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Mutant identification and characterization of the laccase gene family in *Arabidopsis*

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Abstract

Laccases, EC 1.10.3.2 or *p*-diphenol:dioxygen oxido-reductases, are multi-copper containing glycoproteins. Despite many years of research, genetic evidence for the roles of laccases in plants is mostly lacking. In this study, a reverse genetics approach was taken to identify T-DNA insertional mutants (the SALK collection) available for genes in the *Arabidopsis* laccase family. Twenty true null mutants were confirmed for 12 laccase genes of the 17 total laccase genes (*AtLAC1* to *AtLAC17*) in the family. By examining the mutants identified, it was found that four mutants, representing mutations in three laccase genes, showed altered phenotypes. Mutants for *AtLAC2*, *lac2*, showed compromised root elongation under PEG-induced dehydration conditions; *lac8* flowered earlier than wild-type plants, and *lac15* showed an altered seed colour. The diverse phenotypes suggest that laccases perform different functions in plants and are not as genetically redundant as previously thought. These mutants will prove to be valuable resources for understanding laccase functions *in vivo*.

Key words: Abiotic stress, gene expression, laccase, mutant.

Introduction

Laccases are multicopper-containing glycoproteins and have been found to be widespread in higher plants, both in monocots and dicots. Years of past research have associated many physiological functions with laccases in plants. In particular, laccase is known to be associated with lignin synthesis (reviewed by O'Malley *et al.*, 1993; Dean *et al.*, 1998; Gavnholt and Larsen, 2002). Laccase genes were highly expressed in lignifying tissues (Sato *et al.*, 2001).

Immunolocalization of laccases also showed a close association with the lignifying processes (Driouich *et al.*, 1992). Furthermore, isolated laccases from various plants were able to polymerize lignin precursors, monolignol alcohols, in an *in vitro* reaction (Sterjiades *et al.*, 1992; Bao *et al.*, 1993; Ranocha *et al.*, 1999). To provide genetic evidence for laccase involvement in lignification, laccases have either been overexpressed (Dean *et al.*, 1998) or down-regulated (Ranocha *et al.*, 2002). Despite other phenotypes in transgenic plants, an alteration in lignin content was not reported or observed. Thus, genetic evidence for laccase involvement in lignin synthesis is still missing. Instead of playing a role in lignin polymerization, it has recently been demonstrated that a laccase gene (*AtLAC15/TT10*) in *Arabidopsis* is involved in the formation of proanthocyanidin or tannin from the precursor epicatechin (Pourcel *et al.*, 2005).

A difference in subcellular localization can sometimes imply different functions among laccases. Laccases have often been considered cell wall proteins since they were purified from cell walls. In addition, most of the laccases identified in plants had a predicted N-terminal signal peptide which could direct proteins into the secretory pathway and thus into cell walls. A laccase in ryegrass (LpLAC3), however, did not possess a signal peptide, suggesting LpLAC3 could remain inside the cell and might perform a role different from other laccases in cell walls (Gavnholt *et al.*, 2002). Several laccases were expressed in tissues that were rapidly dividing (Gavnholt *et al.*, 2002), which further supported the notion that laccases could have additional roles in plants besides lignification. Laccase-like activity has been detected inside cells. Leaf trichomes of many plants, such as *Lycopersicon* and *Solanum* species, contain high levels of phenols (Kowalski *et al.*, 1992; Yu *et al.*, 1992; Thipyapong *et al.*, 1997). It is believed that polyphenol oxidases, including laccase, are responsible for

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polymerization of those phenolic compounds which protect plants from pathogen and insect attack (Lavid *et al.*, 2001).

Detoxification has also been proposed as a role for plant laccases (Cobbett and Meagher, 2002), since many fungal laccases have the capability of degrading toxins and various environmental pollutants (Mayer and Staples, 2002). This role in detoxification was supported by a recent study in which *Arabidopsis* plants that were over-expressing a cotton laccase were able to degrade organic pollutants, resulting in improved plant growth in a medium containing organic pollutants (Wang *et al.*, 2004). The detoxification capability enables plants to deal with environmental contaminants.

