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► **To cite this version:**

Serge Berthet, Johanne Thévenin, Davy Baratiny, Nathalie Demont-Caulet, Isabelle Debeaujon, et al.. Role of Plant Laccases in Lignin Polymerization. Chapter 5. Lignins - Biosynthesis, Biodegradation and Bioengineering, 61, Academic Press - Elsevier, pp.145-172, 2012, Advances in Botanical Research, 978-0-12-416023-1. 10.1016/B978-0-12-416023-1.00005-7. hal-01203977

HAL Id: hal-01203977

<https://hal.science/hal-01203977>

Submitted on 6 Jun 2020

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Role of Plant Laccases in Lignin Polymerization

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ABSTRACT

Laccases are ubiquitous oxidases present in animals, plants, bacteria, and fungi. In plant species, they occur as large multigenic families. The involvement of peroxidases in lignification is supported by a wealth of literature data. In contrast, the role of laccases in this major plant process is less firmly established. The large number of plant laccases, which argues for a variety of functions in plant development, makes the identification of lignin-specific laccases a challenge. However, in the past decade, the development of new genetic technologies and tools has played a central role towards resolving this issue. In addition, the plant model, *Arabidopsis thaliana*, has recently provided novel insights about the occurrence of laccases involved in stem lignification. Information about lignin-related laccases is also available from other species, such as poplar, or other organs and tissues, such as seed coats. This review brings a short and cutting edge survey of laccases and lignification.

I. INTRODUCTION

Lignins are complex cell wall polymers mainly constituted of *para*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units derived from precursors known as monolignols. These precursors are synthesized in the cytoplasm and then transported to the cell wall where polymerization occurs (Simmons *et al.*, 2010). The oxidative polymerization of monolignols in plant cell walls has been reported to be enzymatically supported by plant oxidases, peroxidases, and/or laccases (Mayer and Staples, 2002). Over the past two decades, efforts have been made to more precisely identify the genes involved in lignin polymerization but until recently the precise role of laccases in lignification was still ill-defined. This review brings a short and up-to-date survey about the involvement of plant laccases in this important plant process.

II. PLANT LACCASES ARE MULTICOPPER OXIDASES SHOWING BROAD *IN VITRO* SUBSTRATE SPECIFICITY

Laccases (EC 1.10.3.2) are a large group of multicopper oxidases occurring in eukaryotes (higher plants, fungi, animals) and in prokaryotes (Claus, 2004). The localization of plant and fungal laccases is extracellular, whereas bacterial laccases are intracellularly localized (Diamantidis *et al.*, 2000).

Laccases are monomeric, dimeric, or tetrameric glycoproteins. These oxidases are copper proteins that are able to catalyse the one-electron oxidation of a broad range of aromatic or non-aromatic compounds (phenols, anilines, arylamines, thiols, monolignols, and lignins) with the concomitant four-electron reduction of molecular oxygen to water. As with other multicopper oxidases, plant laccases contain four copper atoms (Messerschmidt *et al.*, 1992) that are distributed into three types of copper centres, T1, T2, and T3, according to their spectroscopic properties (Bento *et al.*, 2006).

Although a number of 3D structures of bacterial and fungal laccases have been resolved (as reviewed in Chapter 8), there is currently no available crystallographic 3D structure of plant laccases. The only plant laccases structure reported so far has been obtained by a homology modelling approach using bioinformatics compared to fungal and bacterial enzymes (Dwivedi *et al.*, 2011). These laccases models are based on sequence conservation and more particularly on the high conservation of histidine motifs binding copper atoms. Based on these models, the monomers of plant, fungal, and bacterial enzymes would all display a similar architecture organized in three sequentially arranged cupredoxin-like domains mainly formed by beta-barrels (Dwivedi *et al.*, 2011).

Fungal and plant laccases are known to be glycoproteins with a sugar-moiety representing 10–55% of their molecular weight. Plant laccases are more highly glycosylated than fungal laccases. The carbohydrate region contains monosaccharides such as hexosamines, glucose, mannose, galactose, fucose, and arabinose (Rogalski and Leonowicz, 2004) and glycosylation seems necessary for secretion, copper retention, proteolytic susceptibility, thermal stability, and enzymatic activity (Xu, 1999). Putative glycosylation sites are identified by the consensus Asn-X-Thr sequence, even though not all sites are glycosylated. For example, the *Trametes versicolor* laccases (LacIIIb) sequence contains six putative N-glycosylation sites, but only four of them are glycosylated (Bertrand *et al.*, 2002). Fungal laccases have isoelectric points (*pI*) around 4 (reviewed in Rodgers *et al.*, 2010), whereas plant laccases *pI* values are somewhat higher ranging from 5 to 9. Plant laccases show an optimal pH activity in the range of 5–7.5, whereas fungal laccases have a lower optimal pH ranging between 3.5 and 5.5. Some biochemical traits of plant and fungal laccases are summarized in Table I.

TABLE I
Main Biochemical Properties of Some Plant Laccases as Compared to Fungal Laccases

| Laccases source | MW (kDa) | Glycosylation (% or type) | Optimum pH | pI | References |
|---------------------------------|----------|--------------------------------------|------------|-----|--|
| <i>Acer pseudoplatanus</i> | 115 | 40 | 5.2 | 5.2 | Sterjiades <i>et al.</i> (1992) |
| <i>Pinus taeda</i> | 90 | 25 | 5.9 | 9 | Bao <i>et al.</i> (1993) |
| <i>Populus euramericana</i> | 90 | High manose type N-glycan | – | 9.2 | Ranocha <i>et al.</i> (1999) |
| <i>Rhus vernicifera</i> | 109 | 45–55 ^a | – | 8.2 | Yun-Yang <i>et al.</i> (2006) |
| | 120 | | | 8.6 | |
| | 103 | | | 9.1 | |
| <i>Fungal secreted laccases</i> | 55–85 | Up to 25% (3–10 glycosylation sites) | – | ~4 | Reviewed in Rodgers <i>et al.</i> (2010) |

^aAccording to Nitta *et al.* (2002).

It is generally accepted that plant and fungal enzymes would have distinct functions. While it is established that fungal laccases are involved in lignin biodegradation and in detoxification of phenolics, little is known about the functions of plant laccases. Their capability to polymerize various phenolics to form insoluble and protective barriers support a protective role and the secreted laccases from Japanese lacquer tree was suggested to be involved in wound healing (Nakamura, 1958). Similarly, the polymerization of plant phenolics by laccases was also suggested to contribute to plant defence (Walker and Ferrar, 1998). The case study of the laccase-catalysed polymerization of flavonoids in seeds is discussed in Section III.D. For many years, the potential involvement of laccases in lignin polymerization has been repeatedly proposed (Bao *et al.*, 1993; Dean and Eriksson, 1994; Sterjiades *et al.*, 1993). Indeed, the *in vivo* laccase-driven polymerization of plant phenolics, including lignin precursors, has strongly supported the participation of these enzymes in lignin polymerization, but without any direct and conclusive evidence, as discussed in the next section.

