Journal of Experimental Botany, Vol. 64, No. 6, pp. 1769–1781, 2013
doi:10.1093/jxb/ert045 Advance Access publication 15 February, 2013

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Expression of *SofLAC*, a new laccase in sugarcane, restores lignin content but not S:G ratio of *Arabidopsis lac17* mutant

Igor Cesarino[1,2,3]*, Pedro Araújo[1], Juliana Lischka Sampaio Mayer[1], Renato Vicentini[4], Serge Berthet[5], Brecht Demedts[2,3], Bartel Vanholme[2,3], Wout Boerjan[2,3] and Paulo Mazzafera[1]

[1] Departamento de Biologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas, CP 6109, 13083–970 Campinas, SP, Brazil
[2] Department of Plant Systems Biology, VIB, 9052 Gent, Belgium
[3] Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052 Gent, Belgium
[4] Laboratório de Bioinformática e Biologia de Sistemas, Centro de Biologia Molecular e Engenharia Genética, Universidade Estadual de Campinas, Av. Cândido Rondon 400, 13083–875 Campinas, SP, Brazil
[5] CEA, iRTSV, Laboratoire de Physiologie Cellulaire Végétale, UMR 5168 CEA-CNRS-INRA-Université Joseph Fourier, Grenoble, France

* To whom correspondence should be addressed. Email: igces@psb.vib-ugent.be

Received 8 November 2012; Revised 28 January 2013; Accepted 30 January 2013

Abstract

Lignin is a complex phenolic heteropolymer deposited in the secondarily thickened walls of specialized plant cells to provide strength for plants to stand upright and hydrophobicity to conducting cells for long-distance water transport. Although essential for plant growth and development, lignin is the major plant cell-wall component responsible for biomass recalcitrance to industrial processing. Peroxidases and laccases are generally thought to be responsible for lignin polymerization, but, given their broad substrate specificities and large gene families, specific isoforms involved in lignification are difficult to identify. This study used a combination of co-expression analysis, tissue/cell-type-specific expression analysis, and genetic complementation to correlate a sugarcane laccase gene, *SofLAC*, to the lignification process. A co-expression network constructed from 37 cDNA libraries showed that *SofLAC* was coordinately expressed with several phenylpropanoid biosynthesis genes. Tissue-specific expression analysis by quantitative RT-PCR showed that *SofLAC* was expressed preferentially in young internodes and that expression levels decrease with stem maturity. Cell-type-specific expression analysis by *in situ* hybridization demonstrated the localization of *SofLAC* mRNA in lignifying cell types, mainly in inner and outer portions of sclerenchymatic bundle sheaths. To investigate whether SofLAC is able to oxidize monolignols during lignification, the *Arabidopsis lac17* mutant, which has reduced lignin levels, was complemented by expressing *SofLAC* under the control of the *Arabidopsis AtLAC17* promoter. The expression of *SofLAC* restored the lignin content but not the lignin composition in complemented *lac17* mutant lines. Taken together, these results suggest that SofLAC participates in lignification in sugarcane.

Key words: bioenergy, gene expression, genetic complementation, laccase, lignin, sugarcane.

Introduction

Lignins are complex aromatic heteropolymers derived from the oxidative combinatorial coupling of mainly three *p*-hydroxycinnamyl alcohol monomers (monolignols), *p*-coumaryl, coniferyl, and sinapyl alcohols, differing in their degree of methoxylation (Boerjan *et al*., 2003). After incorporation into lignin, these monolignols are called *p*-hydroxyphenyl (H),

Abbreviations: DIG, digoxygenin; G, guaiacyl; H, *p*-hydroxyphenyl; qRT-PCR, quantitative RT-PCR; S, syringyl; SAS, sugarcane assembled sequences; TBA, tertiary butyl alcohol; UTR, untranslated region.

guaiacyl (G), and syringyl (S) units, respectively (Raes *et al.*, 2003). These biopolymers are deposited mainly in the secondarily thickened cell walls of tracheary elements and fibres to provide strength and rigidity for plants to stand upright and hydrophobicity to conducting cells for long-distance water transport (Vanholme *et al.*, 2008; Weng and Chapple, 2010). Cell-wall lignification, as well as the incorporation rate of each monomer, is regulated in a spatio-temporal fashion and varies between primary and secondary cell walls, among tissues and among different taxa (Grabber *et al.*, 2004). In addition, the deposition of lignin not only follows a developmental programme but can also be triggered by a variety of stresses and environmental conditions (Moura *et al.*, 2010).

Monolignols are synthesized in the cytoplasm and transported to the cell wall, where they are oxidized by laccases and/or peroxidases prior to their incorporation into the polymer (Vanholme *et al.*, 2008, 2010a; Bonawitz and Chapple, 2010). However, the broad *in vitro* substrate specificity of the different enzymes makes it difficult to pinpoint their *in planta* substrates, and the high gene redundancy hampers the use of reverse genetics (Cai *et al.*, 2006; McCaig *et al.*, 2005; Cosio and Dunand, 2009, 2010). Nevertheless, the involvement of peroxidases in lignin polymerization has been clearly demonstrated by a small number of studies reporting the generation of transgenic plants in which lignification was affected by the up- or downregulation of a particular peroxidase gene (Fagerstedt *et al.*, 2010). The downregulation of a cationic peroxidase, *NtPrx60*, in tobacco resulted in up to 50% reduction in lignin content, affecting vascular tissue development but not the overall plant growth and development (Blee *et al.*, 2003). It has also been shown that overexpression of a cationic peroxidase led to ectopic lignification in transgenic tomato plants (El Mansouri *et al.*, 1999), whilst transgenic aspen lines downregulated in an anionic peroxidase, *PkPrx03*, had 20% less lignin compared with the wild type (Li *et al.*, 2003). By contrast, laccases have been correlated to lignin biosynthesis based mainly on their ability to oxidize lignin precursors *in vitro* (Karkonen *et al.*, 2002; Liang *et al.*, 2006) and their localization in lignin-forming tissues in various plant species (Ranocha *et al.*, 2002; Caparrós-Ruiz *et al.*, 2006). In an attempt to find genetic evidence of the role of laccases in lignin polymerization, transgenic poplars with reduced laccase expression have been produced and, although they exhibited an increase in total soluble phenolic content, neither lignin content nor composition was affected (Ranocha *et al.*, 2002). *Arabidopsis tt10* (*TRANSPARENT TESTA10*; *LAC15*) mutants have provided evidence for the role of AtLAC15 in the oxidative polymerization of flavonoids in the seed coat (Pourcel *et al.*, 2005). Mutant seeds had a 59% increase in soluble pro-anthocyanidin or condensed tannin and nearly 30% less extractable lignin compared with wild-type seeds (Liang *et al.*, 2006). Only recently, the role of laccases in lignification of stems was demonstrated unambiguously by reverse genetics. By studying laccase T-DNA insertion mutants in *Arabidopsis thaliana*, Berthet *et al.* (2011) demonstrated that single mutants of *AtLAC4* and *AtLAC17* had moderately reduced lignin levels, whereas stems of double mutants displayed up to 40% less lignin compared with the control and had an irregular xylem phenotype. Moreover, tissue-specific alterations were found, as *AtLAC17* is specifically involved in the deposition of G units in fibres. Nevertheless, genetic evidence for the functions of laccases in plants is scarce, especially in monocotyledonous plants.

Although lignin is essential for plant growth and development, the presence of this biopolymer negatively affects the use of lignocellulosic biomass as a source for biofuels and bio-based materials (Chen and Dixon, 2007; Vanholme *et al.*, 2010b). The property of lignin to resist degradation, mainly because of the chemically diverse and poorly reactive linkages and the variety of monomer units, makes this phenolic polymer the major plant cell-wall component responsible for biomass recalcitrance (Weng *et al.*, 2008). Therefore, the identification and characterization of genes responsible for lignin monomer biosynthesis, lignin polymerization, and lignin pathway regulation is a fundamental goal to allow tailoring of energy crops for industrial purposes. Here, we provide evidence for the role of a laccase gene (*SofLAC*) in lignin biosynthesis in sugarcane (*Saccharum* spp.), the most important and productive bioenergy crop, by using a combination of co-expression analysis, tissue-specific expression analysis, and genetic complementation of the *Arabidopsis lac17* mutant.

Material and methods

Plant materials and growth conditions

All experiments were performed using plant material harvested from adult sugarcane plants of cultivar IACSP04-627 cultivated in the greenhouse of the Department of Plant Biology, Institute of Biology, State University of Campinas, Campinas, Brazil. For the genetic complementation of the *Arabidopsis thaliana lac17* mutant, Col-0 plants were grown under long-day conditions in a growth chamber (21 °C, 60% relative humidity). The *lac17* T-DNA mutant (Salk_016748) was obtained from the Salk Institute collection.