Previous studies showed that the transcript level of laccase genes was enhanced by high concentrations of NaCl in both tomato and maize roots (Wei *et al.*, 2000; Liang *et al.*, 2006), suggesting that an increase in transcript level in roots under salinity stress could be a universal response in plants. Thus, laccases may play an important role in roots during acclimation to salinity stress.

Laccases comprise a multigene family in plants (LaFayette *et al.*, 1999; Sato *et al.*, 2001; Gavnholt *et al.*, 2002). In *Arabidopsis*, there are 17 annotated laccase genes that can be divided into four sub-groups (McCaig *et al.*, 2005). This genetic redundancy has presented a major challenge for laccase study in the past, in addition to the complex biochemistry of laccase proteins. The available sequence information for the entire *Arabidopsis* genome and rich genetic resources provide an opportunity to uncover genetic evidence for the roles of laccases *in vivo*. This study reports identification of T-DNA insertional mutants for the *Arabidopsis* laccase gene family and preliminary characterization of mutants for three laccase genes.

Materials and methods

Plant materials and treatments

Seeds of *Arabidopsis thaliana* (Columbia 0 ecotype) were sown in a well-watered potting mix (Enriched Potting Mix, Miracle-Gro Lawn Products, Inc., Marysville, OH), and kept in a cold room (4 °C) for 2 d. Seeds were germinated and seedlings were grown in a growth room at 24 °C under a 16/8 h light/dark photoperiod. Roots, inflorescence stems (stems, flowers, cauline leaves), siliques, and leaves of 6-week-old plants were harvested separately for surveying gene expression in different tissues. To examine the effects of NaCl or PEG treatments on laccase gene expression in roots, seedlings were cultured in square Petri dishes ($10 \times 10 \times 1.5$ cm^3) each containing 35 ml of sterile solid medium to facilitate harvesting of root tissues. The solid medium consisted of $0.5\times$ MS salt, 0.5% sucrose, 10 mM MES, and 0.6% Phytagel (Sigma, St Louis, MO) at pH 5.8. Approximately 150–200 seeds were surface-sterilized and arranged on the solid medium. After a cold treatment (4 °C) for 2 d in the dark, the seeds were germinated and seedlings were grown on a light shelf under a 18/6 h light/dark photoperiod at room temperature. Light was supplied by four cool-white fluorescent bulbs, reaching an intensity of approximately 120 μmol m^{-2} s^{-1} on the surface of the shelf. The plates were placed vertically on a rack so that the roots grew downward on the surface of the growth medium. A salt treatment was imposed by the addition of 20 ml of 150 mM NaCl solution (containing 2 mM CaCl$_2$ and

1 mM KCl) to the growth medium. The plates were slightly tilted so that only the roots were immersed in the solution. The dehydration stress was carried out by treating the roots grown on the surface of the Phytagel plates with 20 ml of 20% (w/v) PEG 8000 solution containing 2 mM CaCl$_2$ and 1 mM KCl. The PEG solution had a water potential equivalent to −0.82 MPa measured with a WP4 Dewpoint PotentialMeter (Decagon Devices, Inc., Pullman, WA). The control roots were treated with a solution containing only 2 mM CaCl$_2$ and 1 mM KCl. All the plant materials were frozen in liquid nitrogen immediately after harvesting and stored at −80 °C until use. All the treatments were performed at least twice on different days but at the same time of day.

Initial T-DNA insertional mutants were identified from the TAIR (www.arabidopsis.org) and the Salk Insertional Mutant Databases (http://signal.salk.edu/tabout.html). Seeds were then obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University.

RNA extraction and cDNA synthesis

Total RNA was extracted from the frozen tissues using RNA$_{WIZ}$ reagent (Ambion Incorporated, Austin, TX). RNA pellets were dissolved in RNase-free water containing RNAsecure (Ambion Incorporated) at 60 °C for 10 min. To eliminate the residual genomic DNA present in RNA samples, 1 μg of total RNA was treated with DNase I following the manufacturer's instructions (RQ1 RNase-free DNase kit, Promega Corporation, Madison, WI). The DNase-treated samples were then converted into cDNA using a Reverse Transcription System Kit (Promega Corporation).