III. TOWARDS THE FUNCTIONAL IDENTIFICATION OF PLANT LACCASES THROUGH FUNCTIONAL GENOMICS

In the past decades, rapid advances in genomic technologies have contributed to a growing knowledge about the roles of plant laccases. These technologies include the genome sequencing of various plant species such as

arabidopsis ([Arabidopsis Genome Initiative, 2000](#)), poplar ([Tuskan *et al.*, 2006](#)), maize ([Schnable *et al.*, 2009](#)), and recently brachypodium ([Vogel *et al.*, 2010](#)), as well as the availability of mutant collections for plant models ([Bechtold *et al.*, 1993](#); [Alonso *et al.*, 2003](#) for *Arabidopsis*; [Vain *et al.*, 2008](#) for Brachypodium), or the improved technologies for specifically silencing specific target genes (for review, see [Matthew, 2004](#)). The plant model *Arabidopsis thaliana*, a small-lignified dicotyledonous species with a short generation time, has proved to be highly relevant for lignification studies ([Vanholme *et al.*, 2010](#)). In addition, the arabidopsis gene expression databases (<https://genevestigator.com>; [Zimmermann *et al.*, 2004](#); <http://genecat.mpg.de>; [Mutwil *et al.*, 2008](#)) provide important information on the spatio-temporal expression patterns of genes. Such information has proved to be particularly useful for selecting the best gene candidates from multigenic families including plant laccases genes ([Berthet, 2010](#); [Berthet *et al.*, 2011](#)). More recently, a database specific for laccases and other multicopper oxidases was developed for improved sequence-based classification <http://www.lcced.uni-stuttgart.de> ([Sirim *et al.*, 2011](#)).

A. FIRST LITERATURE DATA FOR OR AGAINST LIGNIN-SPECIFIC LACCASES

The dehydrogenative polymerization of monolignols into lignins was proposed as early as the 1930s ([Erdtman, 1933](#); [Freudenberg and Nelsh, 1968](#)) and both the main plant oxidases—laccases and peroxidases—were suggested to enzymatically support monolignol coupling. Although both laccases and peroxidase activities were reported in the differentiating xylem of spruce (as reviewed in [Freudenberg and Nelsh, 1968](#)), two different studies led to the conclusion that laccases were not involved in lignification. The laccases purified from *Rhus vernicifera* could not oxidize coniferyl alcohol to synthetic lignin *in vitro* ([Nakamura, 1967](#)) and no laccases activity could be histochemically detected in lignifying tissues of green ash ([Harkin and Obst, 1973](#)). As a result the potential role of laccases to assist peroxidases in lignification was neglected for a long time and it was not until the 1990s that the results of several studies suggested that laccases might indeed be involved in the lignification process. In the absence of H₂O₂, a laccases purified from sycamore maple (*Acer pseudoplatanus*) cell culture could catalyse the *in vitro* polymerization of monolignols ([Driouich *et al.*, 1992](#); [Sterjiades *et al.*, 1992](#)). Another study also described laccases activity in loblolly pine (*Pinus taeda*) xylem ([Bao *et al.*, 1993](#)). [Sterjiades *et al.* \(1993\)](#) suggested that laccases may function during the early stage of lignification to polymerize monolignols, whereas peroxidases may function during later stages of xylem development. In poplar, two laccases isolated from xylem were capable to

oxidize coniferyl alcohol (Ranocha *et al.*, 1999). A 90 kDa H₂O₂-independent phenoloxidase displayed *in vitro* laccases activity and was able to oxidize the three monolignols with the highest affinity for coniferyl alcohol (Ranocha *et al.*, 1999). Moreover, laccases down-regulated transgenic poplars were produced which displayed higher levels of soluble phenolics and irregular fibres, albeit the global lignin level was not reduced (Ranocha *et al.*, 2002). Only two studies have reported changes of lignin levels in transgenic plants showing modified laccases activity (Liang *et al.*, 2006; Wang *et al.*, 2008). A decreased lignin content was reported in one arabidopsis *tt10/lac15* mutant that shows reduced laccases activity particularly in the seeds (Liang *et al.*, 2006). In contrast, an increased lignin level was observed in transgenic poplar plants expressing the cotton laccases *GaLAC1* gene (Wang *et al.*, 2008). These poplar lines showed a 2–13-fold increase in laccases activity when compared to the wild-type (WT) poplar and a 2–19% increase in lignin content measured by the thioglycolic assay.

B. PLANT LACCASES: HOW TO IDENTIFY FUNCTION(S) IN LARGE MULTIGENIC FAMILIES?

Phylogenetic and expression studies of two laccases multigenic families (arabidopsis and poplar) can be used to emphasize the relevancy of these approaches for the functional determination of laccases genes.

In arabidopsis, 17 laccases have been reported (McCaig *et al.*, 2005). The comparison between the phylogenetic and expression trees for these 17 laccases can provide useful indications as to their putative roles (Fig. 1). These laccases are discriminated mainly in five different clusters on the expression tree (Fig. 1A). Among these clusters, three organ-specific groups can be observed: A, C, and D that are stem-specific, root-specific, and seed specific, respectively. The protein alignment allowed us to discriminate seven groups in the arabidopsis laccases gene family (Fig. 1B), unlike previous classifications that have identified six groups in arabidopsis (Caparrós-Ruiz *et al.*, 2006; McCaig *et al.*, 2005; Turlapati *et al.*, 2011). Each of the *AtLAC1*, *AtLAC6*, *AtLAC14*, and *AtLAC15* genes is the unique representative of groups 1, 3, 6, and 7, respectively. According to their geneCAT expression profiles, *AtLAC1*, *AtLAC6*, and *AtLAC14* are weakly expressed without any organ specificity. In contrast, *AtLAC15* is strongly expressed in seeds suggesting a seed-specific function (Pourcel *et al.*, 2005). In spite of its distinct localization in group 5 of the phylogenetic tree (Fig. 1B), *AtLAC5* is coexpressed with *AtLAC15* (group D, Fig. 1A). Both group 4 (with *AtLAC7*, *AtLAC8*, *AtLAC9*) and group 5 (*AtLAC3*, *AtLAC5*, *AtLAC12*, and *AtLAC13*) contain root-specific laccases. The largest group (group 2, Fig. 1B) comprises six

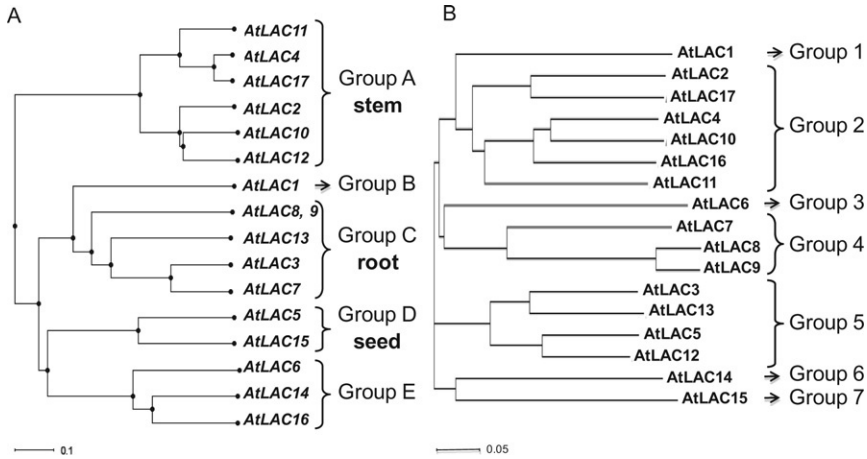


Fig. 1. Expression tree compared to phylogenetic tree for *Arabidopsis thaliana* laccases family. (A) Expression tree for arabidopsis laccases, the scale bar represents the degree of coexpression between any two genes; genes highly coexpressed have a distance close to zero (<http://genecat.mpg.de>). (B) Phylogenetic tree for arabidopsis laccases using protein alignment, the scale bar represents 0.05 substitutions per position (<http://www.ebi.ac.uk/clustalw/>).

laccases (*AtLAC2*, *AtLAC4*, *AtLAC10*, *AtLAC11*, *AtLAC16*, and *AtLAC17*), which are all expressed in stems with the exception of *AtLAC16*, which does not present any organ specificity. The high conservation of the genes encoding for the stem-specific laccases supports the hypothesis that they could have redundant functions and could be potentially involved in lignification.

