lac17* stems were either normal in size or semidwarf. The reason for the dwarfing of mutant stems does not seem to be directly related to

Table 6. Klason Lignin and Acid-Soluble Lignin Content in Extract-Free Mature Stems of *lac17*-Complemented Lines (Expressed as Weight Percentage)

Line	KL%	ASL%
Wild type	20.34 ± 0.05	0.89 ± 0.01
lac17	16.75 ± 0.03	0.85 ± 0.01
lac17c17+3′ 1	22.66 ± 0.13	0.81 ± 0.01
lac17c17+3′ 2	20.39 ± 0.02	0.87 ± 0.02

The analyses were performed for two sets of transgenic lines obtained with the endogenous promoter and genomic sequence of *LAC17* with the 3′ UTR. The two complemented samples were obtained by pooling the stems from transgenic lines in which the S/G ratio was completely (*lac17*c17 +3′ 1) or partially (*lac17*c17 +3′ 2) restored to wild-type levels. Data are mean values and standard errors from duplicate analyses. KL, Klason lignin; ASL, acid-soluble lignin.

their low lignin content. Instead, it seems to be more strongly correlated with the sites at which lignin levels are decreased and to the appearance of an irregular xylem phenotype.

It often has been suggested that lower levels of growth result from the accumulation of flavonol glycosides (Abdulrazzak et al., 2006; Besseau et al., 2007; Mir Derikvand et al., 2008). However, growth reduction in the hypolignified *C3H Arabidopsis* mutant has recently been shown to be independent of flavonoids (Li et al., 2010). The stems of *LAC17* mutants (*lac17*, *lac4-1 lac17*, and *lac4-2 lac17*) contained high levels of sinapoyl malate. This accumulation seems to be correlated with the decrease in lignin levels, providing support for the hypothesis that phenolic intermediates not used for lignification in the *LAC17*-deficient mutants are redirected for sinapoyl malate biosynthesis. This redirection seems to occur even when the flux to lignin is decreased only slightly (by 10% in the *lac17* single mutant). By contrast, the soluble phenolic pool is not affected in the *lac4-1* and *lac4-2* mutants, highlighting the complexity of crosstalk between lignin formation and the biosynthesis of other plant phenolics.

We evaluated lignin structure by thioacidolysis. As the lignin structures generating the H, G, and S thioacidolysis monomers are restricted to those involved in β-O-4 bonds, a higher frequency of resistant bonds (referred to as condensed bonds) in lignins has been demonstrated by low thioacidolysis yields (expressed in μmoles per gram of lignin) in various *Arabidopsis* mutants, such as *COMT*-, *CAD*-, *C3H*-, and *CCR*-deficient mutants (Goujon et al., 2003; Sibout et al., 2005; Abdulrazzak et al., 2006; Mir Derikvand et al., 2008). By contrast, the silencing of *LAC4* or *LAC17* had no effect on the frequency of condensed bonds in stem lignins, except for the semidwarf *lac4-2 lac17* samples (thioacidolysis yield 25% lower than the control level when calculated in μmoles per gram of lignin). This result provides further support for the hypothesis that the semidwarf phenotype of *lac4-2 lac17* is induced by stress, as lignins formed in response to stress are enriched in condensed bonds (Cabané et al., 2004; Betz et al., 2009).

Lignin composition, as reflected by the S/G thioacidolysis ratio, was affected to various extents. No clear change was observed in the *lac4-1* and *lac4-2* mutants. By contrast, the S/G ratio of stem lignins was altered in *LAC17*-deficient mutants to an extent that seemed to be correlated with the decrease in lignin levels. The higher S/G ratio of *LAC17*-silenced mutants resulted principally from a lower recovery of G monomers, whereas the recovery of S monomers from the extract-free cell walls was not affected. In other words, the lower lignin levels of *LAC17*-deficient mutants resulted essentially from lower levels of G lignin unit deposition.

Thioacidolysis of laser capture microdissected vascular bundles and of interfascicular fibers provided conclusive evidence about the tissue specificity of the LAC4 and LAC17 enzymes. Consistent with previous findings (Chapple et al., 1992; Ruel et al., 2009), the interfascicular fibers of the wild type had a higher S/G ratio than the corresponding vascular bundles. This result is consistent with many published data for angiosperm lignins, reporting a higher abundance of S units in supportive tissues, with G-rich lignins found in the xylem. The silencing of *LAC4* and *LAC17* induced a weak or more pronounced increase in fiber S/G ratio, respectively, whereas the analyses of *lac4-1* or *lac4-2* stems did not reveal any effect on S/G. This highlights the importance of using analytical methods capable of providing information about lignin variability in individual tissues.

Unsurprisingly, the stems of *lac4-2 lac17* with 40% lower lignin levels displayed markedly higher levels of saccharification when treated with a commercial cellulase preparation in the absence of pretreatment. Accordingly, laccase silencing may be a promising strategy for increasing the saccharification of plant cell walls when used for lignocellulose-to-ethanol biological conversion. Consistent with the small decrease in lignin content observed in the *lac4-1* and *lac4-2* mutants, only a slight increase in saccharification was observed in these mutants. By contrast, despite having a substantially lower lignin content, *lac17* had a level of saccharification similar to that of the control sample. The reasons for this unexpected result remain unclear, but it shows that factors other than lignin content may affect the susceptibility of cell walls to enzymatic hydrolysis.

The Large Decrease in Lignin Biosynthesis in the Double Mutant Silenced for *LAC4* and *LAC17* May Result from Broader Changes to the Phenylpropanoid Pathway

Disrupting both *LAC4* and *LAC17* had no significant effect on the expression of the other stem-specific laccase genes. By contrast, in *lac4-2 lac17* and, to a lesser extent, in *lac4-1 lac17*, several genes involved in the phenylpropanoid pathway were found to be downregulated. This coordinated downregulation may partly account for the large decrease in metabolic flux to lignins in this mutant. Microarray analyses revealed no other major changes in the expression of genes for other metabolic pathways, by contrast with the *pal1 pal2 Arabidopsis* mutant (Rohde et al., 2004), the *cad c cad d Arabidopsis* mutant (Sibout et al., 2005), and the poplar (Leplé et al., 2007) or tobacco (*Nicotiana tabacum*; Dauwe et al., 2007) lines presenting *CCR* downregulation.

The Final Piece in the Puzzle, Demonstrating the Existence of Lignin-Specific Laccases, Is Provided by Complementation Experiments with a Laccase-Deficient Mutant

For a conclusive demonstration of the involvement of *LAC4* or *LAC17* in the constitutive lignification of *Arabidopsis* stems, it was of the utmost importance to restore the control profile from the altered lignin profile through appropriate complementation experiments. Therefore, we chose to complement the *lac17* mutant, based on the rationale that there are two lignin parameters to be restored: S/G ratio and lignin content. Complete restoration could be obtained for transgenic lines only when the *LAC17* genomic sequence was used under the control of its own 2-kb promoter, including the 3′ UTR region. Complementation was partial in the absence of the 3′ UTR, as already reported for complementation studies of the *Arabidopsis tt10* mutant (Pourcel et al., 2005). This 3′ UTR may help to stabilize the transcript, as proposed by Gutiérrez et al. (1999). The effective complementation of *lac17* by the genomic sequence of *LAC17* under the control of its full-length promoter involved full restoration not only

of S/G ratios but also of Klason lignin levels in stems to control values. This provides definitive proof of the involvement of *LAC17* in the constitutive lignification of *Arabidopsis* inflorescence stems.