Primer design and PCR reaction

Primers for the gene expression study were designed using DNA-STAR software (DNASTAR Inc., Madison, WI) based on the coding sequence for each gene retrieved from the TAIR database. Gene-specific primers were located in two exons flanking an intron. The resulting PCR products were larger if genomic DNA was present in cDNA samples. Primers for mutant screening were designed using a program in SIGnAL iSect Tools (http://signal.salk.edu/isects.html). To confirm an identified homozygous mutant using RT-PCR, the primers were designed to flank the insertion site.

A typical 20 μl PCR reaction contained 1–2 μl cDNA or 1 μl genomic DNA (50 ng), 2 μl 10× buffer, 1 μl each of 10 μM primers, 2 μl 2 mM dNTP, 1 U *Taq* (New England Biolabs, Beverly, MA). The PCR programme consisted of initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, annealing for 30 s at a temperature specific to the primer pairs used for each gene (see supplementary Tables 1 and 2 at JXB online), and elongation at 72 °C for 1 min. The programme ended with an extension at 72 °C for 10 min. The PCR products were visualized on a 1% agarose gel stained with ethidium bromide.

Quantitative PCR (qPCR), semi-qPCR, and data analysis

The relative transcript level was determined mostly by following a method described by Liang *et al.* (2006). Briefly, qPCR was performed using the DyNAmo HS SYBR Green qPCR Kit (FINN-ZYMES, MJ Research Inc., Waltham, MA) by following the manufacturer's instruction. A typical 20 μl reaction contained 10 μl 2× Mix from DyNAmo HS SYBR Green qPCR kit, 1 μl each of 10 μM primers and 1 μl cDNA. The programme consisted of heating at 95 °C for 15 min, followed by 40 cycles of 94 °C for 10 s, annealing for 30 s (the annealing temperature varies with primer pairs), and 72 °C for 1 min. The final step was a 10 min extension at 72 °C. All the fluorescence readings were made at 3 °C lower than the melting point temperature of the PCR product of each cDNA which was predetermined from a qPCR trial. The qPCR products were loaded on a 1% agarose gel to confirm product size and reaction quality.

Table 1. *Summary of laccase mutant screening*

Gene ID	Mutant ID	Insertion site	Phenotypes	RT-PCR confirmation
AtLAC2 (At2g29130)	Salk_025690	exon	**Reduced root elongation under PEG-induced dehydration**	Knockout
AtLAC3 (At2g30210)	Salk_031901	exon	No observed phenotype	Knockout
AtLAC5 (At2g40370)	Salk_093534	exon	No observed phenotype	Knockout
	Salk_063466	exon	No observed phenotype	Knockout
	Salk_092440	exon	No observed phenotype	Knockout
AtLAC8 (At5g01040)	Salk_111587	exon	**Early flowering**	Knockout
AtLAC9 (At5g01050)	Salk_002632	exon	No observed phenotype	Knockout
	Salk_067988	exon	No observed phenotype	Knockout
AtLAC10 (At5g01190)	Salk_083241	exon	No observed phenotype	Knockout
	Salk_017722	exon	No observed phenotype	Knockout
AtLAC11 (At5g03260)	Salk_000192	3′UTR	No observed phenotype	Knockdown
	Salk_063746	exon	No observed phenotype	Knockout
AtLAC12 (At5g05390)	Salk_079491	exon	No observed phenotype	Knockout
	Salk_047456	exon	No observed phenotype	Knockout
	Salk_004019	exon	No observed phenotype	Knockout
AtLAC13 (At5g07130)	Salk_023935	exon	No observed phenotype	Knockout
	Salk_052328	promoter	No observed phenotype	Knockout
AtLAC14 (At5g09360)	Salk_011767	exon	No observed phenotype	Knockout
AtLAC15 (At5g48100)	Salk_002972	exon	**Yellow or pale seed colour**	Knockout
	Salk_128292	intron	**Yellow or pale seed colour**	Knockout
AtLAC16 (At5g58910)	Salk_064093	intron	No observed phenotype	Knockout

Table 2. *Expression of laccase genes in* Arabidopsis *in different tissues*

Gene expression was examined using RT-PCR. The genes whose transcripts were detected in two or more independent biological experiments were considered expressed (ratio in bold).