Up to now, knockout or knockdown T-DNA mutants have been identified for each of these 17 laccases (Table II), with the exception of *AtLAC1*. Different phenotypes were assigned to *lac2*, *lac4*, *lac8*, *lac15* (*tt10*), and *lac17* (Berthet *et al.*, 2011; Brown *et al.*, 2005; Cai *et al.*, 2006; Pourcel *et al.*, 2005), which suggests different functions for these laccases. The *AtLAC15* deficient line presents a seed-associated phenotype whereas mutants for *AtLAC4* and *AtLAC17* show some alterations in stem phenotype (Berthet *et al.*, 2011). The relationship between the stem- or seed-localized phenotype and the expression profile of the corresponding mutated laccases led to the first functional characterization of arabidopsis laccases genes, as discussed below. Currently, no information is available about the function of root-specific laccases.

In *Populus trichocarpa*, 51 putative laccase-encoding genes were identified from the available functional annotation of the poplar genome in Phytozome database <http://www.phytozome.net> (Goodstein *et al.*, 2011). The comparative analyses between poplar and arabidopsis of protein similarity, gene expression and miRNA target prediction can help us to better delineate the

TABLE II
List of *Arabidopsis* Laccases T-DNA Insertion Mutants

| Gene ID | Accession number | Mutant ID | Name | T-DNA insertion site | RT-PCR confirmation | Phenotype ^a | References |
|----------------|------------------|-----------------------|---------------|----------------------|---------------------|--------------------------------|------------------------------|
| <i>AtLAC2</i> | At2G29130 | Salk_025690 | <i>lac2</i> | Exon | Knockout | Reduced root under dehydration | Cai <i>et al.</i> (2006) |
| <i>AtLAC3</i> | At2G30210 | Salk_031901 | <i>lac3</i> | Exon | Knockout | – | Cai <i>et al.</i> (2006) |
| <i>AtLAC4</i> | At2G38080 | Salk_051892 | <i>lac4-1</i> | Promoter | Knockdown | Weak irregular xylem in stem | Brown <i>et al.</i> (2005) |
| <i>AtLAC4</i> | At2G38080 | GabiKat-720 G02 | <i>lac4-2</i> | Exon | Knockout | Altered lignification in stem | Berthet <i>et al.</i> (2011) |
| <i>AtLAC5</i> | At2G40370 | Salk_063466 | <i>lac5</i> | Exon | Knockout | – | Cai <i>et al.</i> (2006) |
| <i>AtLAC6</i> | At2G46570 | V_211D12 ^b | <i>lac6</i> | Exon | Knockout | – | This report |
| <i>AtLAC7</i> | At3G09220 | Salk_070761 | <i>lac7</i> | Exon | Knockout | – | This report |
| <i>AtLAC8</i> | At5G01040 | Salk_111587 | <i>lac8</i> | Exon | Knockout | Early flowering | Cai <i>et al.</i> (2006) |
| <i>AtLAC9</i> | At5G01050 | Salk_002632 | <i>lac9</i> | Exon | Knockout | – | Cai <i>et al.</i> (2006) |
| <i>AtLAC10</i> | At5G01190 | Salk_017722 | <i>lac10</i> | Exon | Knockout | – | This report |
| <i>AtLAC11</i> | At5G03260 | Salk_063746 | <i>lac11</i> | Exon | Knockout | – | This report |
| <i>AtLAC12</i> | At5G05390 | Salk_047455 | <i>lac12</i> | Exon | Knockout | – | This report |
| <i>AtLAC13</i> | At5G07130 | Salk_023935 | <i>lac13</i> | Exon | Knockout | – | Cai <i>et al.</i> (2006) |

| | | | | | | | |
|------------------------------------|-----------|-------------|-------------------------------|--|---|---|------------------------------|
| <i>AtLAC14</i> | At5G09360 | Salk_011767 | <i>lac14</i> | Exon | Knockout | – | Cai <i>et al.</i> (2006) |
| <i>AtLAC15</i> | At5G48100 | Salk_002972 | <i>tt10-4</i> | Exon | Knockout | Transparent testa phenotype | Pourcel <i>et al.</i> (2005) |
| <i>AtLAC16</i> | At5G58910 | Salk_064093 | <i>lac16</i> | Intron | Knockout | – | Cai <i>et al.</i> (2006) |
| <i>AtLAC17</i> | At5G60020 | Salk_016748 | <i>lac17</i> | Promoter | Knockdown | Altered lignification in stem | Berthet <i>et al.</i> (2011) |
| <i>AtLAC4</i> × <i>AtLAC17</i> | | | <i>lac4-1</i> <i>lac17</i> | Promoter | Knockdown | Altered lignification in stem | Berthet <i>et al.</i> (2011) |
| <i>AtLAC4</i> × <i>AtLAC17</i> | | | <i>lac4-2</i> <i>lac17</i> | Exon (<i>lac 4-2</i>) Promoter (<i>lac17</i>) | Knockout (<i>lac 4-2</i>) knockdown (<i>lac17</i>) | Strongly altered lignification in stem with irregular xylem | Berthet <i>et al.</i> (2011) |
| <i>AtLAC12</i> × <i>AtLAC17</i> | | | <i>lac12</i> <i>lac17</i> | Exon (<i>lac12</i>) Promoter (<i>lac17</i>) | Knockout (<i>lac12</i>) knockdown (<i>lac17</i>) | Altered lignification in stem | This report |

All mutants are in the Col0 background, except *lac6* (Wassilewskija accession), and are homozygous knockdown or knockout lines.

^aNo reported phenotype.

^bVersailles collection.

conserved or specific roles of these laccases (Fig. 2). The results of the protein-based similarity analysis showed that both poplar and arabidopsis laccases were grouped in seven main clusters. One major specificity of poplar is the overall higher level of duplicated genes found in the genome when compared to arabidopsis. Important differences were also observed concerning the number of poplar homologs for one particular arabidopsis gene. For example, *AtLAC1*, belonging to group 1, has five putative poplar co-orthologs. In contrast, *AtLAC6* and *AtLAC14* from groups 3 and 6, respectively, have only one predicted ortholog each. An increase in poplar genes was observed to cluster with arabidopsis laccases from groups 1, 2, and 7. Further information was provided by the analysis of poplar *in silico* expression data available through the efp Browser <http://bar.utoronto.ca/> (Wilkins *et al.*, 2009). Expression data from 29 gene models out of the 51 predicted in the genome annotation were collected. Figure 3 presents a hierarchical clustering of the expression data available for the gene models represented on the Affymetrix poplar array. Three main expression clusters were observed: one grouping genes highly, but not exclusively expressed in stem xylem (group X), a second cluster containing genes highly, but not exclusively expressed in roots (group R), and the third cluster represented by weakly expressed genes with no specific tissue expression (group O). Of the five poplar genes belonging to group R, three of them, *PtrLAC33*, 39, and 40 clustered together with arabidopsis laccases *AtLAC3*, 7, 8, 9, and 13 that are mainly expressed in roots (Fig. 1A, group C). One of them, *PtrLAC47*, clustered with the *AtLAC15/TT10* seed-specific laccase. The last one, *PtrLAC43*, is phylogenetically linked to *AtLAC4* and *AtLAC10*, which are more specifically expressed in the stem.