This study demonstrates that both *LAC4* and *LAC17* are involved in the constitutive lignification of *Arabidopsis* floral stems. Based on all the results obtained in expression profile studies, histochemical analyses, and wet chemistry analyses, we can suggest the following. Both the LAC4 and LAC17 laccases play major roles in the lignification of *Arabidopsis* stems, with LAC17 being more specific to interfascicular fibers. The silencing of these lignin-specific laccase genes markedly decreases lignin biosynthesis, with some redirection to sinapoyl malate synthesis. The silencing of *LAC17* specifically affects the deposition of G lignin units in fibers, whereas the unit specificity of *LAC4* is less clear. Previous studies (Sterjiades et al., 1992; Donaldson, 2001) suggested a role for laccases in the early steps of lignin polymerization. It is now well established that lignification begins with the deposition of G lignin units (Terashima and Fukushima, 1993). Therefore, we can hypothesize that LAC17 participates in the early stages of lignification accompanying the deposition of G lignin units. These findings suggest that the genetic engineering of lignin-specific laccases is a potentially innovative and promising tool for the fine-tuning of lignin content and structure.

METHODS

Plant Materials and Growth Conditions

All the plants used were from the Columbia background (Col-0). The *lac4-1* (S_051892) and *lac17* (S_016748) mutants were obtained from the Salk Institute T-DNA insertion collection. The *lac4-2* (GK-720G02) mutant was obtained from the GabiKat collection. The *lac4-1 lac17* and *lac4-2 lac17* double mutants were isolated from the F2 population of the *lac4-1 lac17* and *lac4-2 lac17* crosses, respectively. Homozygous plants were obtained by genomic PCR. The list of primers is reported in Supplemental Table 1 online. Most cultures were performed in long-day conditions (16/8 h light/dark) in a greenhouse or growth chamber (20°C, 60% relative humidity). Some cultures were performed in a growth chamber under continuous light (21°C, 60% relative humidity). For RT-PCR and microarray analyses, plants were arranged at random in the same growth chamber and grown under long-day conditions.

Histology

Cross sections were cut from stems collected at development stage 6.4 (Boyes et al., 2001), corresponding to a plant height of 30 cm. Wiesner staining was performed with phloroglucinol-HCl (Prolabo), and Maüle staining was performed by incubating sections in 1% $KMnO_4$. After incubation in the staining solution for 7 min, sections were washed and acidified by incubation with 30% HCl for 1 min. They were then washed again and incubated with 5% $NaHCO_3$.

GUS Fusion Constructs for the Analysis of Promoter Activity and Histochemical Detection of GUS Activity

We used a 1.95-kb promoter fragment for *LAC4* and a 2-kb promoter fragment for *LAC17*. These promoter fragments were amplified by PCR with primers containing *attB1* and *attB2* GATEWAY recombination sites (see Supplemental Table 1 online) and introduced into the pDONR207 vector by BP recombination. The promoter fragments were transferred into the binary vector pBI101GUS-GTW by LR recombination (Baudry et al., 2006; Dubos et al., 2008). The integrity of the constructs was checked by sequencing and plants were transformed as described by Clough and Bent (1998).

The promoter expression profile was characterized by measuring *GUS* activity in at least five independent lines of transformants. All the plants were genotyped for the presence of the corresponding constructs. The same pattern of promoter activity was observed in T2 plants.

Histochemical analyses of GUS activity were performed as described by Debeaujon et al. (2003). Samples were incubated under a vacuum for 1 h and then for 12 h at 37°C in the dark. Chlorophyll was removed by incubating the samples in 70% (v/v) ethanol/water at room temperature. Tissues were stained by incubation with 5-bromo-4-chloro-3-indolyl glucuronide for 3 h at room temperature. The stained tissues were then cleared by incubation with a chloral hydrate solution (chloral hydrate: distilled water:glycerol [8:2:1,w/v/v]) and observed as whole mounts and stem cross sections. Tissues were observed under a binocular or Axioplan 2 microscope (Zeiss) equipped with bright-field optics and with Nomarski differential interference contrast optics.

Immunolocalization by Indirect Immunofluorescence Analysis

Immunolocalization was performed on stem cross sections from plants at stage 6.2 (Boyes et al., 2001) according to the method of Masclaux-Daubresse et al. (2006), modified as follows: (1) slides were incubated with the same rabbit antibodies as for immunoblots; (2) slides were then incubated in Evans Blue solution (0.001% in PBS: 10 min) to decrease the autofluorescence of lignified cells; and (3) after incubation with the secondary antibodies (goat anti-rabbit IgG labeled with Alexa 488; Molecular Probes), slides were treated with 4',6-diamidino-2-phenylindole. Immunofluorescence was observed with an epifluorescence microscope (DMRB DIC; Leica), and the specificity of the Alexa 488 signal was determined with a spectral confocal laser scanning microscope. No signal was observed when using the preimmune serum.

RNA Extraction and RT-PCR

Stems of plants grown in the same growth chamber were collected at stage 6.2 (Boyes et al., 2001). For each line, we pooled the entire stems of three plants to constitute one sample. Samples were immediately frozen in liquid nitrogen, ground to powder, and stored at −80°C until RNA extraction.

RNA was extracted with the Qiagen RNeasy Plant Minikit according to the manufacturer's instructions. Qiagen DNase was applied to the column to eliminate contaminating DNA. RNA samples were quantified with a Nanodrop spectrophotometer (ND-1000; Labtech). Reverse transcription was performed with 1 μg of total RNA in a final volume of 20 μL, with Superscript Reverse Transcriptase II (Invitrogen), used according to the manufacturer's instructions. PCR was performed in a thermocycler (GeneAmp PCR System 2700).

Transcriptome Studies

Microarray analysis was performed on complete *Arabidopsis thaliana* transcriptome microarrays containing 24,576 GSTs corresponding to 22,089 genes from *Arabidopsis* (Crowe et al., 2003; Hilson et al., 2004). Two independent biological replicates were performed. For each biological replicate and each point, we pooled the RNA from three plants to generate each sample. We collected 20-cm-long stems from three plants at growth stage 6.2 (Boyes et al., 2001). The plants concerned had been grown in a growth chamber under long-day conditions. Total RNA was extracted with the RNeasy Plant Kit (Qiagen) according to the manufacturer's instructions. For each comparison, one technical replicate with fluorochrome reversal was performed for each biological replicate (i.e.,

four hybridizations per comparison). We labeled cRNAs with Cy3-dUTP or Cy5-dUTP (Perkin-Elmer-NEN Life Science Products) and performed hybridization and scanning of the slides as previously described (Lurin et al., 2004).

Statistical Analysis of Microarray Data

Experiments were designed with the statistics group of the Plant Genomics Research Unit. Statistical analysis was performed with normalization based on dye swapping (i.e., four arrays, each containing 24,576 GSTs and 384 controls) as previously described (Gagnot et al., 2008). For the identification of differentially expressed genes, we performed a paired *t* test on log ratios, assuming that the variance of the log ratios was similar for all genes. Spots with extreme variances (too small or too large) were excluded. The raw P values were adjusted by the Bonferroni method, which controls the family-wise error rate (with a type I error equal to 5%) to minimize the number of false positives in a multiple-comparison context (Ge et al., 2003). We considered genes with a Bonferroni P value ≤ 0.05 to be differentially expressed, as previously described (Gagnot et al., 2008).

