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ORIGINAL ARTICLE

The laccase multigene family in *Arabidopsis thaliana*: towards addressing the mystery of their gene function(s)

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Abstract While laccases, multi-copper glycoprotein oxidases, are often able to catalyze oxidation of a broad range of substrates, such as phenols and amines in vitro, their precise physiological/biochemical roles in higher plants remain largely unclear, e.g., *Arabidopsis thaliana* contains 17 laccases with only 1 having a known physiological function. To begin to explore their roles *in planta*, spatial and temporal expression patterns of *Arabidopsis* laccases were compared and contrasted in different tissues at various development stages using RT-PCR and promoter-GUS fusions. Various cell-specific expressions were noted where specific laccases were uniquely expressed, such as *LAC4* in interfascicular fibers and seed coat columella, *LAC7* in hydathodes and root hairs, *LAC8* in pollen grains and phloem, and *LAC15* in seed coat cell walls. Such specific cell-type expression patterns provide new leads and/or strategies into determining their precise physiological/biochemical roles. In addition, there was an apparent redundancy of gene expression patterns for several laccases across a wide variety of tissues, lignified and non-lignified, perhaps indicative of overlapping function(s). Preliminary evidence, based on bioinformatics analyses, suggests that most laccases may also be tightly regulated at both transcriptional (antisense transcripts, histone and DNA methylation) and posttranscriptional (microRNAs) levels of gene expression.

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Introduction

Extracellular glycoprotein laccases (*p*-diphenol:oxygen oxidoreductase, EC 1.10.3.2) are multi-copper containing oxidases able to catalyze oxidation of various phenolic, inorganic and/or aromatic amine substrates (Fig. 1) through simultaneous reduction of molecular oxygen to water (Reinhammar and Malmstroem 1981). The resulting oxidized products are often free-radical species that can undergo either subsequent radical–radical coupling (Lewis et al. 1999; Mayer and Staples 2002) or cross-link to other substances such as proteins (Mattinen et al. 2005) in the extracellular matrix.

Laccases contain four copper (Cu) atoms in three types of coordination centers, namely, Types 1 (T1), 2 (T2) and 3 (T3), with T2/T3 forming a trinuclear cluster. The T1 center, binding the Cu imparting the characteristic laccase blue color (Solomon et al. 1996), is involved in abstraction of substrate electrons that are then transferred to the T2/T3 cluster via a highly conserved His-Cys-His tripeptide motif (Ducros et al. 1998). Residues in the T1 site vicinity, and the bond distances of coordinating histidine moieties to the T1 Cu, also contribute to laccase redox potentials (Piontek et al. 2002). The T2 and T3 centers bind 1 and 2 Cu atoms, respectively, and are arranged in a trinuclear cluster where reduction of molecular oxygen to water occurs (Solomon et al. 1996). While laccases are extensively distributed across bacteria (Claus 2003), fungi (Baldrian 2006), insects (Dittmer et al. 2004) and higher plants (McCaig et al. 2005), definitive biochemical functions/physiological roles for plant laccases remain largely unknown.

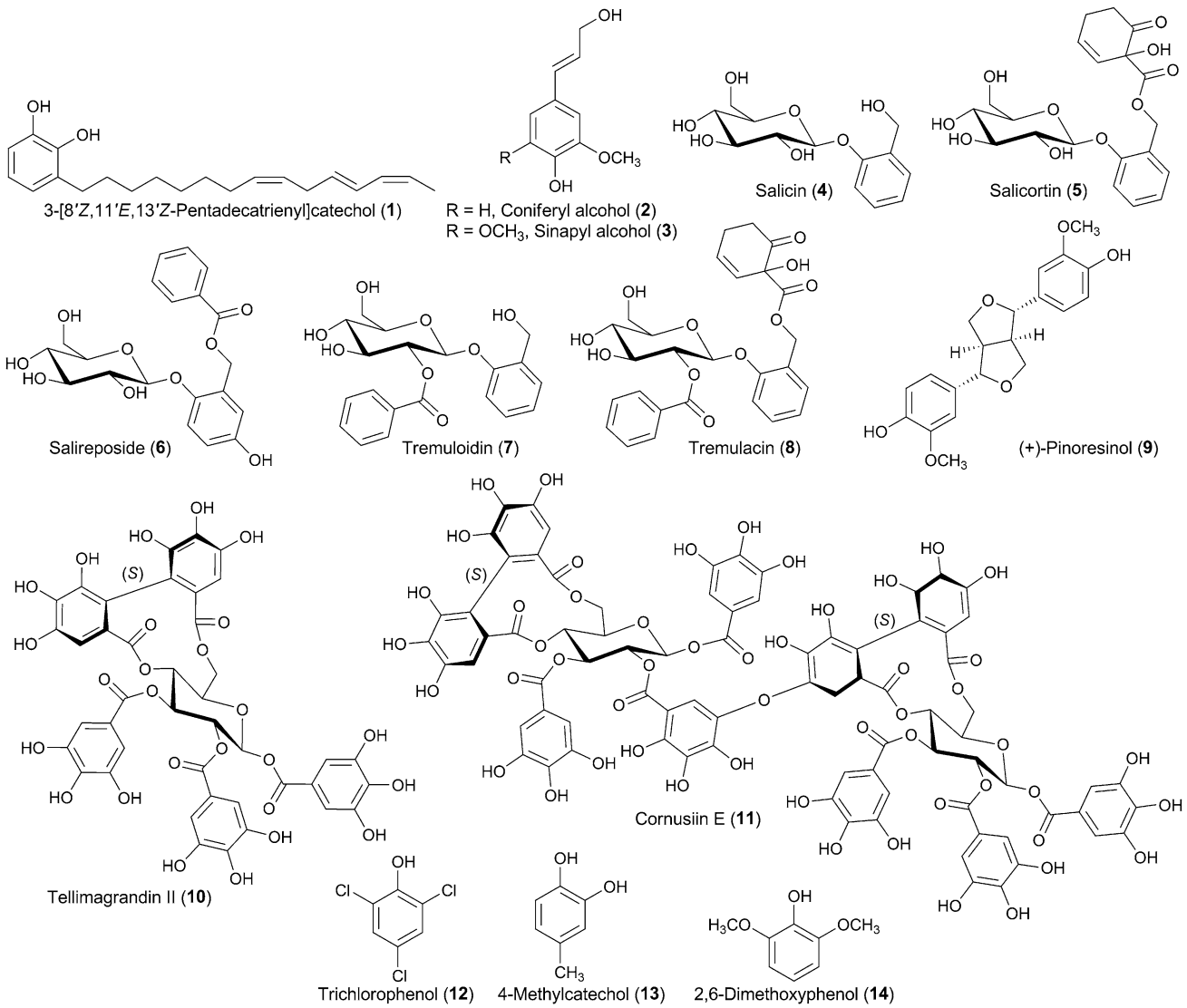

Fig. 1 Miscellaneous aromatics

Laccases were discovered from Japanese lacquer tree (*Rhus vernicifera)* sap almost 127 years ago (Yoshida 1883), where they were purportedly involved in wound healing due to sap hardening released at injury sites. This was later established to result from laccase-mediated oxidation of sap alkylcatechols called urushiols [e.g., (**1**)] (Nakamura 1958). Other contributions, however, proposed putative roles of plant laccases in lignification (Dean and Eriksson 1994, and references therein), but with little definitive evidence. The proposed involvement in lignification arose from both abilities of laccases to oxidize monolignols in vitro, such as coniferyl (**2**) and sinapyl alcohols (**3**) (Higuchi and Ito 1958; Freudenberg 1959; Sterjiades et al. 1992; Bao et al. 1993; Takahama 1995), and detection of laccase-like activities in lignifying cell walls of differentiating xylem (Driouich et al. 1992; Bao et al. 1993; Liu et al. 1994; McDougall 2000; Richardson et al. 2000). However, with laccases subsequently cloned from differentiating xylem cDNA libraries of vascular plants (LaFayette et al. 1995; Ranocha et al. 1999; Sato et al. 2001; Gavnholt et al. 2002), genetic approaches aimed at loss- (Ranocha et al. 2002) or gain-of-function studies (Dean et al. 1998) showed essentially no effect on either lignin contents or monomeric compositions.