Gene	Root	Flower and stem	Leaf	Silique
AtLAC1 (At1g18140)	**3/4**[A]	**2/4**[A]	0/4[w]	0/4
AtLAC2 (At2g29130)	**3/4**[A]	**3/4**[A]	**3/4**	**3/4**[a]
AtLAC3 (At2g30210)	**4/4**[A]	1/4[w]	1/4	**3/4**[a]
AtLAC4 (At2g38080)	**5/5**[A]	**4/5**[A]	**4/5**[A]	**3/5**[a]
AtLAC5 (At2g40370)	**2/4**[A]	**2/4**[A]	1/4	**2/4**[a]
AtLAC6 (At2g46570)	**3/4**[A]	**3/4**[A]	**3/4**	0/4
AtLAC7 (At3g09220)	**4/4**[A]	**2/4**[A]	0/4[A]	0/4[a]
AtLAC8 (At5g01040)	**4/4**[A]	**2/4**[A]	1/4	**2/4**[a]
AtLAC9 (At5g01050)	**4/4**	**2/4**	1/4	0/4
AtLAC10 (At5g01190)	**3/4**[A]	**3/4**[A]	**3/4**[A]	**2/4**[a]
AtLAC11 (At5g03260)	**5/5**[A]	**5/5**[A]	**3/5**[A]	**4/5**[a]
AtLAC12 (At5g05390)	**3/4**[A]	**3/4**[A]	**2/4**	**2/4**[a]
AtLAC13 (At5g07130)	**4/4**[A]	**3/4**[A]	**3/4**[A]	0/4[a]
AtLAC14 (At5g09360)	0/4	1/4	0/4	**2/4**
AtLAC15 (At5g48100)	**2/4**[A]	1/4	0/4	**3/4**[a]
AtLAC16 (At5g58910)	**3/4**[A]	1/4	0/4	1/4
AtLAC17 (At5g60020)	**4/4**[A]	**4/4**[A]	**3/4**[A]	**4/4**[a]

[A] Gene expression was also detected from array experiments in the database (TAIR).
[W] Weak signals were detected from array experiments in the database (TAIR).
[a] Gene expression was detected in the developing seeds from array experiments in the database (TAIR). All array data from the TAIR database were generated using Affymatrix chips. Information on At5g01050 and At5g09360 was not found in the array expression database.

Data from qPCR were analysed using Opticon 2 software (MJ Research) to determine the threshold cycle (C_T) value. The mean C_T value of three PCR reactions for each sample was used to determine the transcript level based on a standard curve that was generated each time with the samples on the same plate. PCR-amplified and then gel-purified cDNA fragments for each gene were sequenced and used as templates in qPCR reactions for the standard curve. To minimize the effect caused by variation in the amount of cDNA among the samples, the laccase transcript level of each sample was normalized to the level of an actin gene (*AtACT2*) in the sample. The normalized transcript level was then compared between treatment and control to obtain the fold change presented in this study.

A semi-quantitative PCR method was also used to evaluate a relative change in transcript level of some of the laccase genes by mainly following a method describe by Wang *et al*. (2003). A PCR reaction was first conducted using PCR-amplified and then gel-purified cDNA as a template after 5–2-fold serial dilution for 30–35 cycles, depending on the genes studied. The PCR products were separated on 1% agarose gel and stained with ethidium bromide. Gel images were acquired by a Fluro-S MultiImage system (Bio-Rad, Hercules, CA) and quantified with Quantity One software (Bio-Rad). A linear range between cDNA concentration (logarithm) and the fluorescence intensity of PCR products was determined, using R^2 ⩾0.95 as a cutoff value. PCR reactions were then performed with samples using the same cycle as used for the serial dilution curve. The cDNA that reached an initial saturation point in the serial dilution curve was included for PCR reactions with samples each time to ensure the products of all the samples fell into the linear range. The mean value of two PCR reactions of each sample was used to determine the transcript level based on the standard curve. The transcript level of each gene was normalized using the transcript level of an actin gene (*AtACT2*) in each sample. A fold change was obtained by calculating the ratio of the transcript level of a treated sample and its control. Several genes, which showed a very low transcript level either in control or in treatment, could not reasonably be quantified for fold changes and were presented as gel images directly (see supplementary Fig. 1b at JXB online).