Data Deposition

Microarray data from this article were deposited at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/, accession number GSE22907) and at CATdb (http://urgv.evry.inra.fr/CATdb/; Project: RA10-01_Laccases) according to Minimum Information about a Microarray Experiment standards.

Purification of *Arabidopsis* Laccases

About 7 g of *Arabidopsis* stems was homogenized in 7 mL of extraction buffer (25 mM BisTris, pH 7, 200 mM $CaCl_2$, 10% [v/v] glycerol, 4 μM sodium cacodylate, and 1/200 [v/v] protease inhibitor cocktail [P-9599; Sigma-Aldrich]) for 5 min in a blender. The homogenate was centrifuged twice at 8°C, for 5 min each, at 3000*g* and once at 4°C for 5 min at 13,000*g*. The supernatant was centrifuged at 8°C for 45 min at 15,000*g*. Proteins were purified by affinity chromatography on a 0.5 $\times$ 3-cm column filled with 1 mL of Concanavalin-A Sepharose (Sigma-Aldrich) and washed with 3 mL of 20 mM Tris-HCl and 0.5 M NaCl buffer, pH 7.4. The soluble protein extract was loaded and the column was washed with 10 mL of buffer. The proteins were eluted with 0.2 M methyl-α-glucopyranoside in the same buffer. The eluates were collected (1 mL per fraction), and 3 or 5 μL samples from each fraction were tested for laccase activity. Pooled fractions showing laccase activity were equilibrated in 25 mM Tris-HCl buffer, pH 7.4, supplemented with 5% glycerol (v/v) and 0.015% Triton X-100 (v/v). Glycerol was added to the buffer to prevent partial inactivation of the enzymes.

SDS-PAGE and Protein Gel Blot Analysis

Protein-denaturating SDS-PAGE was performed with 10% polyacrylamide gels. Standard markers (molecular range 15 to 100 kD; Sigma-Aldrich) were used to determine the approximate molecular masses of purified proteins in Coomassie Brilliant Blue–stained gels. Proteins were transferred onto a 0.45-μm Hybond ECL membrane (Amersham Biosciences) by electroblotting. Proteins were detected with alkaline phosphatase–conjugated primary monoclonal antibodies against laccases and secondary antibodies against alkaline phosphatase conjugate.

In Vitro Laccase Activity Assays

An 11 mg mL^{-1} solution of the substrate, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS), was prepared in DMSO and stored in aliquots at -20°C. Laccase activity was determined at 30°C by the oxidation of ABTS to generate a stable cationic radical assayed by spectrometry at 420 nm. The reaction mixture contained 100 mM acetate buffer, pH 5, 1 mM ABTS, and 20 μL of protein extract in a total volume of 200 μL.

Lignin Analysis

Dry mature stems from control and transgenic lines were harvested. Siliques and leaves were systematically removed. Extract-free samples were obtained by removing all soluble compounds with a Soxhlet apparatus by sequentially extracting the ground material with toluene:ethanol (2/1, v/v), ethanol, and water. The lignin content of dried mature stems was estimated from the extract-free samples by the standard procedures (Dence, 1992). The lignin composition of extract-free material was studied by thioacidolysis, as previously described (Lapierre et al., 1995). The lignin-derived thioacidolysis monomers were identified by gas chromatography–mass spectrometry as their trimethylsilylated derivatives.

Determination of Stem Soluble Phenolic Compounds

The soluble phenolic compounds present in stems collected at stage 6.4 (Boyes et al., 2001) were extracted in 80:20 methanol/water (v/v, HPLC quality) after the addition of the internal standard (morine, 10 μg) and identified by liquid chromatography–mass spectrometry analyses (electrospray ionization, negative mode) as previously described (Goujon et al., 2003; Mir Derikvand et al., 2008). These soluble phenolic compounds were extracted from 1-cm-long sections of 40-cm-high stems obtained from the basal part of the stem (1 cm from the base of the stem) or the upper part of the stem (3 cm below the inflorescence).

Cellulolysis

Cellulolysis assays were performed on extract-free cell walls. Twenty to thirty milligrams of sample was placed in 30 mL of 0.05 M sodium acetate buffer, pH 4.7, supplemented with 2 mg mL^{-1} commercial cellulase (cellulase Onozuka-R10; Serva). After 5 d of incubation at 37°C with magnetic stirring, samples were filtered and weighed again. The Glc released into the filtrate was quantified with the Biomerieux Glucose RTU kit (bioMérieux).

Procedure Used for Laser Capture Microdissection

For this protocol, no specific preparation of the tissues was required. Stem tissues harvested from plants at stage 6.4 (Boyes et al., 2001) were cut into pieces of $\sim$1 to 2 cm each and immediately frozen in a cryotome (CM 1510S Leica) at -20°C, and 40-μm cross sections were cut. These sections were placed directly on slides for microdissection. A Palm Microbeam system (Zeiss microdissector) was used. This system is equipped for laser capture microdissection and pressure catapulting. Selected areas of the cross sections were subsequently cut with a UV A laser (337 nm). After the laser pressure catapulting pulse had been applied to the cut line, the samples were collected in the caps of Eppendorf tubes. Fifteen microliters of Versol water was added to each cap containing 50 microdissected samples. The samples were carefully transferred to a glass tube by repeated rinsing of the Eppendorf with small volumes of water. The samples in the glass tubes were then freeze-dried for thioacidolysis, which was performed as previously described (Ruel et al., 2009).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession

numbers: At2g29130 (*LAC2*), At2g38080 (*LAC4*), At2g40370 (*LAC5*), At2g46570 (*LAC6*), At5g01040 (*LAC8*), At5g01190 (*LAC10*), At5g03260 (*LAC11*), At5g05390 (*LAC12*), At5g48100 (*LAC15*), and At5g60020 (*LAC17*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Laccase Gene Expression Profiles Obtained from the GeneCAT Database.

Supplemental Figure 2. Quantitative RT-PCR Validation of Microarray Data Performed in Triplicate on Stage 6.2 Primary Stems.

Supplemental Figure 3. Cross Sections of *Arabidopsis* Stems (Long-Day Conditions) Stained with Phloroglucinol.

Supplemental Figure 4. Peptide Sequences Used for the Generation of Antibodies against LAC4 and LAC17.

Supplemental Figure 5. Immunolocalization of LAC4 and LAC17.

Supplemental Figure 6. Subcellular Localization of LAC4 in the Fiber Cell Wall and Middle Lamella.

Supplemental Figure 7. Complementation of *lac4-1 lac17* with Pro35S:*LAC17*c (ATG-Stop).

Supplemental Table 1. Primers Used in This Study.

Supplemental Data Set 1. Transcriptome Studies of *lac4-1*, *lac4-2*, *lac17*, *lac4-1 lac17*, and *lac4-2 lac17* by Microarray Analysis.

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REFERENCES

Abdulrazzak, N., et al. (2006). A coumaroyl-ester-3-hydroxylase insertion mutant reveals the existence of nonredundant meta-hydroxylation pathways and essential roles for phenolic precursors in cell expansion and plant growth. Plant Physiol. **140:** 30–48.

Adler, E. (1977). Lignin chemistry. Past, present and future. Wood Sci. Technol. **11:** 169–218.

Bao, W., O'malley, D.M., Whetten, R., and Sederoff, R.R. (1993). A laccase associated with lignification in loblolly pine xylem. Science **260:** 672–674.