In one study, five laccases were isolated from *Populus trichocarpa* differentiating xylem stem tissue, with two (lac90 and lac110) purified from their cell walls and the remaining three (*lac1*, *lac2* and *lac3*) identified by heterologous screening of a xylem cDNA library using *Acer pseudoplatanus* laccase cDNA as probe (Ranocha et al. 1999). While lac90 and lac110 were considered having putative roles in lignification through catalyzing oxidation of coniferyl alcohol (**2**) in vitro, their individual knockdowns in *P. tremula* × *P. alba* transgenic lines exhibited

no effects on estimated lignin contents, ethanol-soluble phenolics, cell wall structure and/or xylem fiber integrity (Ranocha et al. 2002). The *lac3* knock-down transgenic lines, however, apparently accumulated in their stems a two- to three-fold increased level of ethanol-soluble phenolic glycosides, such as salicin (**4**), salicortin (**5**), salireposide (**6**), tremuloidin (**7**) and tremulacin (**8**) as compared to wild type. These transgenic lines reportedly exhibited more readily detachable xylem fiber cell walls. However, *lac1* knock-down transgenic poplar lines did not show any of the above effects. It was thus provisionally proposed that lac3 was involved in cross-linking unidentified cell wall components to help maintain wall structure and xylem fiber integrity; however, for all lines examined, no definite physiological/biochemical function of any isoform was actually established. Indirect evidence of roles other than lignification were also proposed based on laccase expression in non-lignifying tissues (Gavnholt et al. 2002; Caparrós-Ruiz et al. 2006), but with no definitive roles established.

Other involvement of laccases in specific physiological/biochemical processes have also been reported, e.g., in lignan biosynthesis, a laccase was purified along with a dirigent protein from *Forsythia intermedia* stem tissues, with the latter mediating stereoselective bimolecular phenoxy radical coupling in vitro of coniferyl alcohol (**2**)-derived moieties to afford (+)-pinoresinol (**9**) in the presence of this oxidase (Davin et al. 1997). This suggested laccases might have an auxiliary role in stereoselective coupling to 8–8′ linked lignans. In another study, laccase-like phenol oxidases were implicated in regio-specific phenolic coupling yielding monomeric and dimeric ellagitannins, tellimagrandin II (**10**) and cornusiin E (**11**), respectively, in *Tellima grandiflora* (Niemetz and Gross 2003; Niemetz et al. 2003).

Another putative laccase role was proposed in iron uptake in *Liriodendron tulipifera* (yellow-poplar; Hoopes and Dean 2004), where it was reported to catalyze oxidation of ferrous (Fe^{2+}) to ferric (Fe^{3+}) ions in the extracellular cell wall space, with these envisaged to be involved, along with iron-specific permeases, in Fe^{3+} membrane translocation. However, to date there is no *in planta* evidence for this. Yet another potential role for laccases was proposed in *ex planta* phytoremediation, based on studies conducted in *A. thaliana* expressing a *Gossypium arboreum* (cotton) laccase that showed enhanced resistance to presence of environmental pollutants like trichlorophenol (**12**) by transforming them into less toxic substances (Wang et al. 2004). Laccases were also envisaged as redox mediators in maize, (*Zea mays)* through generating quinones from 4-methylcatechol (**13**) that serve as electron acceptors to cytokinin dehydrogenases involved in irreversible cytokinin degradation (Galuszka et al. 2005).

A definitive physiological role of laccases was, however, discovered via a forward (candidate) genetic approach, where a specific *A. thaliana* laccase, *TRANSPARENT TESTA 10* (*TT10/LAC15*), affecting seed coat color was identified in one locus. TT10/LAC15 was considered involved in mediating flavonoid polymerization that led to seed coat color/maturation, but with no unambiguous determination of either substrate(s)/product(s) (Pourcel et al. 2005).

Accordingly, to better understand precise physiological/biochemical roles of laccases, *A. thaliana* was selected for study because of its wealth of genomic information, the various genetic tools available, and since it contains a multi-gene family (genome encodes 17 putative laccases, Table 1). This provided an opportunity to decipher gene expression patterns at tissue and/or cell-specific levels to begin to develop strategies/approaches to identify physiological roles. Herein, using RT-PCR and promoter-GUS analyses, it was established that expression of several laccase gene family members closely overlap across a wide variety of tissues/development stages, whereas others are very unique. Together, these data give new insights into developing more targeted approaches to identify precise physiological/biochemical roles. Additionally, *in silico* analysis of putative promoter sequences of *Arabidopsis* laccases indicated presence of putative *cis* elements potentially involved in normal growth/development gene expression regulation, and in response to dehydration, wound and copper stress.

Materials and methods

Instrumentation and sequence analysis

PCR amplifications used a PTC-0220 DNA Engine Dyad Peltier Thermal Cycler (MJ Research, Waltham, MA, USA) with subsequent sequencing at the Washington State University (WSU) DNA core sequencing facility. RNA/DNA quantifications were obtained using a Lambda 6 UV–visible spectrophotometer (Perkin-Elmer). All sequence alignments and analyses were performed with the BioEdit sequence alignment editor (Tom Hall, Ibis therapeutics, Carlsbad, CA, USA), with a phylogenetic tree constructed using Geneious Pro software (http://www.geneious.com). Putative signal sequences and subcellular localizations were predicted using SignalP and TargetP, respectively (http://www.cbs.dtu.dk/). Potential glycosylation and phosphorylation sites were analyzed using prediction servers available at http://www.cbs.dtu.dk.