Examination of the expression clustering revealed that *PtrLAC2*, *PtrLAC12*, and *PtrLAC21* are the only genes presenting a specific expression in both roots and stem xylem tissue (Fig. 3). Interestingly, these genes are phylogenetically linked to *AtLAC2* (Fig. 2) and more distant from *PtrLAC1* and *PtrLAC31*, which are more specifically expressed in poplar stem xylem as compared to roots. Of the 14 genes belonging to group X, three poplar genes clustered with the arabidopsis seed-specific laccases *AtLAC5* while the other 11 genes are linked to arabidopsis laccases highly expressed in the stem (group A, Fig. 1A). Finally, 10 poplar genes are clustered in group O. Three of them are tightly linked to *AtLAC6*, *AtLAC14*, and *AtLAC16* that are weakly expressed with no particular organ specificity and belong to arabidopsis group E (Fig. 1A). Two are phylogenetically linked to *TT10/AtLAC15*, two are similar to *AtLAC1*, two others clustered together with the stem-specific *AtLAC11* from group A (Fig. 1A), and the last one is linked to the root-specific laccases *AtLAC7*.

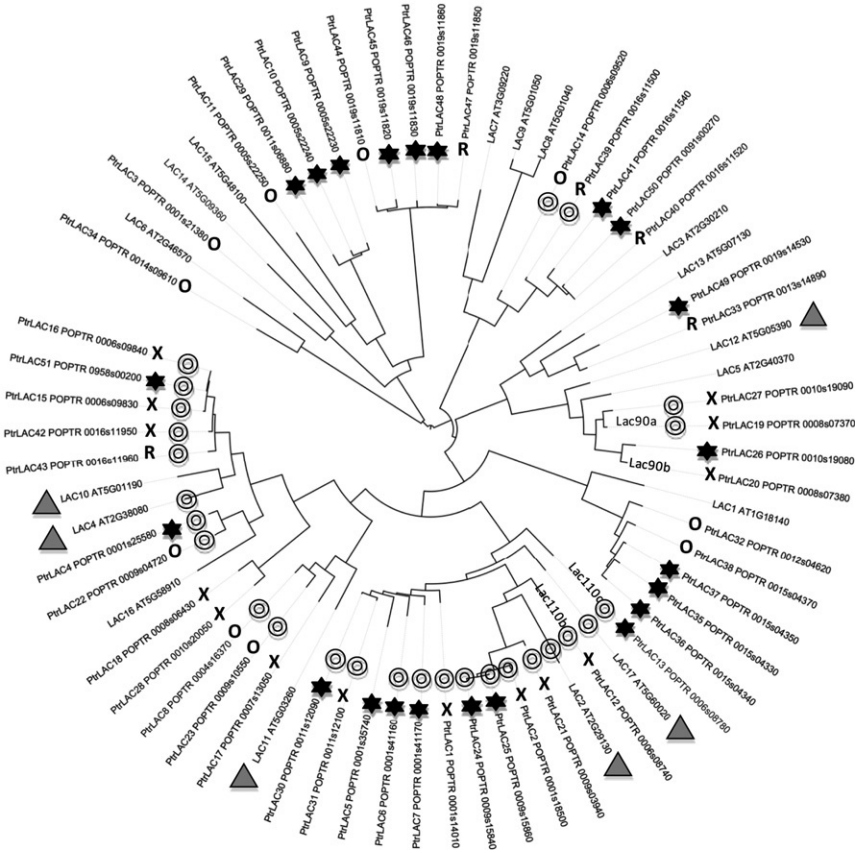


Fig. 2. Phylogenetic tree of poplar laccases whole gene family. All laccases genes were identified on the basis of laccases homologs annotation available from Phytozome database (Goodstein *et al.*, 2011). The phylogenetic tree was built using the web service tool developed by Dereeper *et al.* (2008). R, X, and O letters refer to poplar genes highly expressed in roots, stem xylem, and other tissues, respectively. Star: no expression data on efp Browser for these gene models; triangle: arabidopsis laccases from group A, expressed in stem; double circle: predicted gene targets for *miR397a*. Arabidopsis gene names are underlined. The tree image was produced using iTOL software (Letunic and Bork, 2007). AtLAC1: AT1G18140; AtLAC2: AT2G29130; AtLAC3: AT2G30210; AtLAC4: AT2G38080; AtLAC5: AT2G40370; AtLAC6: AT2G46570; AtLAC7: AT3G09220; AtLAC8: AT5G01040; AtLAC9: AT5G01050; AtLAC10: AT5G01190; AtLAC11: AT5G03260; AtLAC12: AT5G05390; AtLAC13: AT5G07130; AtLAC14: AT5G09360; AtLAC15: AT5G48100; AtLAC16: AT5G58910; AtLAC17: AT5G60020. PtrLAC1: POPTR_0001s14010; PtrLAC2: POPTR_0001s18500; PtrLAC3: POPTR_0001s21380; PtrLAC4: POPTR_0001s25580; PtrLAC5: POPTR_0001s35740; PtrLAC6: POPTR_0001s41160; PtrLAC7: POPTR_0001s41170; PtrLAC8: POPTR_0004s16370; PtrLAC9: POPTR_0005s22230; PtrLAC10: POPTR_0005s22240; PtrLAC11: POPTR_0005s22250; PtrLAC12: POPTR_0006s08740; PtrLAC13: POPTR_0006s08780; PtrLAC14: POPTR_0006s09520; PtrLAC15: POPTR_0006s09830; PtrLAC16: POPTR_0006s09840;

C. STEM LACCASES: FIRST *IN VIVO* EVIDENCE IMPLICATING LACCASES IN LIGNIN POLYMERIZATION

Among the 17 arabidopsis laccases, six are expressed in the inflorescence stem. Homozygous T-DNA insertion lines for the six genes were obtained and lignin contents of mature stems determined (Table III). Only mutants of the *AtLAC4* (*lac4-1* and *-2*) and *AtLAC17* (*lac17*) genes, which are the main laccases expressed in stems, had an approximately 10% decreased lignin content when compared to the WT (Berthet *et al.*, 2011). These lines showed no growth phenotype when plants were grown in greenhouse conditions. A weak irregular xylem phenotype similar to that observed for *irx12* (Brown *et al.*, 2005) was reported for *lac4-1* and *lac4-2* mutants grown under continuous light conditions. As *AtLAC4* and *AtLAC17* are both tightly coexpressed and homologous genes (Fig. 1A and B), they could display some functional redundancy. This hypothesis prompted us to produce double homozygous T-DNA insertion lines (*lac4-1 lac17* and *lac4-2 lac17*). These two double mutants displayed an important reduction (between 19% and 39%) in lignin content in their mature stems when compared to the WT (Berthet *et al.*, 2011). This reduced lignin level was associated with an irregular xylem phenotype characteristic of secondary cell wall deficient mutants (Brown *et al.*, 2005). Alteration of lignin structure (higher S/G ratio) was also observed in these lines, suggesting a higher impact on G-lignin deposition. This result was found to be consistent with the hypothesis that laccases would be involved in the early stages of lignification (Bao *et al.*, 1993; Donaldson, 2001). *AtLAC17* was mainly expressed in interfascicular fibres, whereas *AtLAC4* was expressed in both xylem and interfascicular fibres. According to the literature (Gavnholt and Larsen, 2002; Lewis and Yamamoto, 1990), to assess that an oxidase plays a role in lignin polymerization requires the fulfilment of the three following criteria: (1) a spatial and