Baudry, A., Caboche, M., and Lepiniec, L. (2006). TT8 controls its own expression in a feedback regulation involving TTG1 and homologous MYB and bHLH factors, allowing a strong and cell-specific accumulation of flavonoids in *Arabidopsis thaliana.* Plant J. **46:** 768–779.

Besseau, S., Hoffmann, L., Geoffroy, P., Lapierre, C., Pollet, B., and Legrand, M. (2007). Flavonoid accumulation in *Arabidopsis* repressed in lignin synthesis affects auxin transport and plant growth. Plant Cell **19:** 148–162.

Betz, G.A., Knappe, C., Lapierre, C., Olbrich, M., Welzl, G., Langebartels, C., Heller, W., Sandermann, H., and Ernst, D. (2009). Ozone affects shikimate pathway transcripts and monomeric lignin composition in European beech (*Fagus sylvatica* L.). Eur. J. For. Res. **128:** 109–116.

Boyes, D.C., Zayed, A.M., Ascenzi, R., McCaskill, A.J., Hoffman, N.E., Davis, K.R., and Görlach, J. (2001). Growth stage-based phenotypic analysis of *Arabidopsis*: A model for high throughput functional genomics in plants. Plant Cell **13:** 1499–1510.

Brown, D.M., Zeef, L.A.H., Ellis, J., Goodacre, R., and Turner, S.R. (2005). Identification of novel genes in *Arabidopsis* involved in secondary cell wall formation using expression profiling and reverse genetics. Plant Cell **17:** 2281–2295.

Cabané, M., Pireaux, J.-C., Léger, E., Weber, E., Dizengremel, P., Pollet, B., and Lapierre, C. (2004). Condensed lignins are synthesized in poplar leaves exposed to ozone. Plant Physiol. **134:** 586–594.

Cai, X.N., Davis, E.J., Ballif, J., Liang, M.X., Bushman, E., Haroldsen, V., Torabinejad, J., and Wu, Y.J. (2006). Mutant identification and characterization of the laccase gene family in *Arabidopsis*. J. Exp. Bot. **57:** 2563–2569.

Caparros-Ruiz, D., Fornale, S., Civardi, L., Puigdomenech, P., and Rigau, J. (2006). Isolation and characterisation of a family of laccases in maize. Plant Sci. **171:** 217–225.

Chapple, C.C., Vogt, T., Ellis, B.E., and Somerville, C.R. (1992). An *Arabidopsis* mutant defective in the general phenylpropanoid pathway. Plant Cell **4:** 1413–1424.

Clough, S.J., and Bent, A.F. (1998). Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. **16:** 735–743.

Crowe, M.L., et al. (2003). CATMA: A complete *Arabidopsis* GST database. Nucleic Acids Res. **31:** 156–158.

Dauwe, R., et al. (2007). Molecular phenotyping of lignin-modified tobacco reveals associated changes in cell-wall metabolism, primary metabolism, stress metabolism and photorespiration. Plant J. **52:** 263–285.

Dean, J.F., LaFayette, P.R., Rugh, C., Tristram, A.H., Hoopes, J.T., Eriksson, K.E., and Merckle, S.A. (1998). Laccases associated with lignifying vascular tissue. In Lignin and Lignan Biosynthesis, N.G. Lewis and S. Sarkanen, eds (Washington, DC: American Chemical Society), pp. 96–108.

Dean, J.F.D., and Eriksson, K.E.L. (1994). Laccase and the deposition of lignin in vascular plants. Holzforschung **48:** 21–33.

Debeaujon, I., Nesi, N., Perez, P., Devic, M., Grandjean, O., Caboche, M., and Lepiniec, L. (2003). Proanthocyanidin-accumulating cells in *Arabidopsis* testa: Regulation of differentiation and role in seed development. Plant Cell **15:** 2514–2531.

Dence, C. (1992). Lignin determination. In Methods in Lignin Chemistry, S.Y. Lin and C.W. Dence, eds (Berlin: Springer-Verlag), pp. 33–61.

Do, C.T., Pollet, B., Thévenin, J., Sibout, R., Denoue, D., Barrière, Y., Lapierre, C., and Jouanin, L. (2007). Both caffeoyl Coenzyme A 3-O-methyltransferase 1 and caffeic acid O-methyltransferase 1 are involved in redundant functions for lignin, flavonoids and sinapoyl malate biosynthesis in *Arabidopsis*. Planta **226:** 1117–1129.

Donaldson, L.A. (2001). Lignification and lignin topochemistry - An ultrastructural view. Phytochemistry **57:** 859–873.

Dubos, C., Le Gourrierec, J., Baudry, A., Huep, G., Lanet, E., Debeaujon, I., Routaboul, J.M., Alboresi, A., Weisshaar, B., and Lepiniec, L. (2008). MYBL2 is a new regulator of flavonoid biosynthesis in *Arabidopsis thaliana*. Plant J. **55:** 940–953.

Ehlting, J., et al. (2005). Global transcript profiling of primary stems

from *Arabidopsis thaliana* identifies candidate genes for missing links in lignin biosynthesis and transcriptional regulators of fiber differentiation. Plant J. **42:** 618–640.

Fagerstedt, K., Kukkola, E., Koistinen, V.V.T., Takahashi, J., and Marjamaa, K. (2010). Cell wall lignin is polymerized by class II secretable plant peroxidase in Norway spruce. J. Integr. Plant Biol. **52:** 186–194.

Gagnot, S., Tamby, J.P., Martin-Magniette, M.L., Bitton, F., Taconnat, L., Balzergue, S., Aubourg, S., Renou, J.P., Lecharny, A., and Brunaud, V. (2008). CATdb: A public access to *Arabidopsis* transcriptome data from the URGV-CATMA platform. Nucleic Acids Res. **36** (Database issue): D986–D990.

Ge, Y.C., Dudoit, S., and Speed, T.P. (2003). Resampling-based multiple testing for microarray data analysis. Test **12:** 1–77.

Goujon, T., Sibout, R., Pollet, B., Maba, B., Nussaume, L., Bechtold, N., Lu, F., Ralph, J., Mila, I., Barrière, Y., Lapierre, C., and Jouanin, L. (2003). A new *Arabidopsis thaliana* mutant deficient in the expression of O-methyltransferase impacts lignins and sinapoyl esters. Plant Mol. Biol. **51:** 973–989.

Gutiérrez, R.A., MacIntosh, G.C., and Green, P.J. (1999). Current perspectives on mRNA stability in plants: Multiple levels and mechanisms of control. Trends Plant Sci. **4:** 429–438.

Hilson, P., et al. (2004). Versatile gene-specific sequence tags for Arabidopsis functional genomics: Transcript profiling and reverse genetics applications. Genome Res. **14 :** 2176–2189.

Hoegger, P.J., Kilaru, S., James, T.Y., Thacker, J.R., and Kües, U. (2006). Phylogenetic comparison and classification of laccase and related multicopper oxidase protein sequences. FEBS J. **273:** 2308–2326.

Higuchi, T., and Ito, Y. (1958). Dehydrogenation products of coniferyl alcohol formed by the action of mushroom phenol oxidase, rhus laccase and radish peroxidase. J. Biochem. **45:** 575–579.

Kärkönen, A., Koutaniemi, S., Mustonen, M., Syrjänen, K., Brunow, G., Kilpeläinen, I., Teeri, T.H., and Simola, L.K. (2002). Lignification related enzymes in *Picea abies* suspension cultures. Physiol. Plant. **114:** 343–353.