Analysis of SUCEST Database and bioinformatic analysis

Laccase genes were identified from the Brazilian Sugarcane EST Database (SUCEST; http://www.sucest-fun.org) by performing a local tBLASTn search using laccase protein sequences of *Arabidopsis thaliana* (The Arabidopsis Information Resource; http://www.arabidopsis.org), *Populus trichocarpa* (Joint Genome Institute; http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.info.html), *Oryza sativa* (Phytozome; http://www.phytozome.net/rice.php) and *Sorghum bicolor* (Phytozome; http://www.phytozome.net/sorghum) as queries (Supplementary Table S2 at *JXB* online). The resulting sugarcane hits were then re-blasted against the nr-NCBI database to confirm protein identity. Multiple amino acid sequence alignment and phylogenetic analysis were carried out with MEGA5 software (Tamura *et al.*, 2011). Evolutionary relationships were inferred using the neighbour-joining method with bootstrap tests for 1000 replicates. The evolutionary distances were computed using the Dayhoff matrix-based

method. The representative sugarcane laccase gene (*SofLAC*) was selected based on phylogeny, co-expression analysis, and data from the literature (Casu *et al*., 2007).

In silico *co-expression network between SofLAC and lignin biosynthesis-related genes*

For the co-expression network construction, a set of 37 cDNA libraries developed by the SUCEST project (Vettore *et al*., 2003) and originating from different sugarcane tissue types and developmental stages was used. For all pairs of sugarcane assembled sequences (SASs) the number of libraries in which each SAS was present was determined and the number of common libraries for both SASs was computed, as described by Faccioli *et al*. (2005). To join two SASs by an edge in our network, the one-sided Fisher's exact test (P <0.05) was applied. The final co-expression network, with 50 nodes, was analysed in Medusa software (Hooper and Bork, 2005) using the affinity propagation clustering method.

RNA isolation, cDNA synthesis, and quantitative RT-PCR (qRT-PCR)

Roots, internodes at three developmental stages (young, internodes 1, 2, and 3; developing, internodes 5, 6, and 7; and mature, internodes 15, 16, and 17) and leaves from three biological replicates were harvested and frozen immediately in liquid N_2, ground into fine powder using a pre-cooled IKA® A11 mill and stored at –80 °C until use. Total RNA isolation, cDNA synthesis, and qRT-PCR were performed as described previously (Cesarino *et al*., 2013). Sugarcane *SofLAC*-specific primers (Supplementary Table S1 at *JXB* online) were designed in the 3'-untranslated region (UTR) to discriminate the target laccase gene from other members of the gene family. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a reference gene.

Lignin histochemical staining

Stem material from young internodes (internode 3) was fixed in formalin:acetic acid:ethanol:water (5:5:60:30, v/v/v/v), embedded in Paraplast® X-tra (Fisher) and sections of 20 µm were obtained with a rotary microtome. Lignin deposition and composition were investigated histochemically by staining with Mäule reagent as described previously (Cesarino *et al*., 2012). Mäule reagent gives an indication of lignin composition by staining S-lignin red and G-lignin brown.

In situ *hybridization*

The same pair of primers designed for qRT-PCR was used for the amplification of a 136 bp fragment from the 3'-UTR region of the *SofLAC* clone SCUTST3084C11.g ordered from the Brazilian Clone Collection Center (BCCCenter, FCAV-UNESP, Campus Jaboticabal). The fragment was cloned into pGEM®-T Easy Vector (Promega) using T4 DNA ligase (Promega) and sequenced to confirm identity and check insert orientation. Plasmid linearization was carried out with *Sal*I (Fermentas) and *Nco*I (Fermentas), and probes were synthesized by *in vitro* transcription with SP6 and T7 RNA polymerases using a digoxygenin (DIG) RNA labelling kit (SP6/T7) (Roche Applied Science), according to the manufacturer's protocol. A sense probe was used as a negative control.

Stem material from internode 3 was fixed in 4% paraformaldehyde (w/v) in PBS for 16 h at 4 °C under vacuum, dehydrated in a graded ethanol series, and stored at 4 °C until use. The ethanol was replaced by tertiary butyl alcohol (TBA) in a graded series (70, 85, 95, and 100% TBA), with samples maintained in each solution for 48 h. Incubation with 100% TBA was repeated three times. Solid Paraplast X-tra was added to samples in 100% TBA (3:1, w/v) and incubated at 58 °C to melt the Paraplast and evaporate the TBA. After changing the Paraplast three times (every 12 h), the samples were placed on moulds for solidification. Serial sections of 12 µm thick were cut on a rotary microtome (Leica®), placed on pre-cleaned and electrically charged slides (Probe-On Plus®; Fisher) and distended in a heated plate at 48 °C.

The slides were immersed in xylene for removal of the Paraplast, washed in 100% ethanol, and dried at room temperature. Slides were then incubated at 37 °C in 1 µg ml^{-1} of proteinase K solution (in 50 mM Tris/HCl, pH 7.5) for 10 min, washed in DEPC-treated water for 10 min, and further incubated for 10 min in 100 mM triethanolamine/HCl and 0.5% acetic anhydride (v/v). A hybridization solution containing 50% deionized formamide, 100 mM Tris/HCl (pH 7.5), 50 mM NaCl, 10 mM EDTA, 1× Denhardt's solution, and 10% dextran sulfate was used to dilute 400 ng ml^{-1} of DIG-labelled sense and antisense *SofLAC* probes. Hybridization was carried out overnight at 42 °C. Subsequently, slides were washed twice in 4× SSC (600 mM NaCl, 60 mM tri-sodium citrate dehydrate, pH 7.0) followed by two washes in 2× SSC for 20 min at 42 °C each. The slides were rinsed with 100 mM Tris/HCl (pH 7.5) at room temperature and then incubated with blocking solution [2%, w/v, blocking reagent (Roche) in 100 mM Tris/HCl, pH 7.5] for 10 min at 37 °C. For staining, an alkaline phosphatase-conjugated anti-DIG antibody (Roche) diluted 1:1000 in 100 mM Tris/HCl (pH 7.5), 150 mM NaCl was added and the samples incubated at 37 °C for an additional hour. Slides were washed twice with 100 mM Tris/HCl (pH 7.5) and then incubated for 10 min in 10 mM Tris/HCl (pH 9.0), 10 mM $MgCl_2.6H_2O$ at room temperature. For immunological detection, the slides were maintained in a phosphatase substrate buffer (NBT/BCIP in 100 mM Tris/HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$) at room temperature in the dark overnight. The colour reaction was stopped by washing with 10 mM Tris/HCl (pH 8.0), 50 mM EDTA. Photomicrographs were captured with an Olympus BX 51 photomicroscope equipped with an Olympus DP71 camera.

Complementation of the Arabidopsis thaliana lac17 *mutant*

The full-length open reading frame of *SofLAC* was obtained from the Brazilian Clone Collection Center. The clone SCUTST3084C11.g was used as template for the introduction of *att*B1 and *att*B2 recombination sites by PCR, for

further cloning into the Gateway pDONR221 vector via BP clonase (Invitrogen). *SofLAC*-specific primers were designed also to include the 3'-UTR. For *AtLAC17* promoter cloning, the region directly upstream of the *AtLAC17* (1997 bb; At5g60020) open reading frame was PCR amplified from genomic DNA using gene-specific primers containing the *att*B4 and *att*B1R recombination sites and BP cloned into pDONR-P4P1R. The sequence identity of all pENTR constructs was confirmed by sequencing. The recombination of pENTR-pro*AtLAC17* and pENTR-SofLAC in the destination vector pK7m24GW-FAST was performed via MultiSite LR Clonase Plus (Invitrogen), resulting in the expression clone proAtLAC17::SofLAC, which was introduced into *Agrobacterium tumefaciens* strain C58C1 PMP90 by electroporation. Positive colonies were confirmed by PCR and used for genetic transformation of the *Arabidopsis lac17* mutant plants using the floral dip method (Clough and Bent, 1998). The identification of transformed seeds (T1 line) and further identification of homozygous seeds were based on seed fluorescence (Shimada *et al.*, 2010).

