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 oxidoreductases, 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...
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 (McCag 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 insertion 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 × 10 × 1.5 cm) each containing 35 ml of sterile solid medium to facilitate harvesting of root tissues. The solid medium consisted of 0.5% 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 CaCl2 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 CaCl2 and 1 mM KCl. The PEG solution had a water potential equivalent to −0.82 MPa measured with a WP4 Dewpoint Potential Meter (Decagon Devices, Inc., Pullman, WA). The control roots were treated with a solution containing only 2 mM CaCl2 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/about.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 RNAWiz reagent (Ambion Incorporated, Austin, TX). RNA pellets were dissolved in RNase-free water containing RNase-free RNase (Ambion Incorporated) at 60 ºC 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 DNASTAR software (DNASTAR Inc., Madison, WI) based on the coding sequence for each gene retrieved from the TAIR database. Genespecific 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 ºC for 4 min, followed by 35 cycles of 94 ºC 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 ºC for 1 min. The programme ended with an extension at 72 ºC 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 ºC for 15 min, followed by 40 cycles of 94 ºC for 10 s, annealing for 30 s (the annealing temperature varies with primer pairs), and 72 ºC for 1 min. The final step was a 10 min extension at 72 ºC. All the fluorescence readings were made at 3 ºC 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

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Mutant ID</th>
<th>Insertion site</th>
<th>Phenotypes</th>
<th>RT-PCR confirmation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtLAC2  (At2g29130)</td>
<td>Salk_025690 exon</td>
<td></td>
<td>Reduced root elongation under PEG-induced dehydration</td>
<td>Knockout</td>
</tr>
<tr>
<td>AtLAC3  (At2g30120)</td>
<td>Salk_031901 exon</td>
<td></td>
<td></td>
<td>Knockout</td>
</tr>
<tr>
<td>AtLAC5  (At2g40370)</td>
<td>Salk_093543 exon</td>
<td></td>
<td></td>
<td>Knockout</td>
</tr>
<tr>
<td>AtLAC6  (At2g40370)</td>
<td>Salk_063466 exon</td>
<td></td>
<td></td>
<td>Knockout</td>
</tr>
<tr>
<td>AtLAC7  (At2g40370)</td>
<td>Salk_092440 exon</td>
<td></td>
<td></td>
<td>Knockout</td>
</tr>
<tr>
<td>AtLAC8  (At5g01040)</td>
<td>Salk_111587 exon</td>
<td></td>
<td>Early flowering</td>
<td>Knockout</td>
</tr>
<tr>
<td>AtLAC9  (At5g01050)</td>
<td>Salk_002632 exon</td>
<td></td>
<td></td>
<td>Knockout</td>
</tr>
<tr>
<td>AtLAC10 (At5g01190)</td>
<td>Salk_083241 exon</td>
<td></td>
<td></td>
<td>Knockout</td>
</tr>
<tr>
<td>AtLAC11 (At5g03260)</td>
<td>Salk_000192 3'UTR</td>
<td></td>
<td>No observed phenotype</td>
<td>Knockdown</td>
</tr>
<tr>
<td>AtLAC12 (At5g05390)</td>
<td>Salk_079491 exon</td>
<td></td>
<td></td>
<td>Knockout</td>
</tr>
<tr>
<td>AtLAC13 (At5g09360)</td>
<td>Salk_047456 exon</td>
<td></td>
<td></td>
<td>Knockout</td>
</tr>
<tr>
<td>AtLAC14 (At5g09360)</td>
<td>Salk_004019 exon</td>
<td></td>
<td></td>
<td>Knockout</td>
</tr>
<tr>
<td>AtLAC15 (At5g07130)</td>
<td>Salk_023935 exon</td>
<td></td>
<td></td>
<td>Knockdown</td>
</tr>
<tr>
<td>AtLAC16 (At5g07130)</td>
<td>Salk_052328 promoter</td>
<td></td>
<td></td>
<td>Knockout</td>
</tr>
<tr>
<td>AtLAC17 (At5g58910)</td>
<td>Salk_128292 intron</td>
<td></td>
<td>No observed phenotype</td>
<td>Knockout</td>
</tr>
</tbody>
</table>

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).