Kim, H., Ralph, J., Lu, F., Pilate, G., Leplé, J.-C., Pollet, B., and Lapierre, C. (2002). Identification of the structure and origin of thioacidolysis marker compounds for cinnamyl alcohol dehydrogenase deficiency in angiosperms. J. Biol. Chem. **277:** 47412–47419.

Koizumi, K., Yokoyama, R., and Nishitani, K. (2009). Mechanical load induces upregulation of transcripts for a set of genes implicated in secondary wall formation in the supporting tissue of *Arabidopsis thaliana*. J. Plant Res. **122:** 651–659.

Lapierre, C. (2010). Determining lignin structure by chemical degradations. In Lignin and Lignans: Advances in Chemistry, C. Heitner, D. Dimmel, and J.A. Schmidt, eds (Boca Raton, FL: CRC Press, Taylor & Francis Group), pp. 11–48.

Lapierre, C., Pollet, B., and Rolando, C. (1995). New insights into the molecular architecture of hardwood lignins by chemical degradative methods. Res. Chem. Intermediat. **21:** 397–412.

Leplé, J.C., et al. (2007). Downregulation of cinnamoyl-coenzyme A reductase in poplar: Multiple-level phenotyping reveals effects on cell wall polymer metabolism and structure. Plant Cell **19:** 3669–3691.

Li, X., Bonawitz, N.D., Weng, J.-K., and Chapple, C. (2010). The growth reduction associated with repressed lignin biosynthesis in *Arabidopsis thaliana* is independent of flavonoids. Plant Cell **22:** 1620–1632.

Liang, M.X., Davis, E., Gardner, D., Cai, X.N., and Wu, Y.J. (2006). Involvement of AtLAC15 in lignin synthesis in seeds and in root elongation of *Arabidopsis*. Planta **224:** 1185–1196.

Lurin, C., et al. (2004). Genome-wide analysis of *Arabidopsis* pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. Plant Cell **16:** 2089–2103.

Masclaux-Daubresse, C., Reisdorf-Cren, M., Pageau, K., Lelandais, M., Grandjean, O., Kronenberger, J., Valadier, M.H., Feraud, M., Jouglet, T., and Suzuki, A. (2006). Glutamine synthetase-glutamate synthase pathway and glutamate dehydrogenase play distinct roles in the sink-source nitrogen cycle in tobacco. Plant Physiol. **140:** 444–456.

McCaig, B.C., Meagher, R.B., and Dean, J.F.D. (2005). Gene structure and molecular analysis of the laccase-like multicopper oxidase (LMCO) gene family in *Arabidopsis thaliana*. Planta **221:** 619–636.

Mir Derikvand, M., Sierra, J.B., Ruel, K., Pollet, B., Do, C.-T., Thévenin, J., Buffard, D., Jouanin, L., and Lapierre, C. (2008). Redirection of the phenylpropanoid pathway to feruloyl malate in *Arabidopsis* mutants deficient for cinnamoyl-CoA reductase 1. Planta **227:** 943–956.

Nakano, J., and Meshitsuka, G. (1992). The detection of lignin. In Methods in Lignin Chemistry, S.Y. Lin and C.W. Dence, eds (Berlin: Springer-Verlag), pp 23–32.

Pourcel, L., Routaboul, J.M., Kerhoas, L., Caboche, M., Lepiniec, L., and Debeaujon, I. (2005). TRANSPARENT TESTA10 encodes a laccase-like enzyme involved in oxidative polymerization of flavonoids in *Arabidopsis* seed coat. Plant Cell **17:** 2966–2980.

Ranocha, P., Chabannes, M., Chamayou, S., Danoun, S., Jauneau, A., Boudet, A.-M., and Goffner, D. (2002). Laccase down-regulation causes alterations in phenolic metabolism and cell wall structure in poplar. Plant Physiol. **129:** 145–155.

Richardson, A., and McDougall, G.J. (1997). A laccase-type polyphenol oxidase from lignifying xylem of tobacco. Phytochemistry **44:** 229–235.

Rohde, A., et al. (2004). Molecular phenotyping of the pal1 and pal2 mutants of *Arabidopsis thaliana* reveals far-reaching consequences on phenylpropanoid, amino acid, and carbohydrate metabolism. Plant Cell **16:** 2749–2771.

Ruel, K., Berrio-Sierra, J., Derikvand, M.M., Pollet, B., Thévenin, J., Lapierre, C., Jouanin, L., and Joseleau, J.P. (2009). Impact of CCR1 silencing on the assembly of lignified secondary walls in *Arabidopsis thaliana*. New Phytol. **184:** 99–113.

Sibout, R., Eudes, A., Mouille, G., Pollet, B., Lapierre, C., Jouanin, L., and Séguin, A. (2005). CINNAMYL ALCOHOL DEHYDROGENASE-C and -D are the primary genes involved in lignin biosynthesis in the floral stem of *Arabidopsis*. Plant Cell **17:** 2059–2076.

Sterjiades, R., Dean, J.F.D., and Eriksson, K.E.L. (1992). Laccase from sycamore maple (*Acer pseudoplatanus*) polymerizes monolignols. Plant Physiol. **99:** 1162–1168.

Terashima, N., and Fukushima, K. (1993). Comprehensive model of the lignified plant cell wall. In Forage Cell Wall Structure and Digestibility, H.G. Jung, D.R. Buxton, R.D. Hatfield, and R. Ralph, eds (Madison, WI: American Society of Agronomy), pp. 247–270.

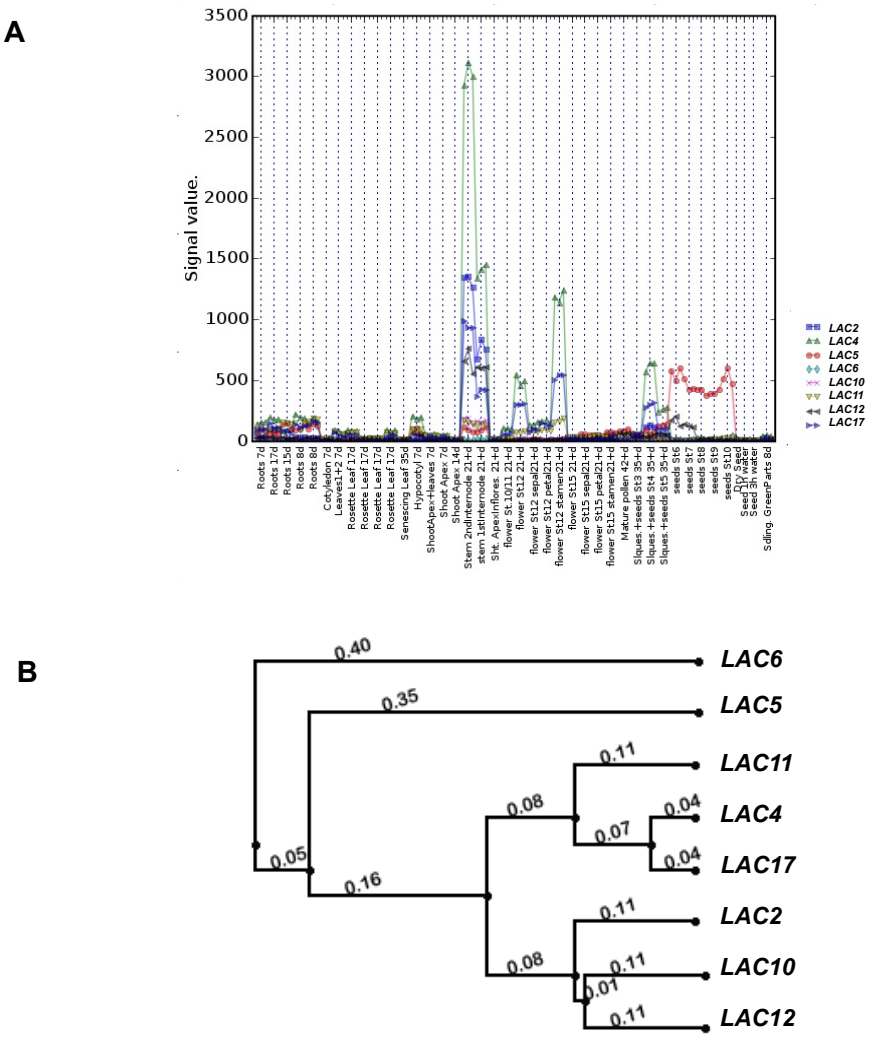
Tognolli, M., Penel, C., Greppin, H., and Simon, P. (2002). Analysis and expression of the class III peroxidase large gene family in *Arabidopsis thaliana*. Gene **288:** 129–138.