Lignin analysis

For preparation of cell-wall material, 5 mg of senescent inflorescence dry stems were sequentially washed with 500 µl of water, ethanol, chloroform and acetone for 30 min each at 98, 76, 59, and 45 °C, respectively. Acetyl bromide lignin extraction was then performed, as described previously (Hatfield and Fukushima, 2005). Lignin composition was investigated using thioacidolysis, as previously described (Lapierre *et al.*, 1999). Lignin-derived compounds were identified by analysis of their trimethylsilyl derivatives using gas chromatography/mass spectrometry. Lignin analyses were performed with four independent transformed lines using four biological replicates for lignin quantification and three biological replicates for analysis of lignin composition. Statistical analysis was performed using Student's *t*-test (P <0.01, n=4 or P <0.05, n=3).

Results

SofLAC *is coordinately expressed with phenylpropanoid genes and is phylogenetically related to* AtLAC17

tBLASTn analysis of the Sugarcane EST Database (SUCEST) based on the annotated *Arabidopsis*, rice, poplar, and sorghum laccase genes resulted in the identification of 12 putative sugarcane laccase sequences. A co-expression analysis using sugarcane cDNA libraries was performed to further select candidate laccase genes putatively involved in cell-wall lignification (Fig. 1). This analysis showed that one specific laccase gene, named *SofLAC* (corresponding to two different tentative consensus numbers, SCVPRZ3027A08.g and SCUTST3084C11.g) was co-expressed with the putative phenylpropanoid genes, *cinnamoyl-CoA reductase* (*CCR1*; SCBFRT1064A01.g), *p-hydroxycinnamoyl-CoA:quinate shikimate p-hydroxycinnamoyltransferase* (*HCT1*; SCUTRZ3072B08.g) and *caffeoyl-CoA O-methyltransferase*

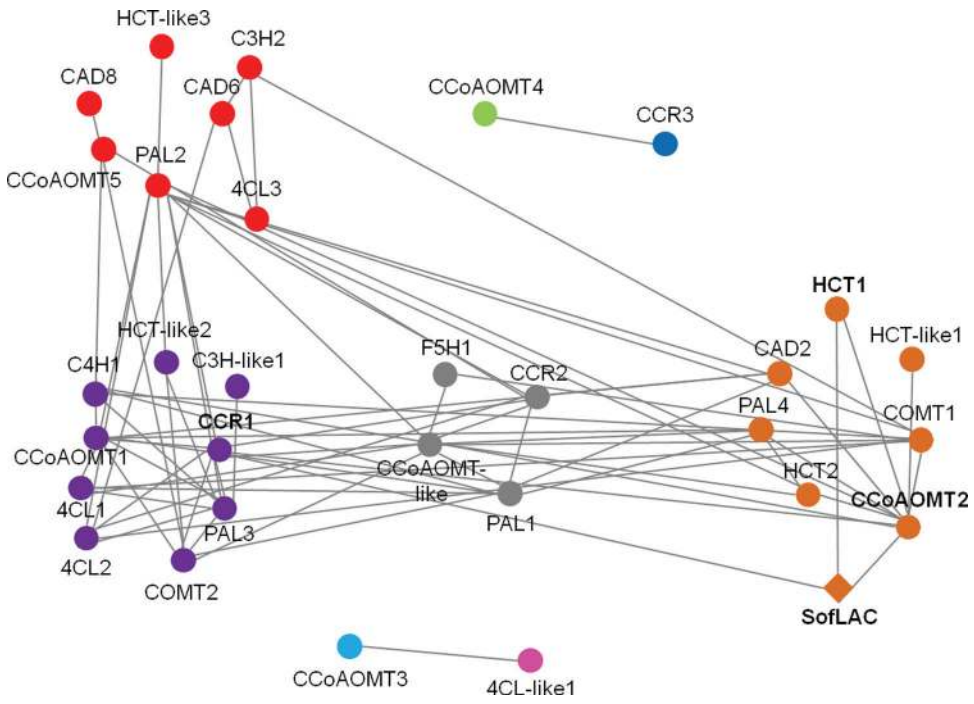

Fig. 1. Co-expression gene network for *SofLAC*. Individual genes/contigs are represented by nodes, whilst edges indicate whether two genes are coordinately expressed above a certain threshold. Genes belonging to the same vicinity are indicated by the same colour. *SofLAC* (diamond) was directly linked with the putative phenylpropanoid genes *HCT1*, *CCoAOMT2*, and *CCR1* (highlighted in bold). Gene abbreviations and SUCEST consensus numbers are given in Supplementary Data at *JXB* online. (This figure is available in colour at *JXB* online.)

(*CCoAOMT2*; SCJLRT2050C09.g). Moreover, several other putative phenylpropanoid biosynthesis-related genes were found in close vicinity of *SofLAC*, including two *hydroxycinnamoyllbenzoyl transferase* (*HCBT*; SCVPRT2082B07.g and SCCCCL4009E02.g), *caffeic acid O-methyltransferase* (*COMT*; SCJLRT1023B09.g), *phenylalanine ammonia-lyase* (*PAL*; SCJFLR1017B11.g) and *cinnamyl alcohol dehydrogenase* (*CAD*; SCEPRZ1011A02.g). In addition, *SofLAC* shared 99% identity with a sugarcane putative laccase gene (GenBank accession no. CA256359) shown previously to be co-expressed with a *cellulose synthase* (*CesA*) gene predicted to be involved in secondary cell-wall synthesis (Casu *et al*., 2007). Thus, *SofLAC* was the best candidate among all 12 identified laccase genes to play a role in secondary cell-wall synthesis in sugarcane.

A phylogenetic analysis was carried out to gain insights into the evolutionary relationships between SofLAC and other plant laccases (Fig. 2). Previously characterized laccases from *Z. mays* (Caparrós-Ruiz *et al*., 2006), *P. trichocarpa* (Ranocha *et al*., 2002), and *G. arboreum* (Wang *et al*., 2008), as well as the laccase protein family from *A. thaliana* and *S. bicolor*, were included in this analysis. Consistent with previous studies, the laccases clustered into six subgroups (McCaig *et al*., 2005; Caparrós-Ruiz *et al*., 2006). Sorghum and *Arabidopsis* laccases were identified in each phylogenetic group, except for subgroup 6, which was formed exclusively by the divergent AtLAC1. Although not the case for all subgroups, monocot laccases generally clustered separately from dicot laccases. In agreement with previous results, there was no clear relationship between sequence and function, as laccases with similar putative functions clustered in different subgroups. SofLAC was clustered within subgroup 1, together with ZmLAC2, ZmLAC4, and ZmLAC5 from maize, PtLAC110 from poplar and *Arabidopsis* AtLAC2 and AtLAC17, as well as several sorghum laccases. The closest protein to SofLAC was its co-orthologue in sorghum, SORBIDRAFT_01g039690, which shared an overall sequence identity of 95%, as expected, as sorghum is the closest relative to sugarcane in the Andropogoneae tribe (Cheavegatti-Gianotto *et al*., 2011). Interestingly, the closest *Arabidopsis* homologue to SofLAC was AtLAC17 (overall sequence identity of 68%), which has been shown to be involved specifically in the deposition of G units in fibres in *Arabidopsis* stems (Berthet *et al*., 2011).

The *SofLAC* coding sequence was 1722 bp and the predicted protein was 574 aa, with a predicted molecular weight of 62.6 kDa and a theoretical pI of 8.28. SofLAC had a putative N-terminal signal peptide for secretion, as predicted by the SignalP program, suggesting that this protein is targeted to the cell wall via the typical secretory pathway using the endoplasmic reticulum–Golgi network. In addition, subcellular localization prediction programs also suggested the apoplastic localization of SofLAC (data not shown). After cleavage, which was predicted to occur between aa 25 and 26, the mature protein contained 548 aa, with a predicted molecular weight of 60 kDa and theoretical pI of 8.31, confirming the basic nature of this laccase. According to typical features of plant laccases (Fig. 3), SofLAC presented four highly conserved copper ligand binding sites, two N-terminal and two C-terminal, and previously reported strictly conserved motifs with unknown function such as a proline-glycine-proline (PGP) motif, upstream of the first copper-binding region, a TQCP motif downstream the first copper-binding region and the NPGxW motif in the C-terminal region (McCaig *et al*., 2005).

SofLAC is preferentially expressed in sclerenchymatic and parenchymatic cells of young internodes

In order to investigate whether SofLAC could be involved in lignification of sugarcane stems, the tissue/developmental expression pattern of *SofLAC* was analysed by qRT-PCR (Fig. 4). Relative expression was determined in roots, internodes at three developmental stages (i.e. young, developing, and mature internodes), and leaves. Comparison among tissues showed that *SofLAC* was expressed preferentially in sugarcanes stems, with lower expression levels in roots and leaves. Analysis during sugarcane stem development showed higher expression of *SofLAC* in young internodes, whilst the expression level decreased with maturity.