Table 1 Prediction of N-terminal signal peptides and glycosylation sites of *Arabidopsis* laccases

Gene	Locus number	Signal peptide length	Cleavage site	Predicted target site	Number of potential glycosylation sites	
					NGlyc	OGlyc
LAC1	At1g18140	25	SSA-ST	Secretory	5	1
LAC2	At2g29130	26	ASA-GI	Secretory	17	1
LAC3	At2g30210	25	ASA-EH	Secretory	7	5
LAC4	At2g38080	24	SES-MV	Secretory	14	1
LAC5	At2g40370	25	AEA-NK	Secretory	8	1
LAC6	At2g46570	29	IGA-AT	Secretory	7	
LAC7	At3g09220	23	TSA-SI	Secretory	11	3
LAC8	At5g01040	25	ASA-AV	Secretory	8	4
LAC9	At5g01050	25	ASA-AI	Secretory	9	5
LAC10	At5g01190	22	VHG-AI	Secretory	10	8
LAC11	At5g03260	23	VDA-AV	Secretory	12	1
LAC12	At5g05390	24	IIA-KV	Secretory	9	2
LAC13	At5g07130	21	VNA-EV	Secretory	7	4
LAC14	At5g09360	33	AEA-EI	Secretory	8	1
LAC15	At5g48100	21	CIA-HH	Secretory	9	
LAC16	At5g58910				8	
LAC17	At5g60020	22	AFG-IT	Secretory	15	

Materials

pCR4®-TOPO/TA cloning vector, SuperScript™ III First-Strand Synthesis Kit for RT-PCR, TRIzol® Reagent, DNase I and *Taq* DNA polymerase were purchased from Invitrogen™ (Carlsbad, CA, USA), whereas pCAMBIA 1305.1 vectors were from Cambia (Canberra, Australia). The QIAquick® Gel Extraction Kit was purchased from Qiagen Inc. (Valencia, CA, USA), with the *Pfu Turbo*® DNA polymerase procured from Stratagene (La Jolla, CA, USA); restriction enzymes and Rapid DNA ligation kit were obtained from Roche Applied Science (Indianapolis, IN, USA). The chromogenic substrate for GUS-staining 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) was purchased from Gold Biotechnology Inc. (St. Louis, MO, USA), and the REDExtract-N-AMP™ Plant PCR Kit was from Sigma–Aldrich® (St. Louis, MO, USA). All primers used were custom synthesized by Invitrogen™, with Wizard® *Plus* SV Miniprep DNA purification System from Promega (Madison, WI, USA).

Plant materials and growth conditions

A. thaliana ecotype Columbia-0 seeds were from Lehle Seeds (Round Rock, TX, USA), with this ecotype used for all transformations. For soil-grown plants, seeds were cold-stratified at 4°C for at least 3 days and subsequently grown in WSU greenhouses. For young tissues (germinating seeds to 21-day-old seedlings), seeds were germinated on a solid medium of 1× Murashige and Skoog (MS) medium as previously described (Kim et al. 2007).

Total RNA isolation and first strand cDNA synthesis

Time points for tissue collection are with respect to sowing time. Total RNA samples were individually obtained from 1- to 2-week-old seedlings grown on 1× MS medium plates and from preanthesis to stage 2 plants grown in soil, respectively. All tissues for total RNA extraction were flash frozen in liquid nitrogen and stored at −80°C until needed. Tissue samples for expression analysis were from different development/growth stages (Altamura et al. 2001). This included collection of: seedlings at 7- and 14-days after germination (DAG); rosette leaves and roots at the bolting stage; rosette leaves, roots, flowers (both opened and closed buds pooled), cauline leaves and stems after bolting at anthesis, stage 1 and stage 2; siliques at stage 1 onset. Since *Arabidopsis* has different leaf morphologies, total RNA was extracted from pooled samples of all leaf types. Root tissues were thoroughly washed in water before freezing in liquid nitrogen. For stem tissues, basal (~2 to 4 cm from hypocotyl, covering first internode) and middle (~2 to 5 cm from terminal flower, spanning middle internodes) sections of inflorescence stems were individually collected. Total RNA was extracted using TRIzol® reagent protocol, with quality and yields determined by measuring absorbances at 280 and 260 nm with visualization via ethidium bromide stained agarose (1%, w/v) gels. Total RNA (5 µg) was treated with DNase I prior to cDNA synthesis, with first strand cDNA synthesized by oligo-dT primers using SuperScript™ III First-Strand Synthesis Kit, all according to the manufacturer's instructions.

Cloning of laccase cDNAs (see supplementary material and supplementary Table 1 for description)

Expression analyses by RT-PCR

First strand cDNA synthesized from different tissues/development stages was analyzed for laccase gene expression profiles, with gene-specific primers designed for each at the exon/intron boundaries and 3′ end of laccases either downstream or upstream of stop codon (Supplementary Table 2). Specificity of each primer pair was checked by BLASTN searches against the *Arabidopsis* genome (both genomic and RNA sequences) to confirm designed primers were laccase-specific. PCR conditions employed for expression analysis were as above. Amplification products were run on 1.2% (w/v) agarose gels and stained with ethidium bromide for visualization. Actin (ACT2, At3g18780) was used as housekeeping gene and as positive control. Gels were scored for presence or absence of appropriate size bands for each laccase, with identity of each verified by sequencing PCR products directly.

Construction of promoter::GUS reporter vectors

The TAIR database was used to retrieve promoter region sequence information for each laccase gene. Specifically, sequences spanning intergenic regions between translation start sites and either translation start or stop codons of neighboring 5′ upstream genes were considered putative promoter regions. In cases where the region was very large, as in *LAC4p* (3,943 bp), *LAC5p* (4,140 bp) and *LAC15p* (8,500 bp), ~2 kb 5′ upstream sequences were amplified, as these presumably encompass the core promoter required for transcription initiation by the basal transcriptional machinery. Additionally, native signal sequences predicted by SignalP for each laccase were individually included in each promoter. Individual laccase promoters were amplified from *Arabidopsis* genomic DNA by PCR amplification using specific primers (Supplementary Table 3), with genomic DNA extracted from leaves of 3-week-old plants using the CTAB (cetyl trimethyl ammonium bromide) method (Stewart and Via 1993). Appropriate restriction enzyme linkers were added at either end of primers to facilitate subsequent cloning into pCAMBIA 1305.1 vectors harboring the *GUS* reporter gene (Supplementary Table 3). In particular, *Sac*I and *Bgl*II restriction enzyme sites were added to forward and reverse primers, respectively, for *LAC3p*, *LAC6p*, *LAC10p–LAC15p* and *LAC17p*. Similarly, restriction enzyme sites corresponding to *Sac*I and *Nco*I for *LAC2p*, *LAC5p*, *LAC7p* and *LAC8p*; *Xba*I and *Nco*I for *LAC4p*, *LAC9p* and *LAC16p*; *Hin*dIII and *Bgl*II for *LAC1p* were added to respective forward and reverse primers. PCR reactions consisted of 0.2 mM dNTPs, 0.2 μM each of forward and reverse primers, 50 ng genomic DNA, 1× cloned *Pfu* reaction buffer, and 2.5 units *Pfu Turbo*® DNA polymerase with amplification conditions as described above, except that extension time varied from 2 to 4 min depending on promoter fragment size. PCR products were analyzed on ethidium bromide stained agarose (0.8%, w/v) gels with fragments gel purified and cloned into pCR4-TOPO vector for sequencing. After sequence verification, each promoter fragment was excised from the pCR4-TOPO vector by digesting with appropriate restriction enzymes and ligated into similarly digested pCAMBIA 1305.1 vector. Ligations were performed using a Rapid DNA Ligation kit per manufacturer's protocol. Clones harboring laccase promoter::GUS cassette in pCAMBIA 1305.1 vector were sequenced to verify fidelity. For convenience, each laccase was numbered as *LAC1* to *LAC17* with corresponding promoter-GUS fusions as *LAC1p::GUS* through *LAC17p::GUS*, following corresponding gene locus accession number notations (Table 1).