Vanholme, R., Morreel, K., Ralph, J., and Boerjan, W. (2008). Lignin engineering. Curr. Opin. Plant Biol. **11:** 278–285.

Wang, G.D., Li, Q.J., Luo, B., and Chen, X.Y. (2004). *Ex planta* phytoremediation of trichlorophenol and phenolic allelochemicals via an engineered secretory laccase. Nat. Biotechnol. **22:** 893–897.

Wang, J., Wang, C.L., Zhu, M.L., Yu, Y., Zhang, Y.B., and Wei, Z.M. (2008). Generation and characterization of transgenic poplar plants overexpressing a cotton laccase gene. Plant Cell Tissue Organ Cult. **93:** 303–310.

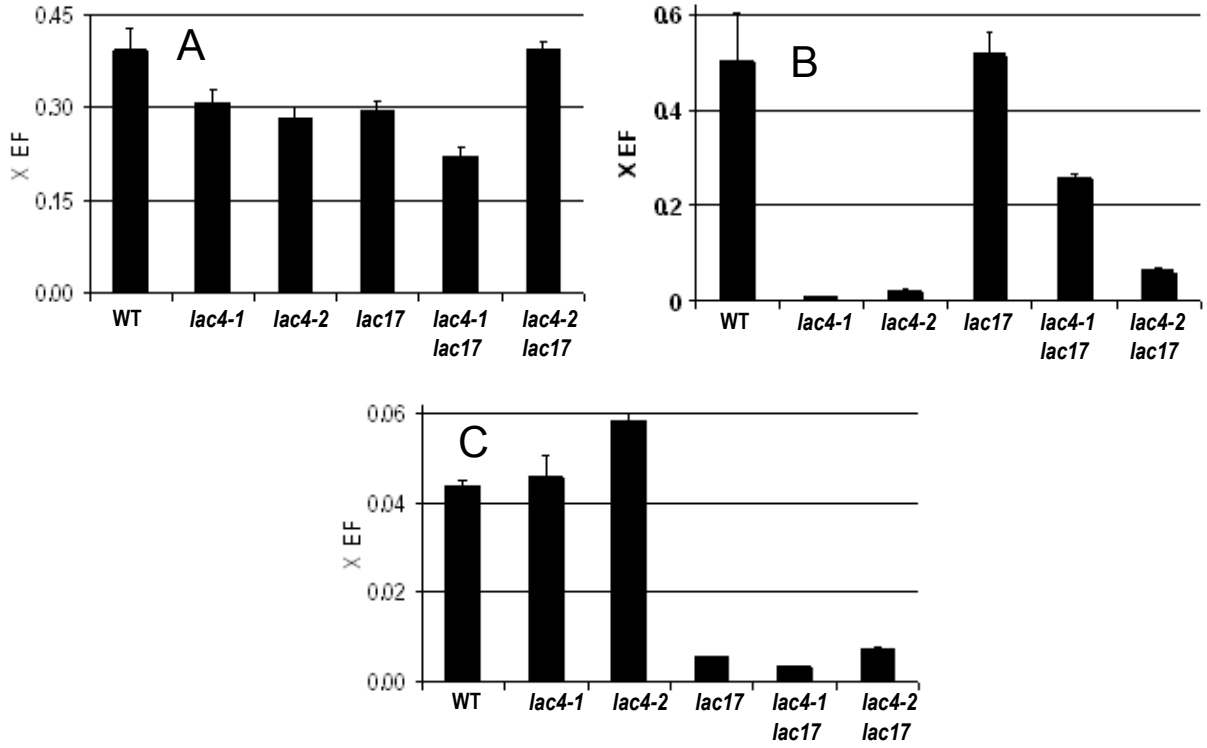
Yoshida, H. (1883). Chemistry of lacquer (urushi). J. Chem. Soc. **43:** 472–486.



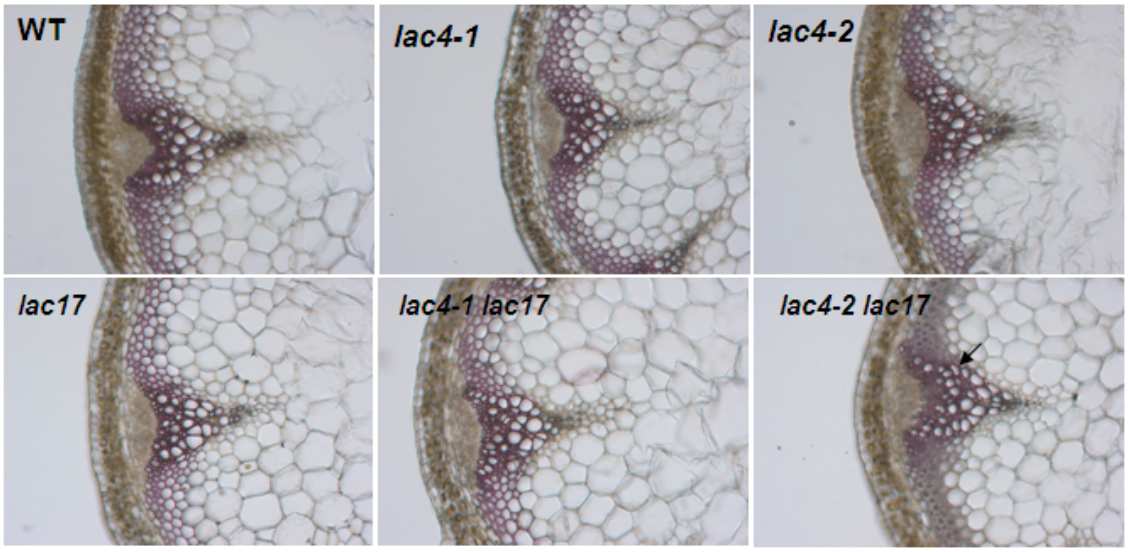
Supplemental Figure 1. Expression profiles of *Arabidopsis* stem-specific laccases

A.Laccases expression profiles obtained from the GeneCAT database (http://genecat.mpg.de). Among the 17 members of *Arabidopsis thaliana* laccases, eight are expressed in stem tissues and were chosen for further analyses.

B. *In silico* tree for expression of the eight major laccase genes expressed in *Arabidopsis* floral stem. The branch length labels (or distances) give the degree of co-expression between any two genes: genes highly co-expressed have a distance close to zero (http://genecat.mpg.de).