In order to localize the expression of *SofLAC* more precisely at the cell-type level, mRNA localization was performed by *in situ* hybridization. Young internodes were chosen for this experiment, as they corresponded to the tissue with the highest accumulation of *SofLAC* mRNA. In addition, histochemical staining with Mäule reagent was carried out to compare developmental lignin deposition with cell-specific *SofLAC* mRNA accumulation. Lignified cell types from internode 3 included the protoxylem and metaxylem elements, sclerenchyma sheath, and, to a lesser extent, parenchymatic cells close to the periphery of the stem (Fig. 5A, C, E). The antisense *SofLAC* probe was detected strongly in inner and outer portions of the sclerenchymatic bundle (Fig. 5B, D, F). A signal was also detected in parenchymatic cells surrounding the vascular bundles (Fig. 5G). No signal was detected when a sense probe (control) was used (Fig. 5H). Therefore, the expression pattern and mRNA localization of *SofLAC* were in agreement with the histochemical staining of lignin and thus with the expected expression pattern of genes involved in the lignification process in sugarcane stems.

Sugarcane SofLAC can complement the Arabidopsis lac17 *mutant*

To investigate whether SofLAC can complement the *Arabidopsis lac17* mutant, which has a lower lignin content, the coding sequence of *SofLAC* including its 3'-UTR was expressed under the control of the endogenous *AtLAC17* promoter in the *lac17* background (Fig. 6A). The 3'-UTR was included, as a previous study revealed its importance for efficient complementation of the *Arabidopsis lac17* mutant by *AtLAC17* (Berthet *et al*., 2011). Several independent transformants were obtained, and *SofLAC* expression was confirmed by RT-PCR (Fig. 6B). Four independent transformants with high *SofLAC* expression were analysed further.

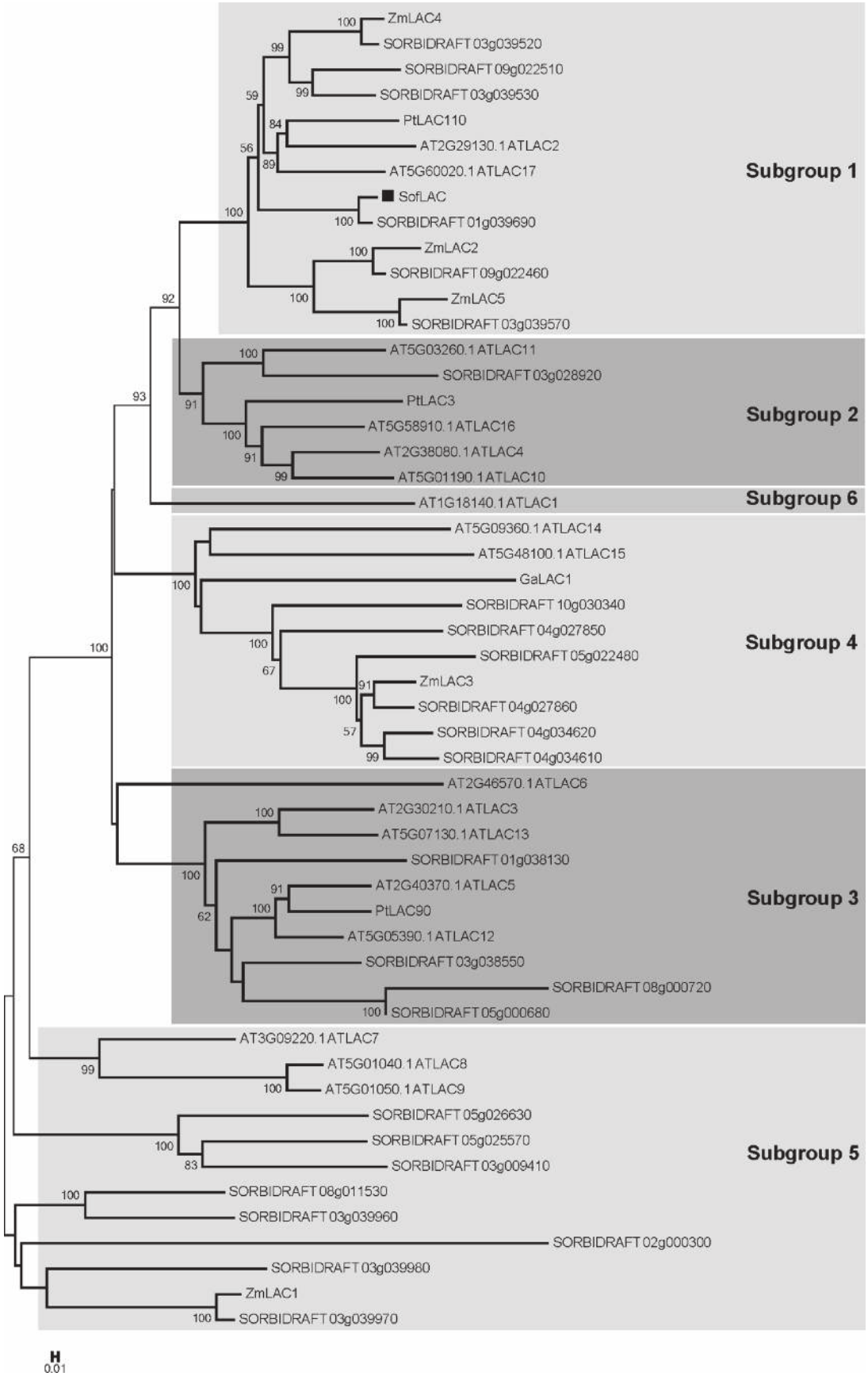

Fig. 2. Phylogenetic tree of SofLAC and other laccase proteins from *A. thaliana*, *Sorghum bicolor*, *Zea mays*, *Populus trichocarpa*, and *Gossypium arboreum*. Different laccase subgroups are based on the results of McCaig *et al*. (2005). SofLAC (black square) clustered in subgroup 1 together with AtLAC17 and other dicot and monocot laccases. The evolutionary relationships were inferred using the