Generation and selection of *Arabidopsis* transformants

LAC1p::GUS through *LAC17p::GUS* expression cassettes were individually transformed into *Agrobacterium tumefaciens* GV3101 strain by the freeze–thaw method (Höfgen and Willmitzer 1988), with transformed colonies selected by screening on LB plates (containing 50 mg l^{-1} rifampicin, 50 mg l^{-1} kanamycin) and verified by colony PCR screening using promoter-specific primers. Subsequently, the modified *Agrobacterium* strains were transformed into *A. thaliana* ecotype Columbia-0 plants using the floral dip procedure (Clough and Bent 1998). For transformant selection and validation, see Kim et al. (2007) for general procedures.

GUS-staining for histochemical studies

Histochemical GUS staining was performed on T_2 plant lines 3 days after plating, and weekly until 8 weeks. Plants analyzed from 3 to 14 days were grown on MS plates and those analyzed from preanthesis to stage 2 were soil grown. GUS activity histochemical staining was as described (Kim et al. 2006). After staining, tissue samples were cleared of chlorophyll by several washes in aqueous ethanol (3:7, v/v), with stained tissues recorded using an Olympus BH-2 photomicroscope, then photographed (Kodak 64T film) and scanned.

Gene expression data sets and promoter element search

Gene expression datasets at Genevestigator (http://www.genevestigator.com) databases were analyzed for laccase

gene expression profiles and compared with data obtained (Hruz et al. 2008); no expression profile for *LAC9* was available in Genevestigator. Root expression patterns were specifically compared with AREX database at http://www.arexdb.org/ (Birnbaum et al. 2003; Brady et al. 2007), whereas expression profiles for laccases regulated upon pathogen attack were analyzed at PathoPlant database (http://www.pathoplant.de/index.php; Bülow et al. 2004). *In silico* analysis of promoter sequences for putative *cis* acting regulatory DNA elements present in promoter sequences was performed using Signal Scan web service (http://www.dna.affrc.go.jp/PLACE/), with DNA binding motifs for transcription factors in promoters searched using Athena database (http://www.bioinformatics2.wsu.edu/Athena/).

Results

Laccase cDNA cloning

The *Arabidopsis* genome encodes 17 putative laccases in the NCBI database, these being distributed across all chromosomes except chromosome 4 (Fig. 2). Fifteen were in 2 gene clusters, with 5 and 10 laccases on the 2nd and 5th chromosomes, with the remaining 2 solitary genes, *LAC1* and *LAC7*, on chromosomes 1 and 3, respectively. While minor discrepancies in splice junctions of annotated sequences for some *Arabidopsis* laccase gene family members were previously reported using computational analysis (McCaig et al. 2005), none at that time were either cloned or their proteins characterized. In this study, full length coding sequences for 12 laccases were obtained by RT-PCR using cDNA synthesized from development stage 2 plants by designing gene-specific primers (Supplementary Table 1), with the 4 others (*LAC4*, *LAC7*, *LAC11* and *LAC15*) from ABRC. However, *LAC16* was neither cloned, nor detected in tissue used for cDNA synthesis, nor available elsewhere. The sequence from the NCBI database for *LAC16* was, nevertheless, used for sequence analysis. Of the 16 laccases obtained, 15 had sequences identical to the database (Fig. 3), with 1 laccase (*LAC14*) differing by a few nucleotide mismatches at the 3′ end. The percentage identity of deduced amino acid sequences also varied greatly from 36 to 89%, with *LAC8* and *LAC9* having highest (89%) homology (Table 2). This low sequence identity among individual members suggests diverse functions, although this is speculative since definitive biochemical/physiological roles are currently lacking.

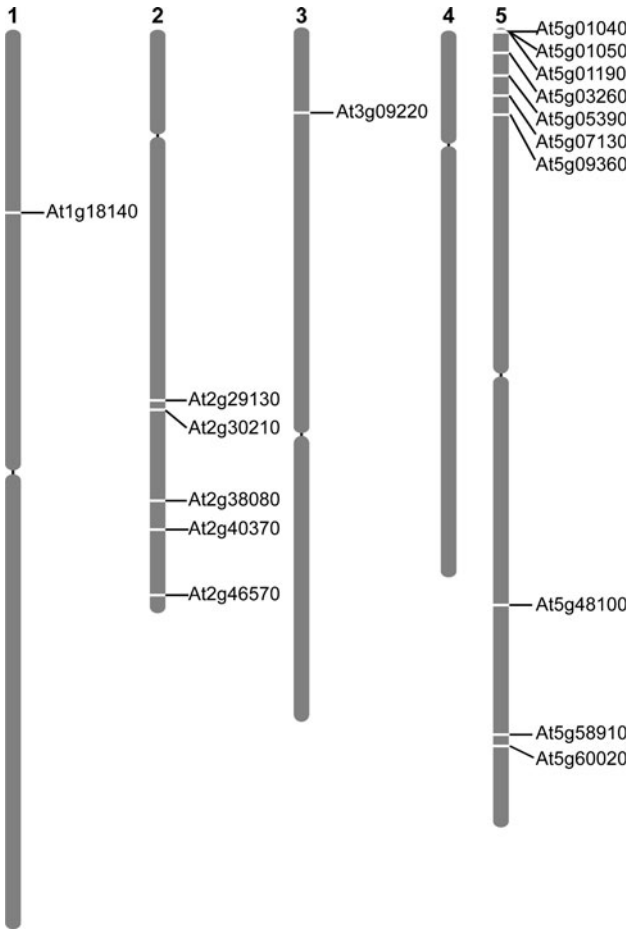

Fig. 2 Chromosome map depicting location of *Arabidopsis* laccases. Most laccases are located on the 2nd and 5th chromosomes, with only one laccase on the 1st and 3rd chromosomes. No laccases are on the 4th chromosome. The map construction utilized the chromosome map tool available on TAIR database (http://www.arabidopsis.org)

Laccase sequence comparisons

The *Arabidopsis* laccase sequences were analyzed to address if any putative functions could be inferred. Since all known laccases contain 4 Cu atoms bound to conserved residues (10 histidines and 1 cysteine), alignment of deduced *Arabidopsis* laccase amino acid sequences also showed these residues were conserved (Fig. 3), except for LAC16 lacking a histidine (H48) at a T3 Cu binding site i.e., H-W-H near the N terminus. However, as no corresponding cDNA was obtained for LAC16, the validity of this sequence could not be confirmed.