Supplemental Figure 2. Q-RT-PCR validation of microarray data performed on triplicates of 6.2 stage (Boyes et al., 2001) primary stems (plants grown under long-day conditions). Error bars indicate SD (n = 3). The elongation factor 1-α (EF) was used as a reference housekeeping gene and the gene expression levels were expressed as fold change relative to EF expression (X EF).
(A) Transcript level of *LAC2* in laccase mutants when compared to the wild type.
(B) Transcript level of *LAC4* in laccase mutants when compared to the wild type.
(C) Transcript level of *LAC17* in laccase mutants when compared to the wild type.



Supplemental Figure 3. Cross-sections of Arabidopsis stems (long-day conditions) stained with phloroglucinol. Scale bar, 100 µm.

Only the *lac4-2 lac17* mutant displayed some weakly collapsed vessels (black arrow).

Ab lac4
52-67 GRYPGPTIYAREDDT

204-21 CPSQGYKLSVENGKT

Ab lac 17
202-217 CSAKDTFRLRVKPGKT

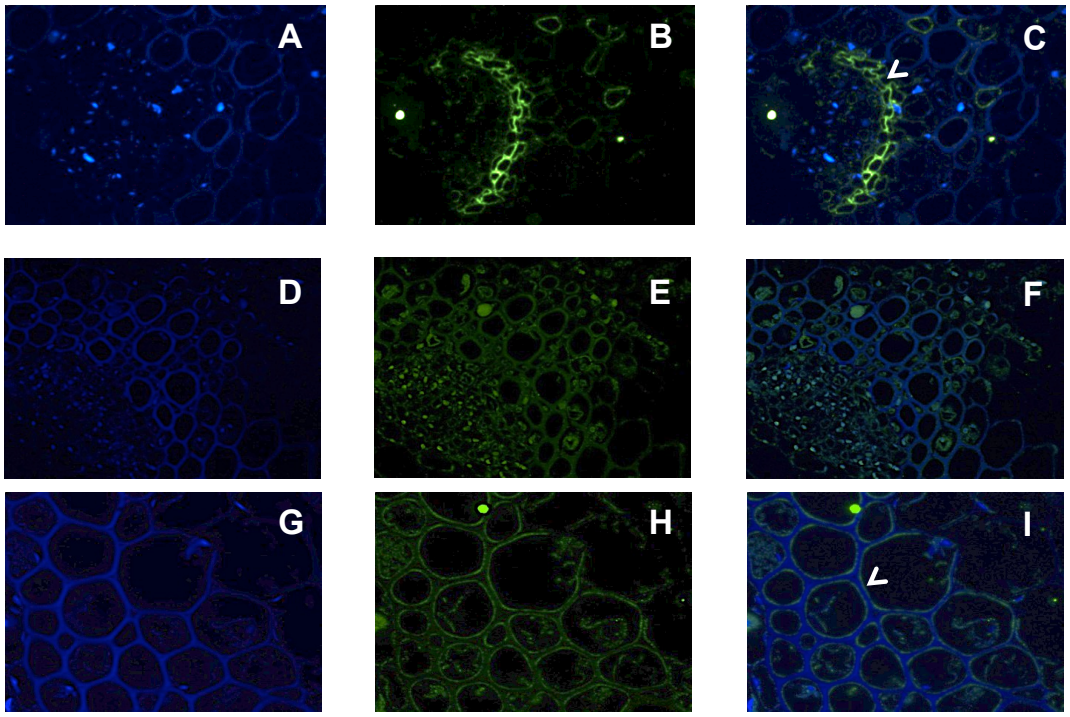
557-570 VLDGDKPDQKLLPP

>O80434|LAC4_ARATH Laccase-4 - *Arabidopsis thaliana* (Mouse-ear cress).
MGSHMVWFLFLVSFFSVFPAPSESMVRHYKFNVVMKNVTRLCSSKPTVTVN**GRYPGPTIY**
AREDDTLLIKVVNHVKYNVSIHWHGVRQVRTGWADGPAYITQCPIQPGQVYTYNYTLTGQ
RGTLWWHAHILWLRATVYGALVILPKRGVPYPFPKPDNEKVIVLGEWWKSDTENIINEAL
KSGLAPNVSDSHMINGHPGPVRN**CPSQGYKLSVENGKT**YLLRLVNAALNEELFFKVAGHI
FTVVEVDAVYVKPFKTDTVLIAPGQTTNVLLTASKSAGKYLVTASPFMDAPIAVDNVTAT
ATVHYSGTLSSSPTILTLPPPQNATSIANNFTNSLRSLNSKKYPALVPTTIDHHLFFTVG
LGLNACPTCKAGNGSRVVASINNVTFIMPKTALLPAHYFNTSGVFTTDFPKNPPHVFNYS
GGSVTNMATETGTRLYKLPYNATVQLVLQDTGVIAPENHPVHLHGFNFFEVGRGLGNFNS
TKDPKNFNLVDPVERNTIGVPSGGWVVIRFRADNPGVWFMHCHLEVHTTWGLKMAFLVEN
GKGPNQSILPPPKDLPKC

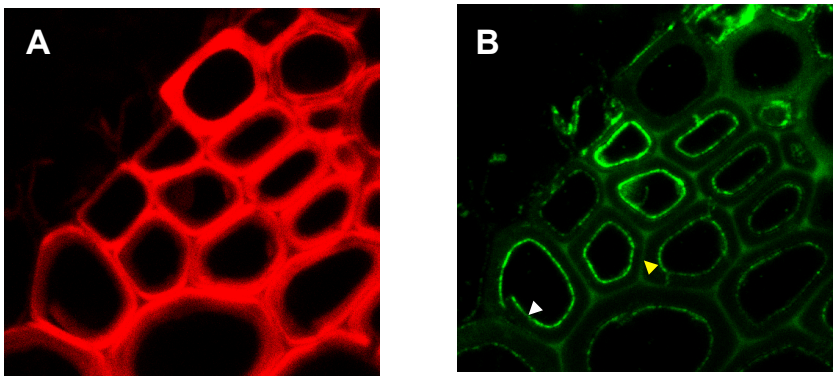
>Q9FJD5|LAC17_ARATH Laccase-17 - *Arabidopsis thaliana* (Mouse-ear cress).
MALQLLLAVFSCVLLLPQPAFGITRHYTLEIKMQNVTRLCHTKSLVSVNGQFPGPKLIAR
EGDQVLIKVVNQVPNNISLHWHGIRQLRSGWADGPAYITQCPIQTGQSYVYNYTIVGQRG
TLWYHAHISWLRSTVYGPLIILPKRGVPYPFAKPHKEVPMIFGEWFNADTEAIIRQATQT
GGGPNVSDAYTINGLPGPLYN**CSAKDTFRLRVKPGKT**YLLRLINAALNDELFFSIANHTV
TVVEADAIYVKPFETETILIAPGQTTNVLLKTKSSYPSASFFMTARPYVTGQGTFDNSTV
AGILEYEPPKQTKGAHSRTSIKNLQLFKPILPALNDTNFATKFSNKLRSLNSKNFPANVP
LNVDRKFFFTVGLGTNPCNHKNNQTCQGPTNTTMFAASISNISFTMPTKALLQSHYSGQS
HGVYSPKFPWSPIVPFNYTGTPPNNTMVSNGTNLMVLPYNTSVELVMQDTSILGAESHPL
HLHGFNFFVVGQGFGNFDPNKDPRNFNLVDPIERNTVGVPSGGWAAIRFLADNPGVWFMH
CHLEVHTSWGLRMAWL**VLDGDKPDQKLLPP**PADLPKC

Supplemental Figure 4. Peptide sequences used for the generation of LAC4 and LAC17 antibodies.