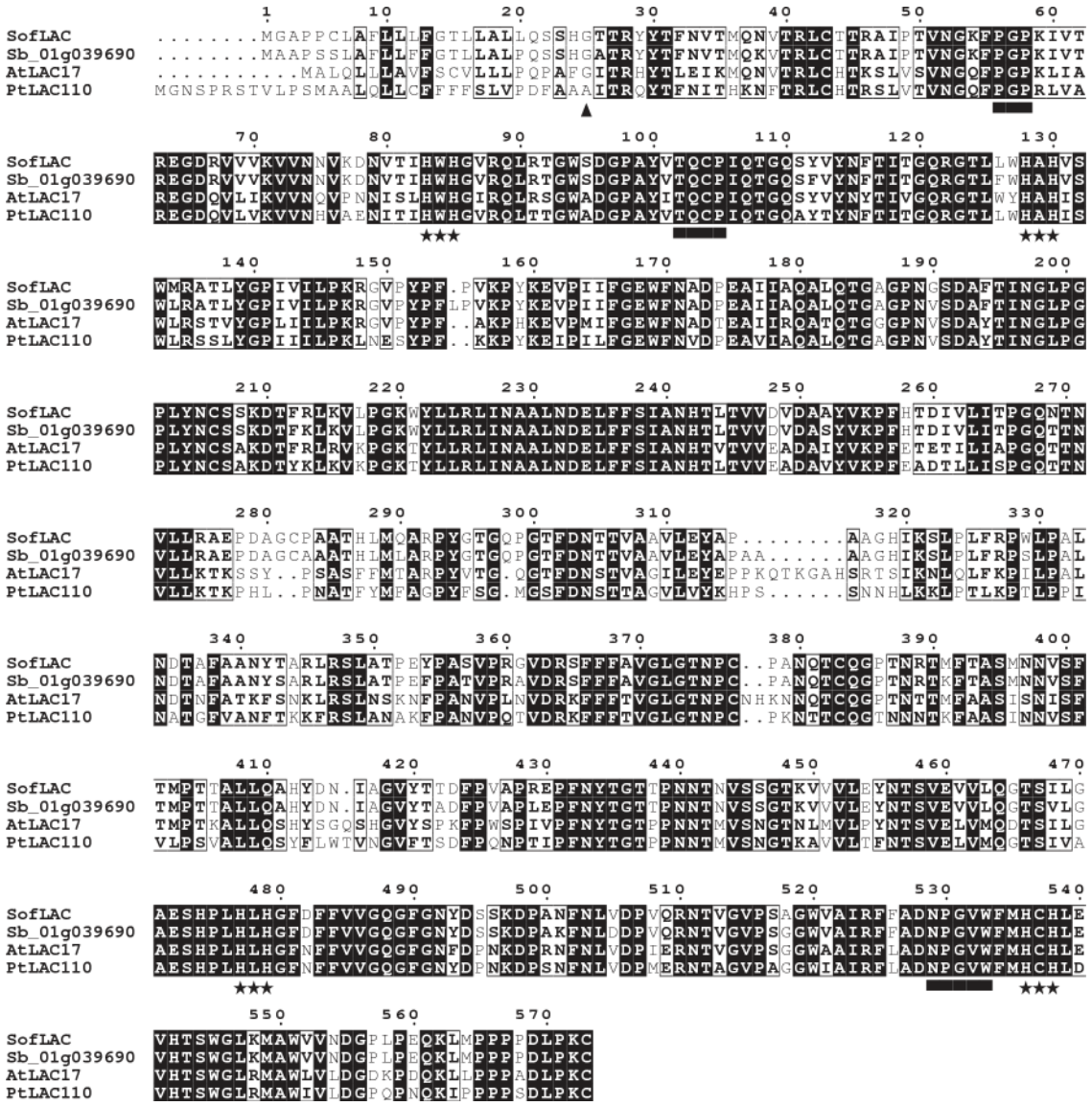

Fig. 3. Multiple amino acid sequence alignment of SofLAC and closely related laccases. Amino acid identity is highlighted by black shading. The overall sequence identity between SofLAC and other proteins was 95% with Sb_01g039690, 68% with AtLAC17, and 66% with PtLAC110. The black triangle indicates the putative cleavage site, black bars correspond to strictly conserved motifs with unknown function, and black stars indicate typical conserved copper ligand binding sites.

The *Arabidopsis lac17* mutant had 14% less Klason lignin than the corresponding control and a higher S:G ratio, as *LAC17* deficiency affects G-unit deposition in interfascicular fibres (Berthet *et al*., 2011). Therefore, the effectiveness of complementation was evaluated by subjecting dry stems to lignin quantification using the acetyl bromide method and to lignin composition analysis using thioacidolysis. Under our conditions, the *lac17* mutant had 19% less lignin than the corresponding WT control and had a higher S:G ratio (0.42 in the *lac17* mutant compared with 0.33 in the WT) (Fig. 6C, D). Expression of *SofLAC* in the *lac17* background resulted in the complete restoration of WT lignin levels in all four transformed lines analysed (Fig. 6C). In contrast, the complemented lines showed a similar (LAC1, LAC2, and LAC4) or even higher (LAC3) S:G ratio when compared with the *lac17* mutant (Fig. 6D). These observations demonstrated that expression of *SofLAC* was sufficient to restore the WT lignin content and suggested that this laccase is potentially involved in lignification in sugarcane young stems. However, in contrast to its *Arabidopsis* homologue, SofLAC might not be specific for the deposition of G units, as the higher S:G ratio found in *lac17* mutants was not restored to the WT level after complementation with the *SofLAC* coding sequence.

neighbour-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. NCBI accession numbers are given in Supplementary Data.

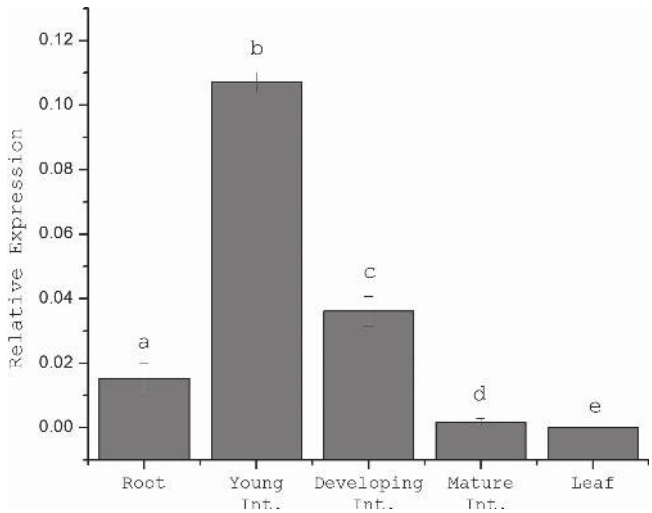

Fig. 4. Tissue-specific expression pattern of *SofLAC* determined by qRT-PCR. *GAPDH* was used as a reference gene. Expression was higher in young internodes, and expression levels decreased with stem maturity. Bars with different letters above were significantly different (Student's *t*-test, $P < 0.01$, $n=3$).

Discussion

Sugarcane is a C4 plant with a high capacity to convert solar radiation into biomass and also with the ability to accumulate up to 50–60% of the stem dry weight as sucrose in the mature tissues (Casu *et al.*, 2004). The worldwide demand for sustainably produced energy has increased the interest in sugarcane in recent years; approximately 25 billion litres of ethanol were produced in Brazil in the 2009/2010 crop season (Cheavegatti-Gianotto *et al.*, 2011). Although the production of sugar-based ethanol, often referred to as first-generation ethanol, represents the most convenient and efficient option for sustainable energy in the case of sugarcane, the biofuel industry could also benefit from recent advances in processing lignocellulosic biomass, especially because sugarcane is among the most efficient biomass producers known (Vermerris, 2011). The so-called second-generation ethanol is produced from the plant biomass composed of the polymers cellulose, hemicellulose, and lignin (Yuan *et al.*, 2008). However, the recalcitrant nature of this mixture negatively affects the conversion of plant cell-wall polysaccharides into fermentable sugars for biofuels (Vanholme *et al.*, 2010a). In this regard, the ability of lignin to resist degradation makes this complex phenolic polymer the major plant cell-wall component responsible for biomass recalcitrance (Weng *et al.*, 2008). Therefore, a deeper understanding of lignin biosynthesis and regulation in monocots is required in order to develop less recalcitrant varieties that are more amenable to biomass processing. Genetic information on lignin biosynthesis in sugarcane is scarce and limited to a few studies reporting on a limited number of phenylpropanoid genes that were expressed differentially during sugarcane stem development or in relation to sucrose accumulation (Casu *et al.*, 2004, 2007). Only recently, the first lignin biosynthetic gene, an orthologue of *COMT*, was functionally characterized in sugarcane through reverse genetics (Jung *et al.*, 2012). Here, a combination of co-expression analysis, tissue- and cell-specific expression analyses, and genetic complementation were employed for the functional characterization of a laccase protein, SofLAC, potentially involved in lignin biosynthesis in sugarcane.

Co-expression approaches have been considered a valuable tool for identifying new genes involved in secondary cell-wall biosynthesis (Brown *et al.*, 2005; Persson *et al.*, 2005; Ruprecht and Persson, 2012). Co-expressed gene pairs, i.e. genes whose expression is correlated over various tissues or conditions, have the potential to be involved in similar processes and to have similar functions (Ruprecht and Persson, 2012). The expression of *SofLAC* was linked directly with the expression of the phenylpropanoid genes *HCT1*, *CCR1*, and *CCoAOMT2*, whilst several other lignin-related genes, although not directly coordinated with *SofLAC* expression, were found in the close vicinity and connected to the same nodes as *SofLAC*. In *Arabidopsis*, non-annotated genes whose expression is highly co-regulated with the expression of *CesA* genes involved in secondary cell-wall synthesis were analysed genetically and their function in the same physiological process was confirmed (Brown *et al.*, 2005; Persson *et al.*, 2005). In the same study (Persson *et al.*, 2005), *AtLAC4* and *AtLAC17* were among the most highly co-regulated genes for these *CesA* genes. Recently, the involvement of AtLAC4 and AtLAC17 in lignin polymerization in *Arabidopsis* stems was demonstrated through reverse genetics (Berthet *et al.*, 2011), validating the effectiveness of co-expression approaches for gene function discovery. In this regard, the transcriptional coordination of *SofLAC* with genes from the lignin biosynthesis pathway and the fact that *Arabidopsis lac17* is the closest laccase homologue to *SofLAC* suggest a potential role of this particular laccase in lignin polymerization in sugarcane.