The axial ligand near the T1 Cu binding site (H-C-H-X_3-H-X_3-G-[LMI(F)]) proximal to the C-terminus putatively partially influences laccase redox potential (Saloheimo et al. 1991; Nitta et al. 2002; Dittmer et al. 2004). Furthermore, based on axial ligand type, *Arabidopsis* laccases

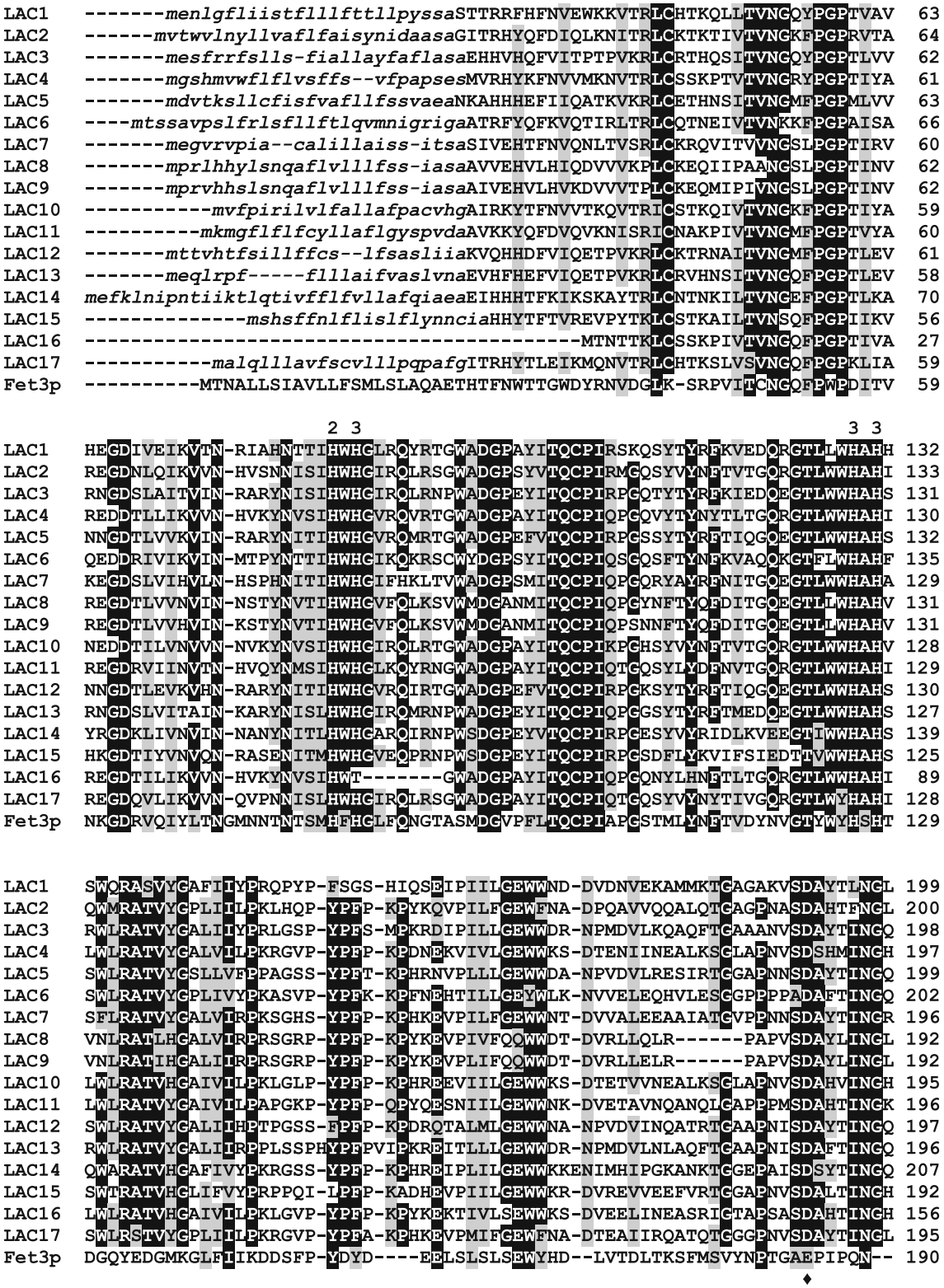

Fig. 3 Multiple alignment of *Arabidopsis* laccases with yeast Fet3p using ClustalW. The C-terminal transmembrane domain (113 amino acids) of Fet3p was removed before aligning. GenBank accession number for Fet3p used for alignment was NP_013774.1. The conserved 10 histidines and a cysteine residue involved in the coordination of different copper types (T1, T2 and T3) are shown. The residues involved in ferroxidase activity (E185, D278, D283, M345, Y354 and D409) for Fet3p are indicated by a diamond, whereas the axial ligand near the C-terminus (H-C-H-X_3-H-X_3-G-[LMI(F)]) putatively partially conferring redox potential to T1 Cu is depicted by a triangle. The N-terminal signal peptide for each laccase is indicated in lower case and italicized, with the Asn residue for pH optimal activity denoted by a *black spade*