PtrLAC17: POPTR_0007s13050; PtrLAC18: POPTR_0008s06430; PtrLAC19: POPTR_0008s07370; PtrLAC20: POPTR_0008s07380; PtrLAC21: POPTR_0009s03940; PtrLAC22: POPTR_0009s04720; PtrLAC23: POPTR_0009s10550; PtrLAC24: POPTR_0009s15840; PtrLAC25: POPTR_0009s15860; PtrLAC26: POPTR_0010s19080; PtrLAC27: POPTR_0010s19090; PtrLAC28: POPTR_0010s20050; PtrLAC29: POPTR_0011s06880; PtrLAC30: POPTR_0011s12090; PtrLAC31: POPTR_0011s12100; PtrLAC32: POPTR_0012s04620; PtrLAC33: POPTR_0013s14890; PtrLAC34: POPTR_0014s09610; PtrLAC35: POPTR_0015s04330; PtrLAC36: POPTR_0015s04340; PtrLAC37: POPTR_0015s04350; PtrLAC38: POPTR_0015s04370; PtrLAC39: POPTR_0016s11500; PtrLAC40: POPTR_0016s11520; PtrLAC41: POPTR_0016s11540; PtrLAC42: POPTR_0016s11950; PtrLAC43: POPTR_0019s11810; PtrLAC44: POPTR_0019s11820; PtrLAC45: POPTR_0019s11830; PtrLAC46: POPTR_0019s11850; PtrLAC47: POPTR_0019s11860; PtrLAC48: POPTR_0019s14530; PtrLAC49: POPTR_0019s00270; PtrLAC50: POPTR_0958s00200.

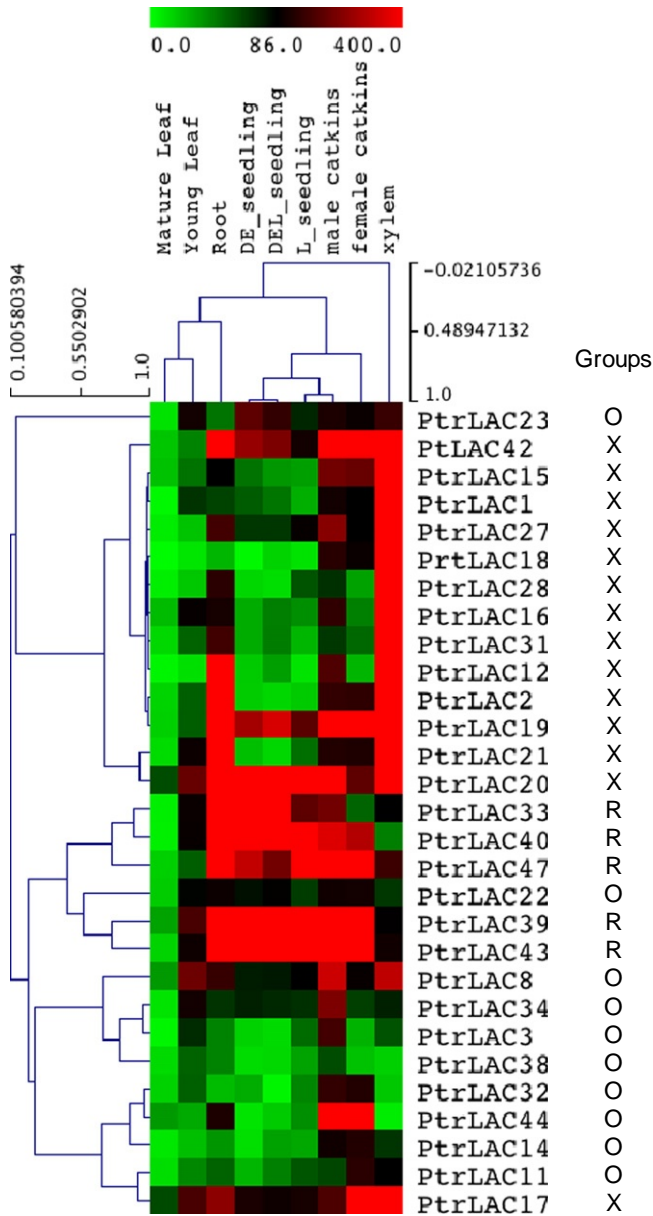


Fig. 3. Hierarchical clustering of poplar laccases gene expression data. Normalized intensities of expression data were collected from the efp Browser web service (Wilkins *et al.*, 2009). When several probesets were matching the same gene model, the one showing the highest overall expression level over the samples was selected. A hierarchical biclustering of both samples and genes was built using the Mev4 software (Saeed *et al.*, 2006). Samples were extracted from seedlings grown under different light regimes, young leaves, mature leaves, roots, xylem, female catkins, and male catkins. DE, dark-grown seedling, etiolated; DEL, dark-grown seedling, etiolated, exposed to light for 3 h; L, continuous light-grown seedling.

TABLE III
Effect of Mutations in Stem-Specific Laccases on the Lignification and Saccharification of Mature Stems as Compared to the Wild Type (Col0)

| Lines | Lignin content relative to the wild-type level (number of cultures) | Thioacidolysis S/G ratio | Saccharification (nmol sugars/mg h) |
|---------------------|---|--------------------------|-------------------------------------|
| WT | 100 | 0.40 ± 0.00 | 63.2 ± 4.6 |
| <i>lac2</i> | 105.0 ± 5.7 (2) | 0.40 ± 0.02 | 63.2 ± 3.5 |
| <i>lac4-1</i> | 87.9 ± 3.5 (8) ^a | 0.44 ± 0.00 | 76.7 ± 3.8 ^a |
| <i>lac4-2</i> | 90.5 ± 3.4 (6) ^a | 0.43 ± 0.02 | 82.3 ± 0.3 ^a |
| <i>lac10</i> | 96.3 ± 5.5 (2) | 0.40 ± 0.01 | 61.8 ± 3.6 |
| <i>lac11</i> | 104.0 ± 10.3 (4) | – | – |
| <i>lac12</i> | 96.5 ± 1.8 (5) ^a | 0.40 ± 0.02 | 64.8 ± 3.1 |
| <i>lac17</i> | 89.7 ± 4.8 (8) ^a | 0.48 ± 0.01 ^a | 72.3 ± 0.3 ^a |
| <i>lac4-1 lac17</i> | 83.0 ± 5.3 (6) ^a | 0.54 ± 0.02 ^a | 79.6 ± 4 ^a |
| <i>lac4-2 lac17</i> | 63.0 ± 2.2 (5) ^a | 0.67 ± 0.03 ^a | 94.1 ± 3.7 ^a |
| <i>lac12 lac17</i> | 84.1 ± 1.7 (2) ^a | 0.55 ± 0.02 ^a | 74.6 ± 2.9 ^a |

The data represent mean values (± standard deviations) between replicates. The relative lignin content of the mutant lines is measured by the Klason protocol (Dence, 1992) and is expressed relative to the corresponding control arbitrarily set at 100% and grown together with the mutant (the number of different cultures is given between brackets; each Klason determination is run on extract-free samples and in duplicates). The thioacidolysis S/G ratio is measured from one culture of all the mutants grown together and with analytical duplicates. Saccharification (run in triplicates) corresponds to the amount of sugars released after enzymatic treatment of pretreated stem segments (alkaline pretreatment NaOH 0.5 mol L⁻¹, 90 °C, 30 min) and is expressed in nanomoles of sugars released per milligram of dry material and per hour.

^aSignificantly different from the corresponding control when $p \leq 0.05$.

temporal correlation of the enzyme with active lignification, (2) altered lignin content and/or structure in plants with modified levels of the enzyme, and (3) a substrate specificity of the enzyme for monolignols. Up to now, the laccases encoded by *AtLAC4* and *AtLac17* have fulfilled the first two criteria. However, the last criteria still remains to be verified and will require purification and enzymatic characterization of the *AtLAC4* and *AtLAC17* proteins. Nevertheless, perhaps the most conclusive demonstration that *AtLAC17* participates to the formation of G units in supportive tissues (fibres) was provided by complementation experiments with the *lac17* mutant. When the full *LAC17* construct was reintroduced in the *lac17* line under the control of its endogenous promoter, both the S/G ratio and the Klason lignin content of the complemented line were restored to WT levels (Berthet *et al.*, 2011).