Similar to peroxidases, functional characterization of plant laccases has been a major challenge due to genetic redundancy among members of the multigene family, the diversity of physiological processes catalysed by them and the broad substrate specificity *in vitro* (Ranocha *et al.*, 2002; Cai *et al.*, 2006; Liang *et al.*, 2006). Transcriptomic approaches seem to offer a valuable option to overcome some of these difficulties (Cosio and Dunand, 2010). Accordingly, a laccase gene whose expression is activated during lignin deposition and is preferentially expressed in plant tissues that undergo lignification is likely to be involved in lignin polymerization in this specific tissue type. *SofLAC* mRNA preferentially accumulated in sclerenchymatic bundle sheaths of sugarcane young internodes. Despite the fact that the lignification process is highly active in developing and mature internodes, secondary walls are already present in cells from young internodes, where lignin is deposited in protoxylem and metaxylem elements and inner and outer portions of sclerenchymatic bundle sheets (Cesarino *et al.*, 2012). Casu *et al.* (2007) reported similar results for this specific laccase gene in sugarcane (i.e. higher expression in young stems), which was similar to the expression pattern of *ShCesA12*, a *CesA* gene putatively involved in secondary cell-wall synthesis in sugarcane. Therefore, the expression pattern of *SofLAC* is in agreement with the hypothesis that laccases play a role in the early stages of lignification in living cells, as

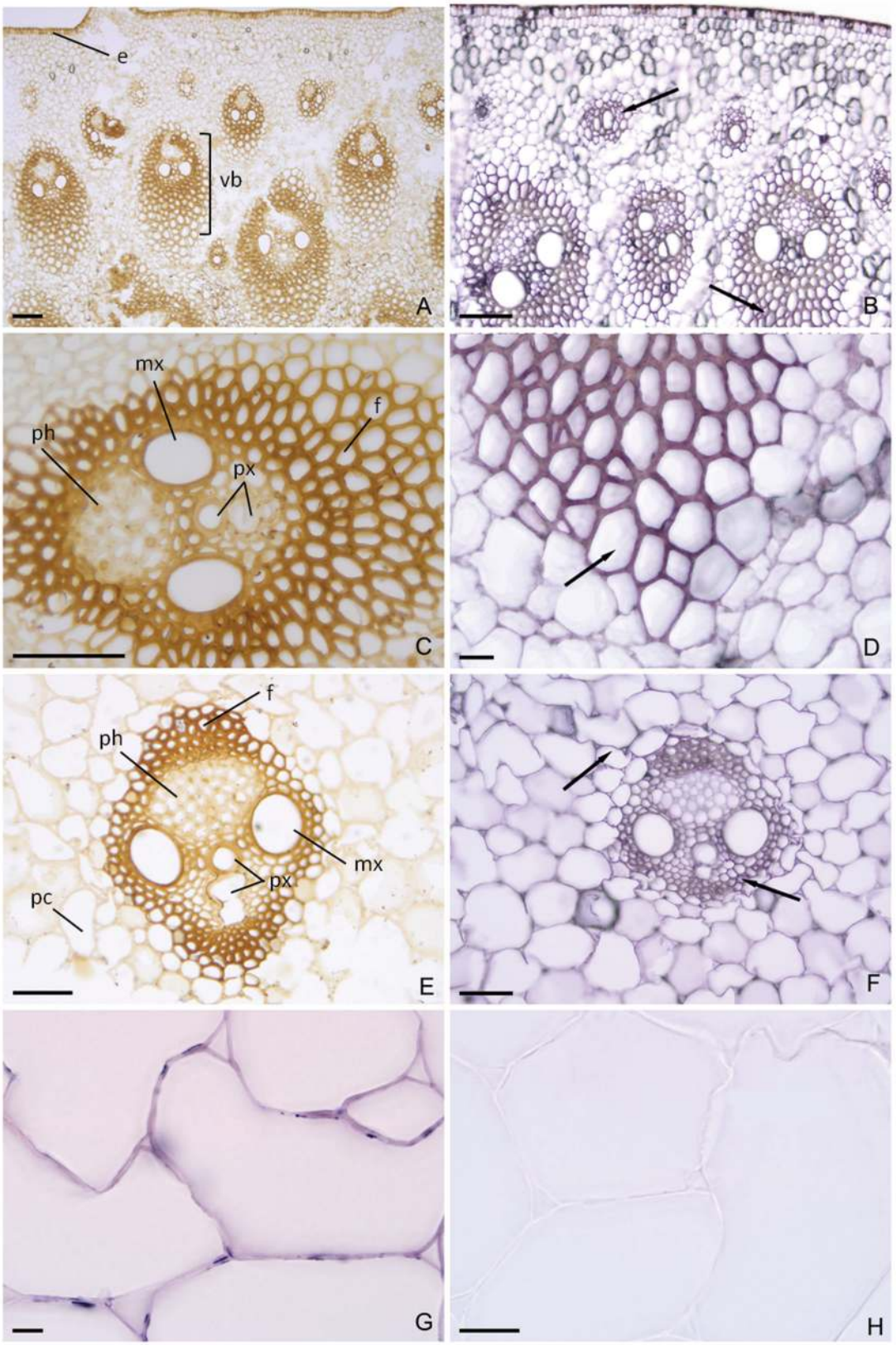

Fig. 5. Histochemical staining of lignin and localization of *SofLAC* mRNA by *in situ* hybridization in sugarcane young internodes. (A, C, E) Developmental deposition of lignin occurred in the protoxylem and metaxylem elements, sclerenchyma sheath, and, to a lesser extent, in parenchymatic cells close to the periphery of the stem. (B, D, F) An antisense probe showed *SofLAC* mRNA accumulation in lignified cell types, especially in sclerenchymatic bundles. (G) A signal was also observed in parenchyma cells. (H) A sense probe was used as a negative control. Arrows indicate the *in situ* hybridization signal. E, epidermis; f, fibres; mx, metaxylem; pc, parenchymatic cells; ph, phloem; px, protoxylem; vb, vascular bundle. (This figure is available in colour at *JXB* online.)