Genomic DNA extraction and T-DNA mutant screening

Homozygous mutants were identified by following the protocol described at the SALK Insertional Mutant Database using a PCR method (Alonso *et al*., 2003). Leaf tissues of soil-grown seedlings were first collected from individual plants. Genomic DNA was extracted using a quick CTAB method (Rogers and Bendich, 1988) and was used for the PCR reaction.
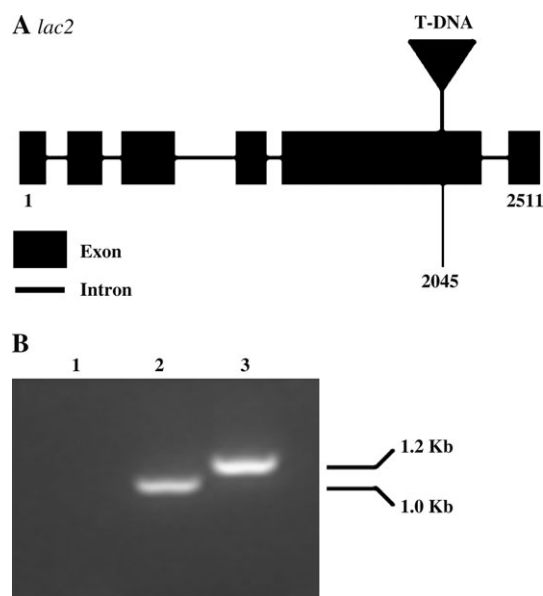
Fig. 1. Schematic diagram of an insertion in *lac2* and confirmation of the null mutation of the gene (*AtLAC2*) using RT-PCR. PCR screening using a primer in T-DNA (LBb1) and a gene-specific primer confirmed that the T-DNA was inserted in the fifth exon (A). RT-PCR analysis using primers flanking the insertion site confirmed that the normal transcript was missing in the mutant (B). Lane 1: mutant (*lac2*); lane 2: wild-type plants; lane 3: PCR product amplified from genomic DNA as a control. An actin gene band was amplified from all cDNA samples as quality control (data not shown).

Primers

The primers used for mutant screening and confirmation are listed in Supplementary Table 1 at JXB online. LBb1 primer sequence is available at the website. The gene-specific primers used for gene expression analysis are listed in Supplementary Table 2 at JXB online. The actin primers were designed from *AtACT2* gene (At3g18780) as described by Himanen *et al.* (2002): 5P=5′-GTTGCACCACCT-GAAAGGAAG and 3P=5′-CAATGGGACTAAAACGCAAAA.

Stress testing of mutants

Seeds of wild-type and mutant plants from the same segregating population were first surface-sterilized and germinated on solid medium (see above). Three-day-old seedlings with uniform root lengths were selected and transplanted into plates containing solid medium with or without 75 mM NaCl, or pretreated with PEG. The PEG plates were prepared as described by van der Weele *et al.* (2000), except for using 0.6% Phytagel as a gelling agent. Briefly, 35 ml of 33.5% PEG solution containing 0.5× MS and 0.5% sucrose at pH 5.8 (autoclaved for 15 min at 15 psi) was added to the plate containing 35 ml of 0.5× MS-salt-Phytagel solid medium and was allowed to equilibrate for 16 h. The plates were ready for use after removal of PEG solution. Similar results concerning root elongation were obtained with filter-sterilized PEG solution in the preliminary testing. The brief autoclaving only decreased water potential slightly. The PEG-pretreated MS-salt-Phytagel reached a water potential of −0.81±0.04 MPa. Each plate ($10×10×1.5$ cm^3) was divided into two halves with 8–10 seedlings of wild-type and mutant plants arranged on each side. Root growth was tracked by marking the position of root tips on the plate with time (Wu *et al.*, 1996).

Flowering timing analysis

Seeds of wild-type and mutant plants were sown in a well-watered potting mix and kept in a cold room (4 °C) for 2 d. The plants were then grown in a growth room under a 16/8 h light/dark photoperiod. The days required for half of the plants in each group to reach flowering (open flowers) were recorded and rosette leaf numbers were counted after all the plants flowered (Lim *et al.*, 2004).