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Root</th>
<th>Flower and stem</th>
<th>Leaf</th>
<th>Silique</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtLAC1 (At1g18140)</td>
<td>3/4</td>
<td>2/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>AtLAC2 (At2g29130)</td>
<td>3/4</td>
<td>3/4</td>
<td>3/4</td>
<td>3/4</td>
</tr>
<tr>
<td>AtLAC3 (At2g30210)</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td>AtLAC4 (At2g38080)</td>
<td>5/5</td>
<td>2/4</td>
<td>1/4</td>
<td>3/5</td>
</tr>
<tr>
<td>AtLAC5 (At2g40370)</td>
<td>2/4</td>
<td>2/4</td>
<td>1/4</td>
<td>2/4</td>
</tr>
<tr>
<td>AtLAC6 (At2g46570)</td>
<td>3/4</td>
<td>3/4</td>
<td>3/4</td>
<td>0/4</td>
</tr>
<tr>
<td>AtLAC7 (At3g09220)</td>
<td>4/4</td>
<td>2/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>AtLAC8 (At5g01040)</td>
<td>4/4</td>
<td>2/4</td>
<td>1/4</td>
<td>2/4</td>
</tr>
<tr>
<td>AtLAC9 (At5g01050)</td>
<td>4/4</td>
<td>2/4</td>
<td>1/4</td>
<td>0/4</td>
</tr>
<tr>
<td>AtLAC10 (At5g01190)</td>
<td>3/4</td>
<td>3/4</td>
<td>3/4</td>
<td>2/4</td>
</tr>
<tr>
<td>AtLAC11 (At5g03260)</td>
<td>5/5</td>
<td>5/5</td>
<td>3/5</td>
<td>4/5</td>
</tr>
<tr>
<td>AtLAC12 (At5g05390)</td>
<td>3/4</td>
<td>3/4</td>
<td>2/4</td>
<td>2/4</td>
</tr>
<tr>
<td>AtLAC13 (At5g07130)</td>
<td>4/4</td>
<td>3/4</td>
<td>3/4</td>
<td>0/4</td>
</tr>
<tr>
<td>AtLAC14 (At5g09360)</td>
<td>0/4</td>
<td>1/4</td>
<td>0/4</td>
<td>2/4</td>
</tr>
<tr>
<td>AtLAC15 (At5g58100)</td>
<td>3/4</td>
<td>1/4</td>
<td>0/4</td>
<td>3/4</td>
</tr>
<tr>
<td>AtLAC16 (At5g58910)</td>
<td>3/4</td>
<td>3/4</td>
<td>3/4</td>
<td>4/4</td>
</tr>
<tr>
<td>AtLAC17 (At5g60020)</td>
<td>3/4</td>
<td>3/4</td>
<td>3/4</td>
<td>4/4</td>
</tr>
</tbody>
</table>

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).
* Gene expression was detected in the developing seeds from array experiments in the database (TAIR).

Data from qPCR were analysed using Opticon 2 software (MJ Research) to determine the threshold cycle (Ct) value. The mean Ct 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 following a protocol described 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 with a Fluro-S Multimager 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.
the plate containing 35 ml of 0.5 M rose at pH 5.8 (autoclaved for 15 min at 15 psi) was added to potential of tial slightly. The PEG-pretreated MS-salt-Phytagel reached a water elongation were obtained with filter-sterilized PEG solution in the and was allowed to equilibrate for 16 h. The plates were ready for potting mix and kept in a cold room (4°C to 8°C). Root growth was tracked by marking the position of root tips on the plate with time (Wu et al., 1996). 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 cm³) 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/organisms 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. 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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial root length (mm)</th>
<th>Final root length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
<td>Mutant</td>
</tr>
<tr>
<td>PEG plate</td>
<td>7.65±0.32</td>
<td>7.55±0.34</td>
</tr>
<tr>
<td>75 mM NaCl</td>
<td>9.23±0.39</td>
<td>8.98±0.33</td>
</tr>
</tbody>
</table>

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 *AtLAC15*, *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., 2002)
on root elongation. Such an inhibition could mask the effect of dehydration plate and of the possible explanations is that a toxic effect of induced dehydration and not salinity stress treatment, growing mutants under dehydration conditions, mised salt tolerance based on a root elongation assay. By mutants identified in this study failed to show a compromised root elongation compared with wild-type plants, suggesting an 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 yet 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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