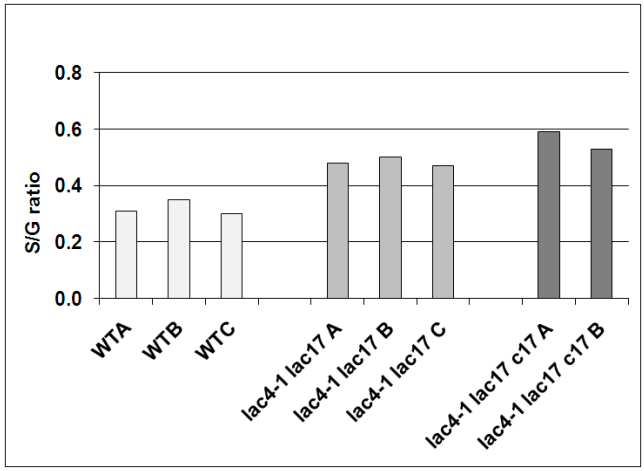
These antibodies were generated by Eurogenetech Society in Belgium.



Supplemental Figure 5. Immunolocalization of LAC4 and LAC17.
Lignin autofluorescence is shown in (A), (D) and (G). Alexa signals corresponding to LAC4 and LAC17 are shown in (B) and [(E), (H)] respectively. Overlap of Alexa signals and autofluorescence is shown in (C), (F) and (I). Scale bar, 50 µm.
(A) to (C) Localization of LAC4 in the vascular bundle. The main localization is in the cambium (white arrow).
(D) to (F) Search for LAC17 in the vascular bundle. No signal can be detected in this region.
(G) to (I) Localization of LAC17 in the interfascicular fibers. The main localization is in the cell wall (white arrow).



Supplemental Figure 6. Immunolocalization of LAC4 in the interfascicular fibers.
Lignin autofluorescence is shown in (A) and Alexa signal corresponding to LAC4 antibody in (B). The main localization is in the secondary cell walls (white arrow) and in the middle lamella (yellow arrow).
Scale bar, 25 µm.



Supplemental Figure 7. Complementation of *lac4-1 lac17* with Pro35S:*LAC17*c (ATG-Stop).

The effectiveness of the complementation was checked by thioacidolysis of mature stems from plants grown under long-day conditions. The thioacidolysis S/G ratio was higher in the *lac4-1 lac17* mutant than in the WT (3 different plants were analyzed for WT and *lac17*). In the complemented samples *lac4-1 lac17 c17* (2 independent lines A and B were characterized), this higher ratio was not restored to the control level.

Supplemental Table 1. Primers used in this study

Sequence of primers used for PCR (F: forward, R: reverse)		
Use for	Oligonucleotide	Primer sequences (5'→3')
Semi quantitative RT-PCR	LAC 2RT F	GCA AGA CAA AAA CAA TCG TGA
	LAC 2RT R	GAA ATC TGA GGG TGG AGG AAG
	LAC 4 RT F	GTT AGA AAC TGT CCA TCT CAA
	LAC 4 RT R	CTC CAC TTG TGT TGA AGT AAT
	LAC 5 RT F	ATC CGG TTG ATG TGT TGA GA
	LAC 5 RT R	AGA GAG ATC GGC TTA TGT TG
	LAC 6 RT F	TAT GCC AAA CAA ACG AGA T
	LAC 6 RT R	CTG CTG GAG GAG GAG GTC
	LAC 10 RT F	TGT AAA GCC GGA AAC TTC TC
	LAC 10 RT R	TTA GGG CCT TTA CCA TTC TC
	LAC 11 RT F	GAG CTA TTC TTC GGG ATT
	LAC 11 RT R	GTC TTT AGG CGG TGG TAG
	LAC 12 RT F	GCC GAC GCA TCT TAC CTC
	LAC 12 RT R	CCA AGA ACG CCA TAG CAA
	LAC 17 RT F	TTC TCT TGT GTT CTT CTT CTT
	LAC 17 RT R	GAA CTT CTT TGT GAG GTT TAG
mutant identification and border sequencing	lac4-1FST F	ATT GTG TAA GCA AAT CGG CAC
	lac4-1FST R	TGG CTT GCT TGA GCA TAA TCT
	lac4-2FST F	TGG TAA CTT TGG ACG ATC AGG
	lac4-2FST R	AGT AAT GAA CAG TTGCGG TGG
	lac17FST F	TCG AAG AGG GTC AAA GAG TTT
	lac17FST R	TCT TAG CCA TGA AAT GTG AGC

Sequence of primers used for PCR (F, forward; R, reverse)		
Used for	Oligonucleotide	Primer sequences (5'→3')
quantitative PCR	q2F	TAC ACG GGT ACG CCA CCA AAC
	q2R	GGG CCT CAA TAC CCA AGA TGC
	q4F	TCC TTC AAG ATA CCG GCG TCA
	q4R	ACC GGA TGG AAC TCC GAT TGT
	q17F	CGA TAA ACG GGC TTC CTG GTC
	q17R	AAC CGT GTG ATT TGC GAT GCT
	qCAD-DF	TGC GTT TGA GAG ACT CGA GAA GAA
	qCAD-DR	TCA AGC GTC GAG ATT GCT TCC
	qMT1C F	TTG GCA TTG TGT TGC GTA AT
	qMT1C R	CAG CAG GAA CAA GTC AAG CA
	EF1 F	CTG GAG GTT TTG AGG CTG GTA T
	EF1 R	CCA AGG GTG AAA GCA AGA AGA
promoter amplification for GATEWAY® cloning	pLAC2 F1	TTGATCGAATCATGAGTTGG
	pLAC2 R1	ATTGTTTGAATATTGAAGG
	pLAC3 F1	AAGTCAAACTTCACGATTTACG
	pLAC3 R1	TGTTTGCTTCACTGTGAGAAG
	pLAC4 F2	GTTTGATCCAGTTTTGGTTCG
	pLAC4 R1	CTCCCTCTCTATCTTTCTCTTC
	pLAC5 F2	CAAATTCAGATGGGAGGTGG
	pLAC5 R1	TGATGTGATTTCTGTAAGCTTC
	pLAC12 F1	GGAATGGTTGATTACTATTAAGC
	pLAC12 R1	TAAGTGTGTTTAAGCTTTGAG
	pLAC17 F1	CACTTTACTACGTAGTAATTG
	pLAC17 R1	AGATTGTGTATATATATTAGTTTTGG

Sequence of primers used for PCR (F, forward; R, reverse)			
Used for		Oligonucleotide	Primer sequences (5'→3')
Complementations with GATEWAY Cloning	ATG-STOP	L17ATGSF	ATGGCGTTACAGCTACTCCT
		L17ATGSR	GCATTTGGGCAAGTCTGCAGG
	17g -3'	pLAC17F1	CACTTTACTACGTAGTAATTG
		L17ATGSR	GCATTTGGGCAAGTCTGCAGG
	17g + 3'	pLAC17F1	CACTTTACTACGTAGTAATTG
		L17+3'R	AGATTGTGTATATATTAGTTTTGG