Results

Identification of T-DNA insertional mutants of laccase genes

There are 17 annotated laccase genes in *Arabidopsis* (www.arabidopsis.org). Searching the TAIR database and the SALK Insertional Mutation Database, putative T-DNA insertional mutants (Alonso *et al.*, 2003) were identified for 16 laccase genes. For some of the genes, multiple insertional lines are available. In this case, at least two mutant lines were screened for each gene with preference to the lines that have inserts in the exons. For *AtLAC1*, *AtLAC3*, *AtLAC4*, *AtLAC7*, *AtLAC14*, and *AtLAC16*, the only mutant line available in the SALK collection was screened. From the 35 putative mutant lines obtained from the ABRC, PCR screening confirmed 21 real mutations in 12 genes. RT-PCR confirmed 20 mutants for 12 genes as true null-expression or knockout mutants (Table 1). One example of RT-PCR confirmation for the *lac2* mutant is shown in Fig. 1 as an example. A mutant for *AtLAC11* (Salk_000192) has an insertion in the 3′-UTR and only showed a decrease in transcript level (knockdown mutant, data not shown).

Mutant and wild-type plants that segregated from the same heterozygous plant were used for examination of phenotype when plants were cultured under normal or stress conditions. Four mutants for three laccase genes showed altered phenotypes (Table 1).

Altered root elongation of lac2 under dehydration conditions

Previous studies showed that the transcript levels of laccase genes in the roots of various species were increased by treatment of high concentrations of salt and heavy metal ions (Wei *et al.*, 2000; Liu and Yang, 2003; Liang *et al.*, 2006), suggesting laccase may play roles in stress response in the roots. To test this hypothesis, the laccase mutants were subjected to salinity stress. Laccase was suggested to be closely associated with lignin synthesis in vascular tissues and thus important for water transport (Croteau *et al.*, 2000; Boerjan *et al.*, 2003). Mutant plants were thus also treated with dehydration stress. To help observe root growth under these stress conditions, a root assay system was employed on solid medium (MS-salt-Phytagel) either containing 75 mM NaCl or pre-equilibrated with 33.5% PEG (PEG-induced dehydration) in Petri dishes (Wu *et al.*, 1996; van der Weele *et al.*, 2000).

Experiments were performed first to determine which genes in the laccase family were expressed in the roots using the RT-PCR method. As shown in Table 2, 16 of the

17 genes were expressed in the roots. *AtLAC16* was only expressed in the roots. Compared with other tissues/organs studied, roots have the highest number of expressed laccase genes. Mutants for the root-expressed laccase genes were then subjected to stress testing on the solid medium. Based on the root elongation, no mutants showed a difference from wild-type plants on either control or NaCl-containing medium. Under PEG-induced dehydration conditions, *lac2* (Salk_025690) showed a small but statistically significant reduction in root elongation (Table 3). The mutant roots showed no difference in elongation from the wild-type roots under normal growth conditions. *lac2* has a T-DNA insert in the fifth exon (Fig. 1a) and showed no normal transcripts (Fig. 1b). The transcript level of *AtLAC2* was enhanced in the roots after roots were treated with 150 mM NaCl or 20% PEG solution (Fig. 2).

Table 3. *Change in root length of wild-type and mutant (*lac2*) plants under stress*

Three-day-old seedlings were transplanted to MS-salt-Phytagel plates containing either 75 mM NaCl or pretreated with 33.5% PEG (see Materials and methods). Root elongation was measured 6 d after PEG treatment and 8 d after 75 mM NaCl treatment. Data in the table are means ±SE from five experiments. An asterisk indicates a significant difference at a 95% confidence interval (P=0.015) from the Student t test for means of a paired sample.

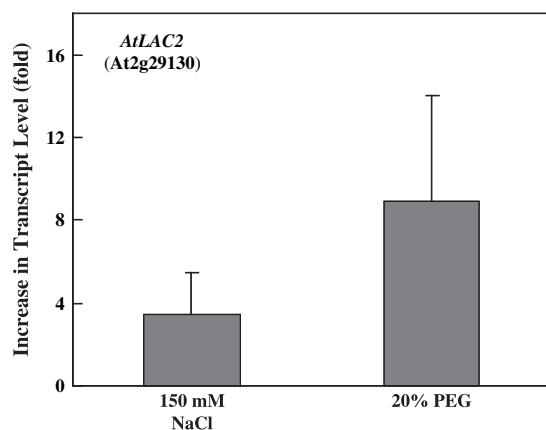| Treatment	Initial root length (mm)		Final root length (mm)	
	Wild type	Mutant	Wild-type	Mutant
PEG plate	7.65±0.32	7.55±0.34	49.66±2.02	44.84±1.92*
75 mM NaCl	9.23±0.39	8.98±0.33	47.20±2.16	46.10±2.13