Deciphering the role of other stem-specific laccases needs further investigations. *AtLAC11* belongs to the same functional cluster as *AtLAC4* and *AtLAC17* (Fig. 1A). In addition, the expression study of promoter-*uidA* gene (beta-glucuronidase, GUS) fusion constructs revealed that *AtLAC11* was

expressed in both xylem and interfascicular fibres (Turlapati *et al.*, 2011). However, the *lac11* single mutant displayed neither reduced lignin content (Table III) nor altered lignin structure (data not shown). The situation was similar for *AtLAC2* and *AtLAC10*: while these genes were found to be expressed in stem lignified tissues (Turlapati *et al.*, 2011), their silencing did not induce any change in lignin content or structure (Table III). These results do not rule out the possibility that *AtLAC2*, *AtLAC10*, or *AtLAC11* might make a minor contribution to the lignin polymerization process. Double and even multiple knockout mutants would be necessary to confirm or reject this hypothesis. In contrast, the hypothesis that *AtLAC12* is involved in stem lignification was supported not only by its expression pattern in stem lignified tissues (Turlapati *et al.*, 2011) but also by a weakly, but consistently decreased lignin content of the *lac12* stems (Table III). Further support for the hypothesis was provided by the observation that the double mutant *lac12 lac17* showed greater alterations in lignin content and structure than the single *lac12* and *lac17* mutants (Table III). Taken together, these results show that both *AtLAC4* and *AtLAC17* indisputably contribute to lignin polymerization in arabidopsis stems, and it is very likely that *AtLAC12* might assist these enzymes.

D. FLAVONOID POLYMERIZATION IN SEEDS: OTHER ROLES FOR ARABIDOPSIS LACCASES, NAMELY, *TRANSPARENT TESTA 10/ATLAC15*

In addition to their role in lignification, it is possible that laccases might be involved in other physiological processes. Arabidopsis seeds accumulate two types of flavonoids, that are, proanthocyanidins (PAs) and flavonols (Lepiniec *et al.*, 2006). Colourless PAs (condensed tannins), the major contributors to the brown WT seed colour when oxidized, are localized specifically in the inner integument (mostly in the endothelium) and in the chalaza zone of the seed (Debeaujon *et al.*, 2003). Arabidopsis tannins are polymers of flavan-3-ols of the epicatechin type. Colourless to yellow flavonols accumulate in the seed envelopes (seed coat and endosperm) and in the embryo as glycosylated derivatives (Routaboul *et al.*, 2006).

The *TRANSPARENT TESTA 10* gene (*TT10*, At5g48100) encodes the TT10/*AtLAC15* laccases (Pourcel *et al.*, 2005). It was cloned through a candidate gene approach using the *tt10-1* mutant originally isolated by Koornneef (1990), mapped and first described by Shirley *et al.* (1995). The *tt10* mutant alleles are characterized by delayed seed browning caused by the inability to perform enzymatic oxidation of tannins (Pourcel *et al.*, 2005; Shirley *et al.*, 1995). The consequence is an increased amount of soluble (unoxidized) versus insoluble (more oxidized) tannins. Detailed metabolomic analysis revealed that *tt10* is also affected in flavonol composition, characterized by a higher

ratio of quercetin rhamnoside monomers to dimers, when compared to wild-type seeds (Pourcel *et al.*, 2005).

There might be also some role for TT10 in primary root development as mutant roots seem to be significantly shorter than wild-type roots when grown *in vitro* (Liang *et al.*, 2006). On the other hand, the root hair density and the number of inflorescence stems were higher in *tt10* than in WT (Buer and Djordjevic, 2009). All these phenotypes need further confirmation using several *tt10* alleles.

Public microarray data (Genevestigator server, <https://genevestigator.com>) show that the *TT10* gene is expressed strongly in siliques, moderately in flowers and weakly in roots, but is undetected in seedlings, leaves, and inflorescence stems (Turlapati *et al.*, 2011). Several reports using RT-PCR or RT-qPCR confirm silique and flower expression (Cai *et al.*, 2006; Kleindt *et al.*, 2010; McCaig *et al.*, 2005; Pourcel *et al.*, 2005; Turlapati *et al.*, 2011), but some also detect *TT10* mRNA in seedlings, stems (Pourcel *et al.*, 2005), and leaves (McCaig *et al.*, 2005; Turlapati *et al.*, 2011) but not in roots (Pourcel *et al.*, 2005).

Histochemical analysis of *TT10* promoter (*pTT10*) activity was performed in several studies using arabidopsis transformants expressing promoter fusions with the *uidA* gene that encodes the GUS reporter protein. At the seed level, Pourcel *et al.* (2005) showed that *pTT10* is active not only in the tannin-producing and flavonol-producing cells of the inner and outer integuments, respectively (colocalization with the flavonoid substrates), but also in early aborted seeds. Promoter activity was also detected in funiculus, replum, and transmitting tissue of the silique (Pourcel *et al.*, 2005; Turlapati *et al.*, 2011), and in root, leaf, and stem vessels (Turlapati *et al.*, 2011). On the other hand, Liang *et al.* (2006) did not detect any activity in roots and leaves. Interestingly, the *TT10* promoter construct studied by Liang *et al.* (2006) was also shown to be active in the seed coat of canola (*Brassica napus*), but activity was restricted to outer integument (El-Mezawy *et al.*, 2009).

Discrepancies between laboratories in the results concerning *TT10* gene expression and promoter activity might be explained by different environmental conditions used for plant growth or PCR conditions and various promoter fragments and vectors used in the *GUS* gene reporter studies. Indeed, there is experimental evidence showing that the *TT10* developmental spatiotemporal pattern of gene expression can be modulated by external stimuli such as drought stress (Turlapati *et al.*, 2011) or copper nutrition (Abdel-Ghany and Pilon, 2008).

Candidate transcription factors (TFs) regulating *TT10* expression still wait to be discovered. As explained above, the TT10 laccases was demonstrated to use flavonoids as *in vivo* substrates (Pourcel *et al.*, 2005). This activity is

not specific to plant laccases as both fungal and bacterial laccases are also capable of catalysing flavonoid polymerization (Fowler *et al.*, 2011; Ghidouche *et al.*, 2008). Zhao *et al.* (2010) hypothesize that TT10 may be the initial polymerizing enzyme for PA formation. However, seeds of *tt10* fed with epicatechin catalyse the formation of epicatechin oligomers (e.g. dehydrodiepicatechin A) that differ from PAs by the interflavan linkage (Pourcel *et al.*, 2005). It remains to be determined, whether epicatechin is the true *in vivo* substrate. On the other hand, the TT10 protein is predicted to be secreted in the apoplast. The experimental confirmation of this subcellular localization may rule out the hypothesis that TT10 is a potential vacuolar-condensing enzyme.

As laccases are known to have a broad substrate range (Mayer and Staples, 2002), TT10 may not be specific for flavonoid substrates. As a matter of fact, Liang *et al.* (2006) showed evidence of TT10 involvement in monolignol polymerization. They analysed extractable lignin content in the mature seeds using the TGA assay and detected a 30% reduction of lignin content in *tt10* mutant seeds. Moreover, they also suggest that TT10 is involved in lignin polymerization, based on the results of *in vitro* assays where they observed a decrease in monolignol polymerization in the mutant compared to the WT. As genes encoding catechol oxidases are not present in the arabidopsis genome (Pourcel *et al.*, 2005), it is possible that TT10 laccases activity has acquired the capacity to oxidize flavonoids in addition to oxidizing monolignols.