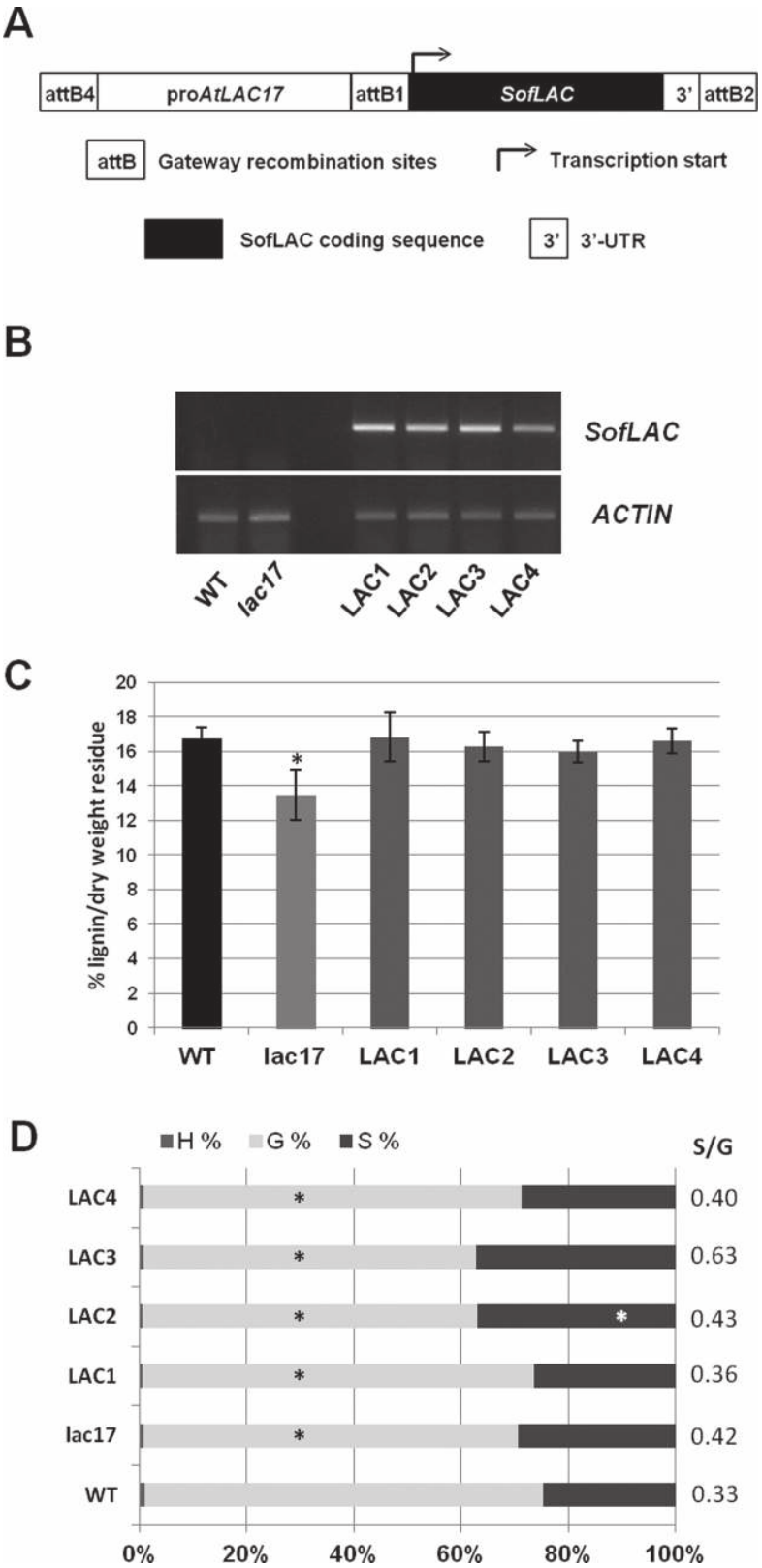

Fig. 6. Genetic complementation of the *Arabidopsis lac17* mutant with the sugarcane *SofLAC* coding sequence. (A) Schematic representation of the Gateway construct used for complementation of the *lac17* mutant. (B) RT-PCR analysis of *SofLAC* and *ACTIN* transcript levels in wild-type (WT), *lac17*, and complemented lines (LAC1–4). (C) Acetyl bromide lignin content in dry stems of WT, *lac17*, and complemented lines (LAC1–4). Error bars indicate the standard deviation of four biological replicates and an asterisk indicates the statistically significant value (Student's *t*-test, P <0.01, n=4). (D) Lignin composition of WT, *lac17*, and complemented lines (LAC1–4) as measured by thioacidolysis. Data are percentage values of total monomeric composition and the S:G ratio is shown on the right. Statistically significant values are indicated by asterisks (Student's *t*-test, P <0.05, n=3).

they operate in the absence of toxic H_2O_2 (Sterjiades *et al*., 1993). Interestingly, most expressed phenylpropanoid genes in maize had maximum expression in young ear internodes, with lower levels during the following stages of development, whilst lignin content remarkably increased with maturity (Riboulet *et al*., 2009). Finally, transcripts and proteins of AtLAC17, SofLAC's closest homologue in *Arabidopsis*, could only be detected in interfascicular fibres and not in vessels (Berthet *et al*., 2011), which resembles the cell-type-specific localization of *SofLAC* expression. Taken together, the results from expression analyses suggest that SofLAC is involved in lignification mainly of sclerenchymatic sheaths during early stages of sugarcane stem development.

Although co-expression networks and expression analyses can be used to link a laccase gene to a specific process, transgenic approaches are in principle more reliable for elucidation of gene function (Cai *et al*., 2006; Cosio and Dunand, 2009). However, genetic transformation of sugarcane has continued to be a major bottleneck. Sugarcane transformation has mostly been performed by particle bombardment of embryogenic calli, but transgene expression is generally switched off in mature plants due to both transcriptional and post-transcriptional gene silencing (Arruda, 2012). Expression instability in mature sugarcane plants is attributed to gene silencing induced by integration of high copy numbers of transgenes, as well as to the high ploidy levels of the sugarcane genome, which is normally about 12× in modern cultivars (Manners and Casu, 2011; Arruda, 2012). Alternatively, *Agrobacterium*-mediated transformation has been employed, but this approach is still limited by the low transformation efficiency, high variability between experiments, and genotype specificity (Anderson and Birch, 2012). Furthermore, regardless of the transformation protocol, tissue culture and plant regeneration are prerequisites for sugarcane genetic transformation, which are laborious and time-consuming. Finally, an additional drawback of sugarcane is the lack of mutants compared with *Arabidopsis* and other model plants. To overcome these limitations and to provide strong genetic evidence of the involvement of SofLAC in lignin biosynthesis, genetic complementation of the *Arabidopsis lac17* mutant was performed with the coding sequence of *SofLAC* under the control of the *AtLAC17* promoter. The *Arabidopsis lac17* mutant was chosen because: (i) AtLAC17 is the closest homologue to SofLAC in *Arabidopsis*; (ii) a knockdown T-DNA line is available, which has a lower lignin content and an altered S:G ratio; (iii) the cell-type specificity of *SofLAC* expression seems to be similar to that observed for *AtLAC17* in *Arabidopsis* stems; and (iv) SofLAC substrate specificity towards S and G monomers could be assessed *in vivo* by analysing the S:G ratio of the complemented mutant lines.

The expression of *SofLAC* under the control of the endogenous *AtLAC17* promoter in the *lac17* background led to complete recovery of lignin levels to those of the WT. These results provide strong genetic evidence of the involvement of SofLAC in the constitutive lignification of sugarcane stems. In addition, the genetic complementation approach allowed us to gain insight into the specificity of SofLAC towards lignin monomers *in vivo*. Whilst the reintroduction of *AtLAC17* in the *lac17* mutant under the control of its endogenous promoter decreased the elevated S:G ratio of the mutant to WT levels, *SofLAC* expression resulted in similar or even higher S:G ratios when compared with the *lac17* mutant. As grass cell walls differ dramatically from dicot cell walls in terms of types and relative abundance of hemicellulosic polysaccharides, levels of pectin and structural proteins, and lignin monomeric composition (Vogel, 2008), the interpretation of SofLAC specificity in a heterologous cell-wall environment must be done with care. Grass lignin not only accumulates up to 15% of H units but also contains substantial amounts of ferulic acid and *p*-coumaric acid (Grabber *et al*., 2004). Therefore, the S:G ratio in dicots might not easily be restored by laccases of grasses, which may have evolved to accommodate the different substrates available in grass cell walls. Nevertheless, it seems obvious that SofLAC presents a different monomeric specificity *in planta* from its corresponding *Arabidopsis* homologue AtLAC17 and thus does not preferentially use G units during lignin polymerization.

In conclusion, here we have reported, for the first time to our knowledge, on the functional characterization of a laccase gene in sugarcane and also provided additional confirmation of the involvement of laccases in the oxidative coupling of monolignols. The potential use of sugarcane lignocellulosic material as biofuel feedstock will require an enhanced understanding of the major factors affecting biomass recalcitrance. In this regard, the identification and characterization of genes involved in all aspects of lignin deposition in the cell walls of bioenergy crops is essential for the rational metabolic engineering of plant feedstock for bioenergy purposes.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Data S1. List of NCBI protein accession numbers and gene abbreviations used in this work.

Supplementary Table S1. List of primers used in this work.

Supplementary Table S2. SUCEST consensus numbers.

Acknowledgements

This work was financially supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; grant BIOEN 08/58035–6). The authors thank CAPES-Brazil for doctoral fellowships (to I.C. and P.A.), FAPESP for a post-doctoral fellowship (to J.L.S.M.), and CNPq-Brazil for a sandwich PhD fellowship (to I.C.; grant 201660/2010–5) and research fellowship (to P.M.). B.V. and W.B are supported by the Ghent University Multidisciplinary Research Partnership 'Biotechnology for a Sustainable Economy'.

References

Anderson D, Birch R. 2012. Minimal handling and super-binary vectors facilitate efficient, *Agrobacterium*-mediated, transformation of sugarcane (*Saccharum* spp. hybrid). *Tropical Plant Biology* **5,** 183–192.

Arruda P. 2012. Genetically modified sugarcane for bioenergy generation. *Current Opinion in Biotechnology* **23,** 315–322.

Berthet S, Demont-Caulet N, Pollet B, *et al.* 2011. Disruption of *LACCASE4* and *17* results in tissue-specific alterations to lignification of *Arabidopsis thaliana* stems. *Plant Cell* **23,** 1124–1137.

Blee KA, Choi JW, O'Connell AP, Schuch W, Lewis NG, Bolwell GP. 2003. A lignin-specific peroxidase in tobacco whose antisense suppression leads to vascular tissue modification. *Phytochemistry* **64,** 163–176.

Boerjan W, Ralph J, Baucher M. 2003. Lignin biosynthesis. *Annual Review of Plant Biology* **54,** 519–546.

Bonawitz ND, Chapple C. 2010. The genetics of lignin biosynthesis: connecting genotype to phenotype. *Annual Review of Genetics* **44,** 337–363.