Fig. 2. Change in transcript level of *AtLAC2* in roots treated with NaCl or PEG solution. Roots of 10-d-old seedlings grown on MS-salt-Phytagel were treated with 20% PEG or 150 mM NaCl, or a control solution (see Materials and methods). The transcript level was determined using a semi-quantitative PCR method. The transcript level of each gene was first normalized with the transcript level of an actin gene. The fold change in transcript level presented in the figure is the ratio between the normalized transcript level of PEG or NaCl-treated samples and the control. Data are means ±SE of four experiments.

lac8 *flowered earlier*

The early flowering phenotype was reproducibly observed from *AtLAC8* mutants (*lac8*) compared with wild-type plants. *lac8* has an insert in the last or sixth exon of the gene. As showed in Fig. 3, *lac8* reached flowering with fewer leaves (Fig. 3A) in less time (Fig. 3B).

lac15 *showed altered seed colour*

Two independent insertion lines were identified for *At-LAC15*, *lac15-1* (Salk_002972), and *lac15-2* (Salk_128292). The seeds of both mutants showed yellow or pale seed colour, resembling a *transparent testa* phenotype. It is known that the *transparent testa* is usually caused by a defect in proanthocyanidin synthesis (Debeaujon *et al.*, 2000). A recent study confirmed that *lac15-1* and *lac15-2* are allelic to the *tt10* mutant (Pourcel *et al.*, 2005). Biochemical analyses of these mutants indicated the mutants accumulated soluble proanthocyanidin (Pourcel *et al.*, 2005) and showed a reduction in lignin content which will be described in detail elsewhere (M Liang, Y Wu, unpublished data).

Discussion

Laccase in roots responding to dehydration stress

Among the 17 annotated laccase genes (www.arabidopsis. org) or laccase-like multicopper oxidases (McCaig *et al.*,
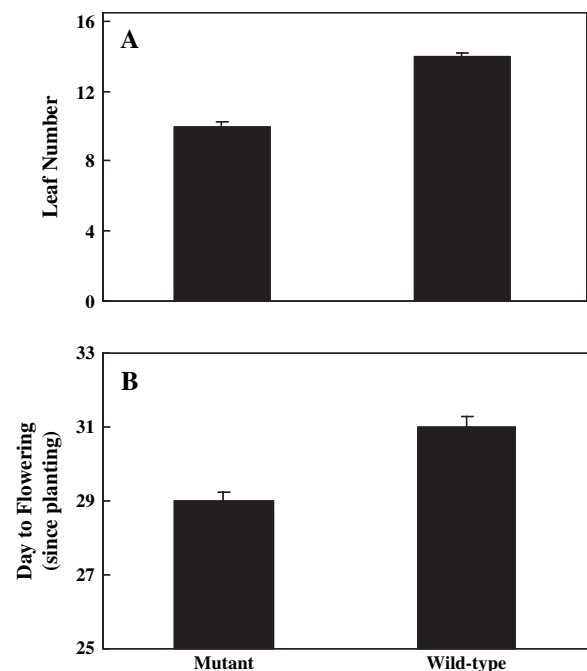

Fig. 3. Change in flowering timing in *lac8*. (A) The leaf number when flowering. (B) Average day to flowering since planting. Data are means ±SE of one experiment. The experiment was repeated at least two times with similar results.

2005), 16 are expressed in the roots. Thus the roots have the greatest number of expressed laccase genes. The results confirmed the information presented by McCaig *et al.* (2005) who collected the laccase gene expression information from the massively parallel signature sequencing (MPSS) database. In addition, the expression information collected from the MPSS database implicated a higher expression level of these laccase genes in the roots than other tissues, based on the number of the sequence tag represented in different libraries (McCaig *et al.*, 2005). The greater number of expressed genes and greater abundance of relative gene expression levels in the roots imply the important involvement of laccase in root physiology. In this study, *AtLAC16* appeared to be specifically expressed in the roots. However, its sequence tag was detected in the MPSS analysis in other tissues, including leaves and inflorescences (McCaig *et al.*, 2005). The number of the sequence tag in other tissues was much lower compared with that in the roots, which could explain why its transcript was not detected in other tissues in this study.