In conclusion, the physiological roles of the TT10 laccases are still a matter of debate. As TT10 is accumulated mainly in the seed coat during seed development, it could play a role in protecting the embryo and remaining endosperm by providing a physicochemical barrier against various stresses through quinone production (Aniszewski *et al.*, 2008; Pourcel *et al.*, 2007). However, the absence of TT10 laccases in *tt10-1* (Landsberg background) does not affect seed dormancy, germination, longevity, and testa permeability to tetrazolium salts, contrarily to what is observed for *tt* mutants lacking tannins (Debeaujon *et al.*, 2000). These results need to be confirmed using several *tt10* alleles in other ecotype backgrounds.

IV. TRANSCRIPTIONAL REGULATION OF PLANT LACCASES

Lignification and secondary cell wall formation is regulated via a complex network of TFs acting upon genes encoding monolignols biosynthetic enzymes (Zhao and Dixon, 2011; Zhong *et al.*, 2010). It is also becoming

apparent that the regulation of laccases gene expression controls lignification at the oxidative polymerization step. Like many other plant genes, laccases are regulated by different developmental and environmental cues. For example, fungal laccases are regulated by aromatic compounds derived from lignin degradation, by the nitrogen concentration in the culture media, by the carbon source and by the presence of different metallic ions (Aro *et al.*, 2005; Galhaup *et al.*, 2002).

A recent *in silico* analyses of arabidopsis laccases promoters identified a number of different potential regulatory sequences (Turlapati *et al.*, 2011). All laccases promoters have potential binding sites for MYB and/or MYC TFs, as well as ARF binding sites, and T-box promoter motifs and W-box elements. There is a statistically significant over-representation of binding sites for *MYB4* and *MYB1AT* factors. Analyses also showed that six laccases promoters (*AtLAC1*, *AtLAC2*, *AtLAC4*, *AtLAC10*, *AtLAC11*, *AtLAC17*) contained at least one AC element characteristic of phenylpropanoid gene promoters and known to be involved in MYB factor mediated transcriptional regulation of these genes.

Experimental information on transcriptional regulation of laccases genes was obtained from studies of plants up-/down-regulated for different MYB TFs suspected to be involved in controlling lignin and secondary cell wall biosynthesis. Comparative transcriptomics by affymetrix microarrays and Illumina sequencing (Ko *et al.*, 2009) of arabidopsis plants over-expressing *AtMYB46* indicated that ectopic secondary cell wall formation was associated with upregulation of a large number of cellulose, hemicellulose, and lignin biosynthetic genes suggesting that *AtMYB46* is a master regulatory gene in this process. Transcriptomic data indicated that *AtLAC4* (*IRX12*), *AtLAC10*, and *AtLAC11* genes were also up-regulated and suggested that this MYB protein also regulates the expression of laccases genes. However, subsequent transient activation analyses revealed that *AtMYB46* was incapable of directly activating *AtLAC10* expression and suggested that this laccases gene was activated indirectly via the *AtMYB46*-activation of another TF gene (*AtC3H14*). The direct effects of *AtMYB46* on *AtLAC4* and *AtLAC11* expression were not evaluated in this study. Dominant repression and over-expression of two other MYB factor genes (*AtMYB58* and *AtMYB63*) in arabidopsis was shown to be associated with reduced lignification and ectopic lignification, respectively (Zhou *et al.*, 2009). Electrophoretic mobility shift assays experiments showed that *AtMYB58* and *AtMYB63* were able to bind to AC elements in the *4CL* lignin gene promoter and that the *AtMYB58* protein was able to directly activate *AtLAC4* but not *AtLAC17*. Taken together, these results clearly suggest that laccases genes in arabidopsis are transcriptionally regulated by MYB proteins and by other TFs.

Different MYB TFs (*AtMYB4*, *EgMYB1*, and *ZmMYB31/42*) have also been shown to function as repressors of lignin biosynthesis (Fornale *et al.*, 2006, 2010; Jin *et al.*, 2000; Legay *et al.*, 2010). For example, ChIP assays indicated that the *ZmMYB31* protein interacts directly with the *ZmCOMT* and *ZmF5H* genes *in vivo* (Fornale *et al.*, 2010). Over-expression of the *ZmMYB31* gene in arabidopsis induced a dwarf phenotype, reduced lignification, and downregulated several lignin biosynthesis genes. Similarly, over-expression of *EgMYB1* in poplar and arabidopsis is associated with reduced lignin and downregulates several lignin biosynthesis genes (Legay *et al.*, 2010). However, in these studies laccases gene expression was not determined. It would obviously be of interest to see whether MYB proteins capable of repressing lignin biosynthesis genes are also capable of directly inhibiting laccases gene expression. The observation that potential binding sites for the *MYB4* repressor are statistically over-represented in arabidopsis laccases promoters could support such a hypothesis. Differential expression of laccases genes, as well as monolignols biosynthesis genes has also been implicated in the natural hypolignification of flax bast fibres (Fenart *et al.*, 2010; Huis *et al.*, 2012). Flax bast fibres, present in outer stem tissues of this species possess thick cellulose-rich secondary cell walls and are naturally hypolignified in contrast to inner stem (xylem) tissues that are normally lignified (Day *et al.*, 2005). Recent comparative transcriptomics of flax stem inner- and outer-tissues show that transcripts of lignin biosynthesis and laccases genes are significantly more abundant in lignified inner tissues than in outer-tissues suggesting that bast fibre secondary cell wall hypolignification is regulated at both the monolignols production step and at the polymerization step. Interestingly, a MYB TF (*LuMYB4*) belonging to the subgroup 4 that includes the *AtMYB4*, *EgMYB1* and *ZmMYB31/42* repressors was significantly upregulated in stem outer-tissues. Although functional analyses are obviously necessary, it is possible that this MYB factor might play a role in flax bast fibre hypolignification.

The expression of MYB genes is regulated by other TFs. For example, *AtMYB52* and *AtMYB7* are directly activated by *AtMYB46* (Ko *et al.*, 2009). MYB genes can also be regulated by other TFs and the *AtMYB46* gene has been shown to be a direct target of the NAC (*NAM*, *ATAF1*, and *CUC2*) factor *SND1* (SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1) (Zhong *et al.*, 2007). NAC proteins contain a conserved domain, the NAC domain, and are encoded by a large multigene family consisting of approximately 100 genes in arabidopsis (Olsen *et al.*, 2005). RNAi inhibition of *AtSND1* and its homolog *AtNST1* in arabidopsis down-regulates the expression of the lignin-specific *AtMYB58* and *AtMYB63* while overexpression of *AtSND1* leads to up-regulation of *AtMYB58* (but not

AtMYB63) and up-regulation of *AtNST1* leads to up-regulation of both *AtMYB58* and *AtMYB63*. These results clearly indicate that NAC factors play an important role in regulating lignin biosynthesis. More recently, certain NAC factors have been shown to directly regulate the expression of poplar laccases homologs of *AtLAC4* and *AtLAC11* (Ohtani *et al.*, 2011). Subsequent transient activation assays revealed that 10/12 PtVNS/PtWND (*P. trichocarpa* VND-, NST/SND-, SMB-related proteins/*P. trichocarpa* wood associated NAC domain TFs) genes are able to activate the poplar ortholog of *AtLAC11*. The capacity of the *PtVNS/PtWND* genes to activate the poplar *AtLAC4* gene was not evaluated in this study. Interestingly, certain *PtVNS/PtWND* genes activated both the poplar laccases gene as well as a cationic peroxidase gene. Expression profiling in flax (Huis *et al.*, unpublished) has also shown that an ortholog of the arabidopsis NAC protein ANAC002/ATAF1 is co-regulated with 2 *PAL* unigenes and the flax orthologs of *AtLAC4* and *AtLAC17* suggesting that the regulation of both monolignols production and oxidative polymerization in flax involve NAC TFs.