Brown DM, Zeef LA, Ellis J, Goodacre R, Turner SR. 2005. Identification of novel genes in Arabidopsis involved in secondary cell wall formation using expression profiling and reverse genetics. *Plant Cell* **17,** 2281–2295.

Cai X, Davis EJ, Ballif J, Liang M, Bushman E, Haroldsen V, Torabinejad J, Wu Y. 2006. Mutant identification and characterization of the laccase gene family in *Arabidopsis*. *Journal of Experimental Botany* **57,** 2563–2569.

Caparrós-Ruiz D, Fornalé S, Civardi L, Puigdomènech P, Rigau J. 2006. Isolation and characterisation of a family of laccases in maize. *Plant Science* **171,** 217–225.

Casu RE, Dimmock CM, Chapman SC, Grof CP, McIntyre CL, Bonnett GD, Manners JM. 2004. Identification of differentially expressed transcripts from maturing stem of sugarcane by *in silico* analysis of stem expressed sequence tags and gene expression profiling. *Plant Molecular Biology* **54,** 503–517.

Casu RE, Jarmey JM, Bonnett GD, Manners JM. 2007. Identification of transcripts associated with cell wall metabolism and development in the stem of sugarcane by Affymetrix GeneChip Sugarcane Genome Array expression profiling. *Functional & Integrative Genomics* **7,** 153–167.

Cesarino I, Araújo P, Paes Leme AF, Creste S, Mazzafera P. 2013. Suspension cell culture as a tool for the characterization of class III peroxidases in sugarcane. *Plant Physiology and Biochemistry* **62,** 1–10.

Cesarino I, Araújo P, Sampaio Mayer JL, Paes Leme AF, Mazzafera P. 2012. Enzymatic activity and proteomic profile of class III peroxidases during sugarcane stem development. *Plant Physiology and Biochemistry* **55,** 66–76.

Cheavegatti-Gianotto A, de Abreu HM, Arruda P, *et al.*, 2011. Sugarcane (*Saccharum officinarum*): a reference study for the regulation of genetically modified cultivars in Brazil. *Tropical Plant Biology* **4,** 62–89.

Chen F, Dixon RA. 2007. Lignin modification improves fermentable sugar yields for biofuel production. *Nature Biotechnology* **25,** 759–761.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16,** 735–743.

Cosio C, Dunand C. 2009. Specific functions of individual class III peroxidase genes. *Journal of Experimental Botany* **60,** 391–408.

Cosio C, Dunand C. 2010. Transcriptome analysis of various flower and silique development stages indicates a set of class III peroxidase genes potentially involved in pod shattering in *Arabidopsis thaliana*. *BMC Genomics* **11,** 528.

El Mansouri I, Mercado JA, Santiago-Dómenech N, Pliego-Alfaro F, Valpuesta V, Quesada MA. 1999. Biochemical and phenotypical characterization of transgenic tomato plants overexpressing a basic peroxidase. *Physiology Plantarum* **106,** 355–362.

Faccioli P, Provero P, Herrmann C, Stanca AM, Morcia C, Terzi V. 2005. From single genes to co-expression networks: extracting knowledge from barley functional genomics. *Plant Molecular Biology* **58,** 739–750.

Fagerstedt KV, Kukkola EM, Koistinen VV, Takahashi J, Marjamaa K. 2010. Cell wall lignin is polymerised by class III secretable plant peroxidases in Norway spruce. *Journal of Integrative Plant Biology* **52,** 186–194.

Grabber JH, Ralph J, Lapierre C, Barriere Y. 2004. Genetic and molecular basis of grass cell-wall degradability. I. Lignin-cell wall matrix interactions. *Comptes Rendus Biologies* **327,** 455–465.

Hatfield R, Fukushima RS. 2005. Can lignin be accurately measured? *Crop Science* **45,** 832–839.

Hooper SD, Bork P. 2005. Medusa: a simple tool for interaction graph analysis. *Bioinformatics* **21,** 4432–4433.

Jung JH, Fouad WM, Vermerris W, Gallo M, Altpeter F. 2012. RNAi suppression of lignin biosynthesis in sugarcane reduces recalcitrance for biofuel production from lignocellulosic biomass. *Plant Biotechnology Journal* **10,** 1067–1076.

Karkonen A, Koutaniemi S, Mustonen M, Syrjanen K, Brunow G, Kilpelainen I, Teeri TH, Simola LK. 2002. Lignification related enzymes in *Picea abies* suspension cultures. *Physiology Plantarum* **114,** 343–353.

Lapierre C, Pollet B, Petit-Conil M, *et al.* 1999. Structural alterations of lignins in transgenic poplars with depressed cinnamyl alcohol dehydrogenase or caffeic acid *O*-methyltransferase activity have an opposite impact on the efficiency of industrial kraft pulping. *Plant Physiology* **119,** 153–164.

Li Y, Kajita S, Kawai S, Katayama Y, Morohoshi N. 2003. Down-regulation of an anionic peroxidase in transgenic aspen and its effect on lignin characteristics. *Journal of Plant Research* **116,** 175–182.

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

Manners J, Casu R. 2011. Transcriptome analysis and functional genomics of sugarcane. *Tropical Plant Biology* **4,** 9–21.

McCaig BC, Meagher RB, Dean JF. 2005. Gene structure and molecular analysis of the laccase-like multicopper oxidase (LMCO) gene family in *Arabidopsis thaliana*. *Planta* **221,** 619–636.

Moura JCMS, Bonine CAV, Viana JDF, Dornelas MC, Mazzafera P. 2010. Abiotic and biotic stresses and changes in the lignin content and composition in plants. *Journal of Integrative Plant Biology* **52,** 360–376.

Persson S, Wei H, Milne J, Page GP, Somerville CR. 2005. Identification of genes required for cellulose synthesis by regression analysis of public microarray data sets. *Proceedings of the National Academy of Sciences, USA* **102,** 8633–8638.

Pourcel L, Routaboul JM, Kerhoas L, Caboche M, Lepiniec L, 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.

Raes J, Rohde A, Christensen JH, Van de Peer Y, Boerjan W. 2003. Genome-wide characterization of the lignification toolbox in Arabidopsis. *Plant Physiology* **133,** 1051–1071.

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

Riboulet C, Guillaumie S, Mechin V, *et al.* 2009. Kinetics of phenylpropanoid gene expression in maize growing internodes: relationships with cell wall deposition. *Crop Science* **49,** 211–223.

Ruprecht C, Persson S. 2012. Co-expression of cell wall related genes: new tools and insights. *Frontiers in Plant Science* **3**:83.

Shimada TL, Shimada T, Hara-Nishimura I. 2010. A rapid and non-destructive screenable marker, FAST, for identifying transformed seeds of *Arabidopsis thaliana*. *Plant Journal* **61,** 519–528.

Sterjiades R, Dean JFD, Gamble G, Himmelsbach DS, Eriksson KEL. 1993. Extracellular laccases and peroxidases from sycamore maple (*Acer pseudoplatanus*) cell-suspension cultures. *Planta* **190,** 75–87.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28,** 2731–2739.

Vanholme R, Demedts B, Morreel K, Ralph J, Boerjan W. 2010*a*. Lignin biosynthesis and structure. *Plant Physiology* **153,** 895–905.

Vanholme R, Morreel K, Ralph J, Boerjan W. 2008. Lignin engineering. *Current Opinion in Plant Biology* **11,** 278–285.

Vanholme R, Van Acker R, Boerjan W. 2010*b*. Potential of *Arabidopsis* systems biology to advance the biofuel field. *Trends in Biotechnology* **28,** 543–547.

Vermerris W. 2011. Survey of genomics approaches to improve bioenergy traits in maize, sorghum and sugarcane. *Journal of Integrative Plant Biology* **53,** 105–119.

Vettore AL, da Silva FR, Kemper EL, *et al.* 2003. Analysis and functional annotation of an expressed sequence tag collection for tropical crop sugarcane. *Genome Research* **13,** 2725–2735.

Vogel J. 2008. Unique aspects of the grass cell wall. *Current Opinion in Plant Biology* **11,** 301–307.

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

Weng JK, Chapple C. 2010. The origin and evolution of lignin biosynthesis. *New Phytologist* **187,** 273–285.

Weng JK, Li X, Bonawitz ND, Chapple C. 2008. Emerging strategies of lignin engineering and degradation for cellulosic biofuel production. *Current Opinion in Biotechnology* **19,** 166–172.

Yuan JS, **Tiller KH**, **Al-Ahmad H**, **Stewart NR**, **Stewart CN Jr**. 2008. Plants to power: bioenergy to fuel the future. *Trends in Plant Science* **13,** 421–429.