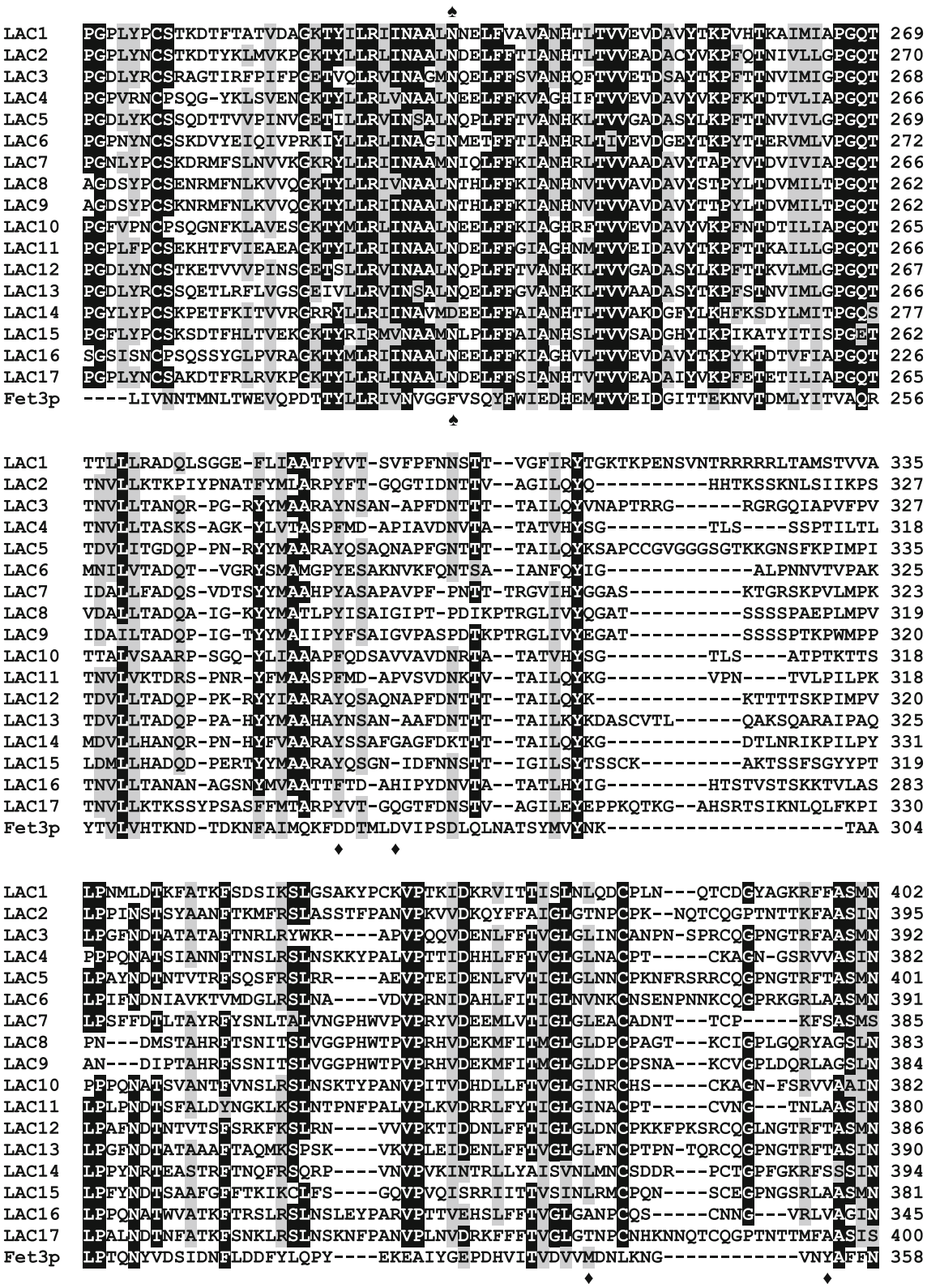

Fig. 3 continued

are provisionally grouped into two redox potential classes: low (Met as axial ligand; LAC6, LAC14 and LAC15) and high redox potential [(Ile; LAC8 and LAC9) or (Leu; remaining laccases)] (Fig. 3). However, Piontek et al. (2002) suggest that the distances between the T1 Cu and its ligands are more important.

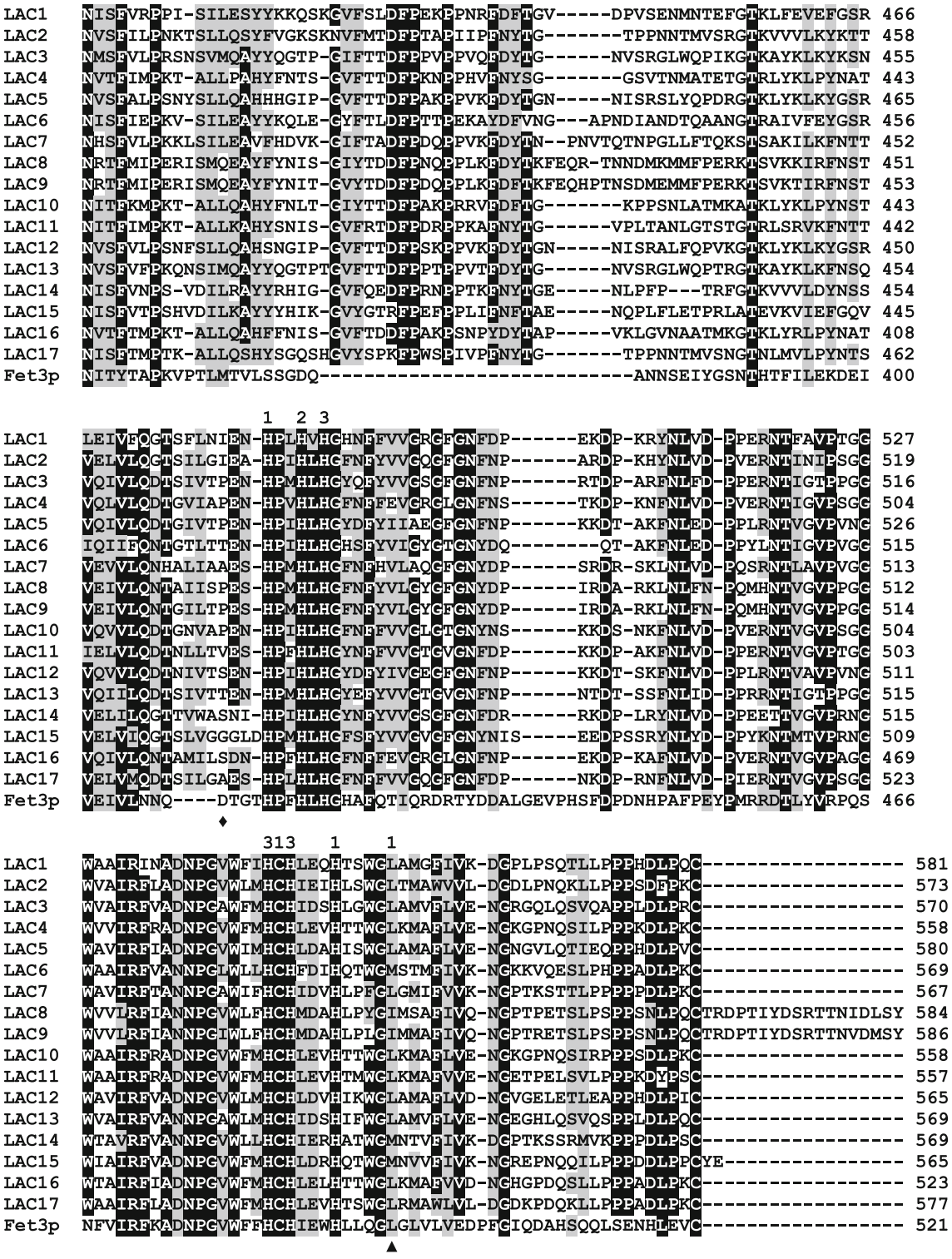

Fig. 3 continued

Laccase sequences were also compared for putative substrate binding residues, as an aspartate residue is highly conserved in fungal laccases (e.g., Asp206 in *Trametes versicolor*) and strongly interacts with substrates as well as influencing enzyme activity pH optimum (Madzak et al. 2006). Indeed, Asp206Asn mutation of the *T. versicolor* laccase caused a 3.4 to 4.8 pH optimum shift using 2,6-dimethoxyphenol (**14**) as potential substrate without

Table 2 Percent sequence identity matrix of *Arabidopsis* laccase amino acid sequences

LAC	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1		46	46	46	44	41	43	36	38	45	48	46	44	42	45	43	49
2			48	52	48	42	44	38	38	50	53	50	46	43	41	49	66
3				47	62	44	46	41	40	46	46	63	73	48	44	44	47
4					46	42	43	39	37	73	60	48	67	42	39	67	54
5						44	46	41	42	46	45	76	63	48	45	43	46
6							41	37	39	43	43	44	45	41	38	40	40
7								54	55	44	45	47	46	41	40	42	44
8									89	39	39	41	41	36	37	39	36
9										39	39	41	41	36	37	39	37
10											57	47	44	41	41	63	52
11												49	47	43	41	55	55
12													65	50	45	46	48
13														48	43	43	44
14															48	39	42
15																39	41
16																	50
17																	

apparently affecting transformation rate. By contrast, plant laccases generally have Asn at this comparable position, and this holds for the *Arabidopsis* laccase gene family, except LAC14 with an Asp (Fig. 3, black spade).

The putative involvement of plant laccases in Fe transport was reported based on ferroxidase-like activities (Hoopes and Dean 2004). Thus, the *Arabidopsis* laccases were compared with Fet3p, an extensively studied membrane-bound multi-copper oxidase from *Saccharomyces cerevisiae*, which contains residues (E185, D278, D283, M345, Y354 and D409) involved in ferroxidase activity (Taylor et al. 2005). Based on this sequence comparison, no evidence was obtained for *Arabidopsis* laccases harboring residues for ferroxidase-like activity (Fig. 3).