In silico analyses of arabidopsis laccases promoters (Turlapati *et al.*, 2011) reveals that all promoters, except for *AtLAC6* and *AtLAC14* genes, contain copper-response elements suggesting that laccases gene expression in plants is regulated by copper levels. Copper can activate gene expression in plants through either oxidative stress- or metal-mediated mechanisms leading to activation of MAPK signalling pathways induced stress (Jakubowicz *et al.*, 2010). Several studies have shown that the addition of copper to the culture medium increases laccases transcript levels in lignin-degrading fungi (Canessa *et al.*, 2008; Galhaup *et al.*, 2002). More detailed studies indicated that the copper ion was recruited by an ACE (activation of cup1) TF and that the metallic ion is necessary for DNA binding by stabilization of the protein tertiary structure (Canessa *et al.*, 2008). A link between copper and laccases gene expression is also supported by the observation that the expression of genes involved in copper metabolism (and water stress) is affected in the laccases *Atlac4*, *Atlac7* double mutant (Berthet *et al.*, 2011).

Copper has also been shown to play a central role in regulating the expression of different laccases genes in arabidopsis through a post-transcriptional mechanism implicating different microRNAs (miRNAs) (Abdel-Ghany and Pilon, 2008). miRNAs are endogenous small non-coding RNAs involved in regulating gene expression at the post-transcriptional level (Axtell *et al.*, 2007). After processing, a miRNA-AGO1 (Argonaute 1) complex associates with its mRNA target and blocks gene expression by cleaving mRNA or repressing translation (Baumberger and Baulcombe, 2005). Gene target specificity is determined by the nucleotide sequence of the miRNA that is complementary to a sequence in the mRNA. A miRNA (miR398) was

previously shown to down-regulate the expression of a copper/zinc superoxide dismutase gene in arabidopsis in response to low copper levels (Yamasaki *et al.*, 2007). Subsequent computational analysis predicted that genes encoding copper-containing laccases are also targeted by three different miRNAs (miR408, miR397, and miR857) in arabidopsis (Abdel-Ghany and Pilon, 2008). The *miR408* is predicted to target *AtLAC3*, *AtLAC12*, and *AtLAC13*; *miR397a/b* are predicted to target *AtLAC2*, *AtLAC4*, and *AtLAC17*; and *miR857* is predicted to target *AtLAC7*. Plants were grown in hydroponic conditions under three different copper regimes (Cu-deficient, Cu-limited, and Cu-replete) and expression profiles of miRNA precursors and potential target genes were evaluated. Transcripts for *miR408*, *miR397*, and *miR857* accumulate when copper is limiting and disappear when copper is sufficient. In contrast, transcripts for target laccases genes showed a reciprocal profile, that is, transcripts decrease when copper is limiting and accumulate when copper is sufficient. Interaction between miRNAs and target genes was confirmed by the identification of specific cleavage products. MicroRNAs have also recently been shown to play a role in regulating the expression of laccases and other genes in poplar (Lu *et al.*, 2011). Another study (Lu *et al.*, 2009) has also recently underlined the potential role of RNA degradation mechanisms in regulating laccases gene expression via the controlled elimination of miRNAs.

Using the PsRNA target tool (Dai and Zhao, 2011), 25 poplar genes are predicted to be putative targets for *miR397a* (default parameter values). Of these predicted targets, only one gene does not encode a putative laccases (not shown). All of the poplar genes clustering with *AtLAC4* and *AtLAC17*, except *PtrLAC5*, are predicted targets of *miR397a* (Fig. 2). As shown in Fig. 2, a tight correlation is observed between the expression clads, the phylogenetic clads, and the prediction of predicted *miR397a* targets. As for *AtLAC4* and *AtLAC17*, *miR397a* may play an important role in the control of poplar laccases, particularly those related to xylem formation.

Bioinformatic analyses of laccases genes have also indicated the existence of other potential regulatory mechanisms (Turlapati *et al.*, 2011). For example, natural antisense transcripts are present at the loci for the genes *AtLAC6* and *AtLAC17*. These *cis*-antisense strands are transcribed from the complementary stand and provoke the degradation of the sense transcript via an RNAi-mediated process and result in post-transcriptional regulation of gene expression (Jin *et al.*, 2008). Similarly, evidence for epigenetic modifications (histone acetylation and/or DNA methylation) were identified in several laccases gene promoters and/or coding sequences (Turlapati *et al.*, 2011). These modifications are known to affect gene expression and therefore represent another potential mechanism by which laccases gene expression may be modified.

In conclusion, it appears that laccases gene expression is coordinated with that of monolignols biosynthesis genes and involves not only a complex regulatory network of TFs but also copper-dependent miRNA-based mechanisms and other post-transcriptional mechanisms.

V. CONCLUSION AND PERSPECTIVES

Laccases are widespread oxidases represented by large gene families in plants. The definite establishment of their involvement in the polymerization of lignins has been difficult to establish for three main reasons: (1) the broad range of *in vitro* laccases substrates, which makes the identification of specific *in vivo* substrate(s) difficult; (2) the broad individual expression profile of most laccases expressed in model plants, that is, very few laccases show a strict spatial or temporal specificity; and (3) the redundancy of gene expression patterns of different laccases, which probably underlies a functional redundancy. As recently shown (Turlapati *et al.*, 2011), deciphering the spatial and temporal expression patterns of laccases might contribute to elucidating their functional roles. Up to now, the physiological roles of laccases have only been assessed via a forward genetics approach for a few members of the 17-member arabidopsis multigene family. The seed-specific laccases (*AtLAC15/TT10*) is involved in the polymerization of seed coat flavonoids and the production of brown and insoluble polymers with a putative protective role (Pourcel *et al.*, 2005). The stem-specific laccases *AtLAC4* and *AtLAC17* are definitely involved in lignification (Berthet *et al.*, 2011), with the possible assistance of *AtLAC12* (this study). There are thus many other arabidopsis laccases genes with still unknown functions. As clearly demonstrated for the *AtLAC4* and *AtLAC17* genes, the most appropriate approach to determine the function of these 14 laccases would probably be the production of multiple mutants in order to suppress putative functional redundancy. The recent observation showing that *AtLAC17* silencing in arabidopsis mainly affects the deposition of G-lignin units in the interfascicular fibres of stems (Berthet *et al.*, 2011) provides new clues into elucidating the precise role of this laccases that significantly and specifically contributes to lignification of supportive tissues. Nevertheless, the spatiotemporal processes governing the lignin polymerization process seem more mysterious than ever and the question as to why G unit deposition in the fibres of *lac17* is mainly affected still remains to be addressed. Such specificity might be due to the spatiotemporal specificity of *AtLAC17* expression or alternatively, by a higher substrate specificity of this laccases towards coniferyl alcohol. Further investigation of the complex network of

post-transcriptional regulation mediated by miRNAs should also contribute to a better understanding of the biological role(s) of plant laccases (Berthet, 2010).

ACKNOWLEDGEMENTS

The technical assistance of Philippe Le Bris, Frédéric Legée, and Laurent Cézard (IJPB, Versailles) is gratefully appreciated. The work was supported by a PhD grant from INRA to S. B. and was partly funded by the EU RENEWALL program.

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