Previous gene expression studies showed an increase in laccase transcript levels in roots in response to salt stress (Wei *et al.*, 2000; Liang *et al.*, 2006). This is also demonstrated in the present study (see supplementary Fig. 1 at JXB online), which showed the enhancement of transcript levels of more than a dozen laccase genes in *Arabidopsis* roots treated with NaCl. However, laccase mutants identified in this study failed to show a compromised salt tolerance based on a root elongation assay. By growing mutants under dehydration conditions, *lac2* showed a reduction in root elongation compared with wild-type plants, suggesting an involvement of *AtLAC2* in the dehydration response. Gene expression analysis showed an increase in *AtLAC2* transcript level in roots treated with PEG in addition to NaCl treatment. Previous studies showed that the transcript level of a maize laccase, *ZmLAC1*, only responded to salinity treatment and not to PEG treatment (Liang *et al.*, 2006). Thus, the results suggest that different laccases could be regulated differently at the transcriptional level. At this point, it is not clear why the *lac2* mutants were only sensitive to PEG-induced dehydration and not salinity stress treatment, since 75 mM NaCl can also cause dehydration stress. One of the possible explanations is that a toxic effect of 75 mM NaCl used in this study severely inhibited root elongation, despite having a higher water potential than the PEG-treated plate (-0.61 ± 0.12 MPa for 75 mM NaCl plate and -0.81 ± 0.04 MPa for the PEG-treated plate). Such an inhibition could mask the effect of dehydration on root elongation.

Laccase and flowering timing

It is interesting that *lac8* mutants flower earlier than wild-type plants. The cause of the early flowering, however, could be a complex issue. Flowering time in plants is controlled by environmental stimuli such as day length (photoperiod pathway), light quality, exposure to low temperatures (vernalization pathway), and internal factors such as plant age or stage of development (autonomous and gibberellic acid pathways) (reviewed by Levy and Dean, 1998; Mouradov *et al.*, 2002; Putterill *et al.*, 2004). In addition, it has been reported that some intermediates in the phenylpropanoid pathway, such as *t*-cinnamic acid, benzoic acid and dihydrokaempferol-7-*O*-glucoside, could promote flowering in plants (Nakanishi *et al.*, 1995; Hatayama and Takeno, 2003). Further studies are needed to understand why *lac8* flowers earlier. For example, it will be interesting to examine whether *lac8* mutants accumulate these intermediates in the phenylpropanoid pathway.

Laccase in proanthocyanidin (PA) and lignin synthesis

The pale seed colour mutants, *lac15/tt10*, were identified using various different approaches, such as forward genetics (Koornneef, 1990; Debeaujon *et al.*, 2000) and reverse genetics in this study. Pourcel *et al.* (2005) have demonstrated that *AtLAC15* is involved in polymerization of PA from its monomer epicatechin. The mutant seeds accumulated PA precursor. However, the mutant seeds also accumulated soluble PA, which suggests that other laccases or enzymes are also involved in PA synthesis in *Arabidopsis* seeds and that *AtLAC15* could potentially be involved in PA oxidation too and thus contributing to the brown seed colour in wild-type plants. Studies with these mutants revealed a reduction in lignin content in *lac15/tt10* seeds, suggesting an additional role of *AtLAC15* in *Arabidopsis* seeds, i.e. in lignin synthesis (M Liang, Y Wu, unpublished data).

In summary, this study has provided new insights into the potential roles of laccase in *Arabidopsis*. Identification of three mutants with observable phenotypes in the same family suggests that laccases play versatile roles in plants. Further study of these mutants could lead to an understanding of the specific functions of these laccases in plants. Even so, mutants for many other laccases did not yield any noticeable phenotype. This could be because, either other laccases play different roles in plants and require other assays to reveal phenotypes, or genetic redundancy exists among these laccase genes. To address the latter possibility, mutants with mutations in more than one laccase gene are currently being generated and examined.

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