Most plant laccases are predicted to have an N-terminal cleavable signal peptide targeting them to the secretory pathway (McCaig et al. 2005); there is however a report of a monocot laccase (*Lolium perenne*) with an uncleavable signal peptide that presumably targets it to peroxisomes (Gavnholt et al. 2002). Sequence analysis of *Arabidopsis* laccases, using currently available web-based bioinformatics tools (http://www.cbs.dtu.dk), indicated all have cleavable N-terminal signal peptides (Fig. 3), except LAC16 which has no signal peptide perhaps suggesting an intracellular localization (Table 1).

Most secretory plant laccases are glycoproteins with *N*-glycosylation being common. While all *Arabidopsis* laccases have putative *N*-glycosylation sites (N-X-[ST]), the potential number of sites vary extensively. In addition, several potential *O*-linked glycosylation sites were identified for most (Table 1). Analysis for other post-translational modifications, such as phosphorylation using the PhosPhAt database (Heazlewood et al. 2008) and the NetPhos 2.0 server, predicted that all *Arabidopsis* laccases also have a number of potential serine, threonine and tyrosine phosphorylation sites.

Molecular phylogeny of plant laccases

To better understand laccase sequence divergence and similarities among *Arabidopsis* gene family members and other plants, a provisional molecular phylogenetic tree was constructed using multiple sequence alignment from various plant species. The phylogenetic tree indicated that *Arabidopsis* laccases cluster into six arbitrary groups (Fig. 4), a finding consistent with other studies based on sequence identities and taxonomic clustering (McCaig et al. 2005; Pourcel et al. 2005; Caparrós-Ruiz et al. 2006). *Arabidopsis* laccase gene family members were dispersed throughout the phylogenetic tree, reflecting, at least in part, relatively low sequence homology. Interestingly, all putative low redox potential *Arabidopsis* laccases (LAC6, LAC14, and LAC15) cluster in group 4, while those putatively of high redox potential, with either Ile or Leu as axial ligand, are distributed across different groups (Fig. 4). Additionally, group 1 includes a monophyletic cluster of laccases from *L. tulipifera* along with *Arabidopsis* LAC2 and LAC17, whereas group 2 consists of *Arabidopsis* LAC4, LAC10, LAC11 and LAC16, with other plant laccases in this group mainly cloned from differentiating xylem. Group 3 includes *Arabidopsis* LAC3, LAC5, LAC12 and LAC13, with group 5 including LAC7,

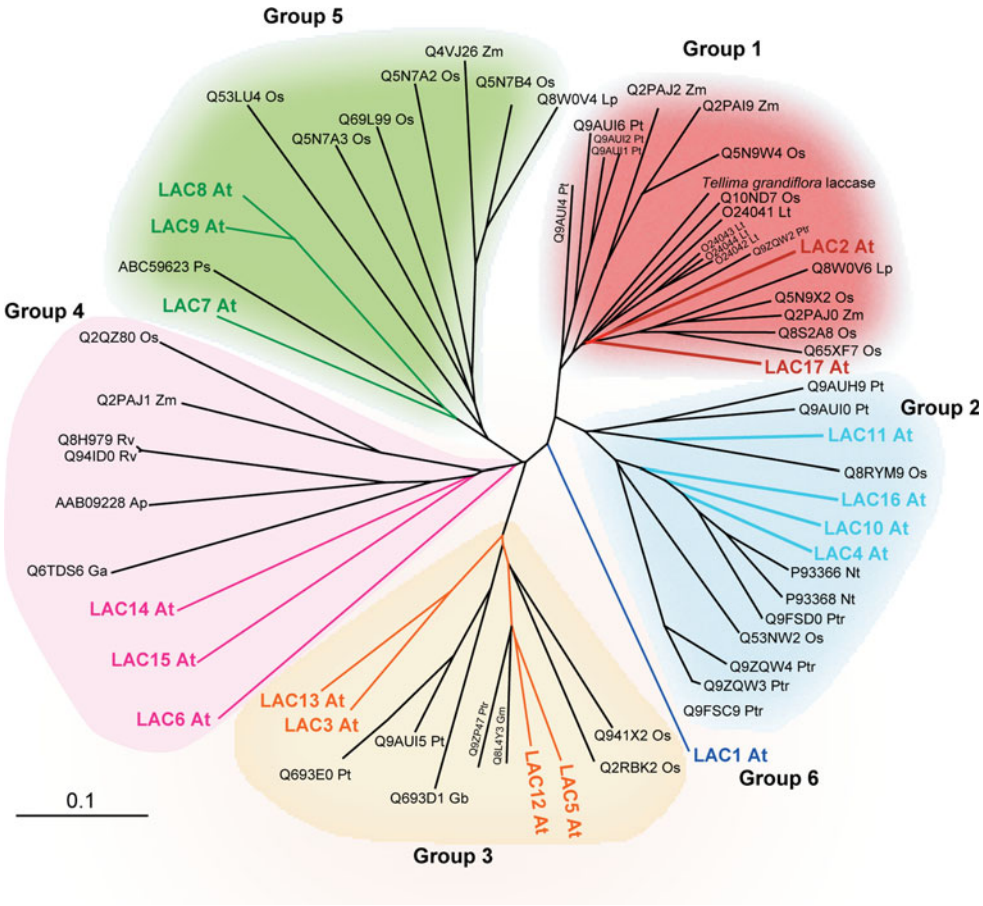

Fig. 4 Provisional unrooted neighbor-joining phylogenetic tree of plant laccases. The GenBank accession numbers are depicted for each species in the phylogeny. Ap, *Acer pseudoplatanus*; At, *Arabidopsis thaliana*; Ga, *Gossypium arboretum*; Gb *Ginkgo biloba*; Gm *Glycine max*; Lt *Liriodendron tulipifera*; Lp *Lolium perenne*; Nt *Nicotiana tabaccum*; Os *Oryza sativa*; Ps *Pisum sativum*; Pt *Pinus taeda*; Ptr *Populus trichocarpa*; Rv *Rhus vernicifera*; Zm *Zea mays*. *Tellima grandiflora* laccase was not in the NCBI database

LAC8 and LAC9 of *Arabidopsis* along with uncharacterized laccases from other plants. Lastly, putative high redox *Arabidopsis* laccase, LAC1, did not cluster with any plant laccase and formed a separate group 6.

Gene expression analyses

To better understand if gene expression patterns followed similar grouping patterns to this molecular phylogeny, and if they exhibited any tissue/development specific expression patterns, RT-PCR using gene-specific primers was initially employed (Supplementary Table 2) followed by promoter-GUS analysis. The development stages investigated are broadly defined in three stages with respect to reproductive growth after appearance of the first flower i.e., anthesis that usually occurs by 3–4 weeks growth. Following anthesis, these are: stage 1, first silique differentiation corresponding to 4–8 days later; stage 2, appearance of immature green siliques with floral buds still present, corresponding to 6–20 days later; and stage 3, when all siliques are dehiscent, this occurring 27–30 days later (Altamura et al. 2001; Patten et al. 2010).

Based on RT-PCR analysis, 15 laccases displayed some level of expression in most tissues, with the remaining 2 (*LAC6* and *LAC16*) not detected in any tissues (Fig. 5). Furthermore, since expression patterns did not follow any similar clustering pattern as shown in the provisional phylogeny, they could instead be broadly classified into three groups. Firstly, expression in all tissues examined was observed for *LAC2*, *LAC4*, *LAC5*, *LAC10–LAC12* and *LAC17* (Fig. 5). Secondly, expression in roots and reproductive organs was noted for *LAC1*, *LAC3*, *LAC7*, *LAC8* and *LAC15*. Besides these organs, expression at specific development stages was observed in stems (*LAC3* at stage 2; *LAC7* at end of stage 2) and leaves (*LAC8* in 3-week-old rosette leaves and stage 1 cauline leaves, as well as *LAC15* in cauline leaves at end of stage 2). Additionally, in young seedlings, *LAC15* did not show any expression and *LAC1* expression was not detected in siliques. Thirdly, tissue- and development-specific expression patterns in roots were noted for *LAC9* and *LAC13*, with *LAC14* expression being restricted to reproductive organs.

Cloning of promoter-GUS fusion constructs

To gain more definitive insight into cell/tissue-specific expression, laccase promoter-GUS fusions were individually constructed and their spatial/temporal expression patterns investigated.

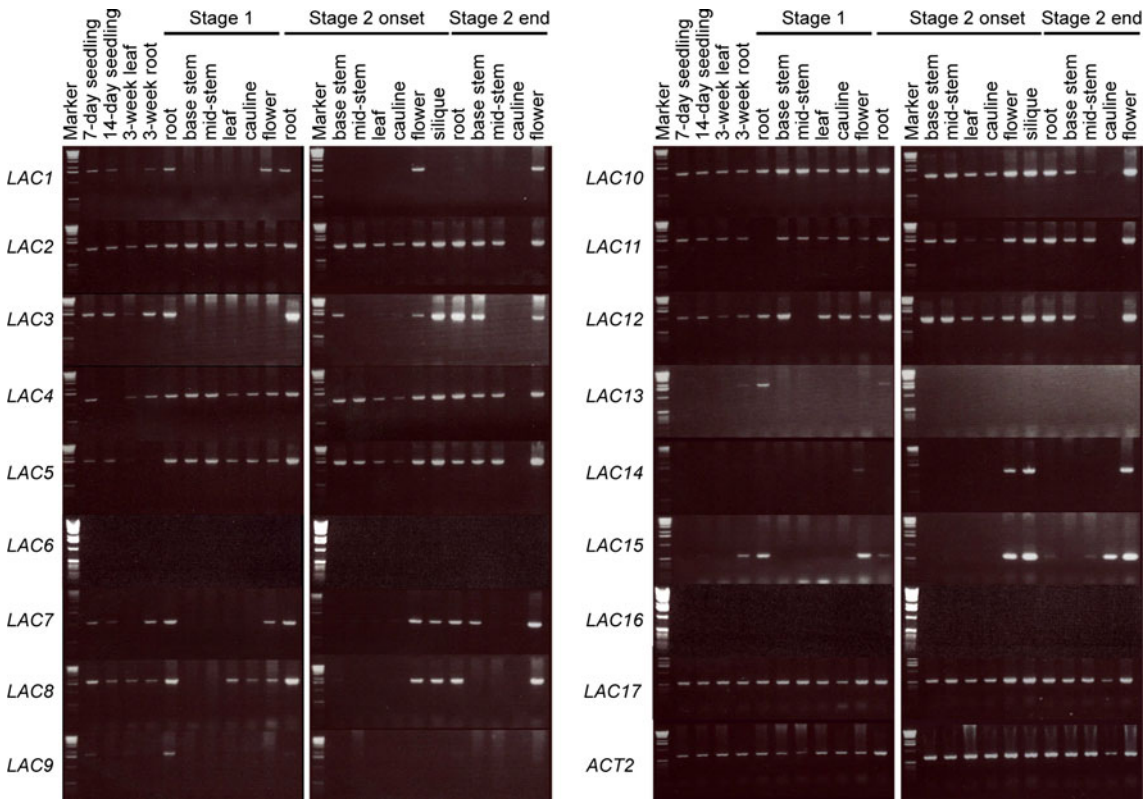

Fig. 5 RT-PCR expression profiles of *Arabidopsis* laccases across different tissue and development stages. Actin (ACT2) was used as housekeeping gene and as positive control

The presence of these constructs in T_2 plants was then verified by PCR screening using gDNA isolated from leaves of each *LACp::GUS* transgenic plant as template and vector-specific primers for amplification of GUS reporter gene. GUS-reporter visualization was carried out at various development stages across distinct tissues as described below.

GUS-staining in germinating seeds

Emergence of the radicle and undifferentiated cotyledons, leaf-like structures, occurs within 24–48 h after germination. At this stage, *LAC3p::GUS–LAC5p::GUS*, *LAC7p::GUS–LAC9p::GUS*, *LAC11p::GUS*, *LAC12p::GUS*, *LAC14p::-GUS*, *LAC15p::GUS* and *LAC17p::GUS* expression patterns were readily observed (Fig. 6c–e, g–i, k, l, n, o, q). Conversely, no expression was noted at this developmental stage for the remainder (Fig. 6a, b, f, j, m, p). More specifically, expression was prominent in the radicle vasculature, as well as in cotyledons either towards tips (*LAC3p::GUS* and *LAC4p::GUS*) (Fig. 6c, d) or edges (*LAC5p::GUS*) (Fig. 6e). *LAC7p::GUS* expression was also prominent near root hairs and in cotyledon vasculature (Fig. 6g). Interestingly, *LAC8p::GUS* displayed a specific-expression pattern in the root cap and meristem of the emerging radicle, although staining was observed in other regions, such as cortex (Fig. 6h). The root cap is composed of living parenchyma cells that helps protect the root apical meristem and aids in gravity perception/root penetration. In addition, it secretes mucilage consisting of pectins, proteins and other metabolites to assist in root growth (Tsugeki and Fedoroff 1999). *LAC9p::GUS*, *LAC11p::GUS* and *LAC12p::GUS* expression was also mostly visible in the radicle, although faint expression was noted in cotyledon vasculature (Fig. 6i, k, l), whereas *LAC14p::GUS* and *LAC17p*::*GUS* expression was readily detectable in the vascular apparatus of cotyledons and radicle (Fig. 6n, q). Interestingly, *LAC15p::GUS* was also observed specifically in the hilum, where seeds are attached to the replum in siliques (Fig. 6o).

GUS-staining in young seedlings

At about 14 days post-germination, cotyledons and the first set of true leaves (i.e., rosettes) have matured further and a new set of young leaves, leaf primordia, originating from shoot apical meristem (SAM) at the shoot tip are now observed. At this stage, the rosette leaf vascular apparatus is readily observed, with a venation pattern of the reticulate type and development of trichomes on the leaf epidermis (Dharmawardhana et al. 1992). In *Arabidopsis*, trichomes

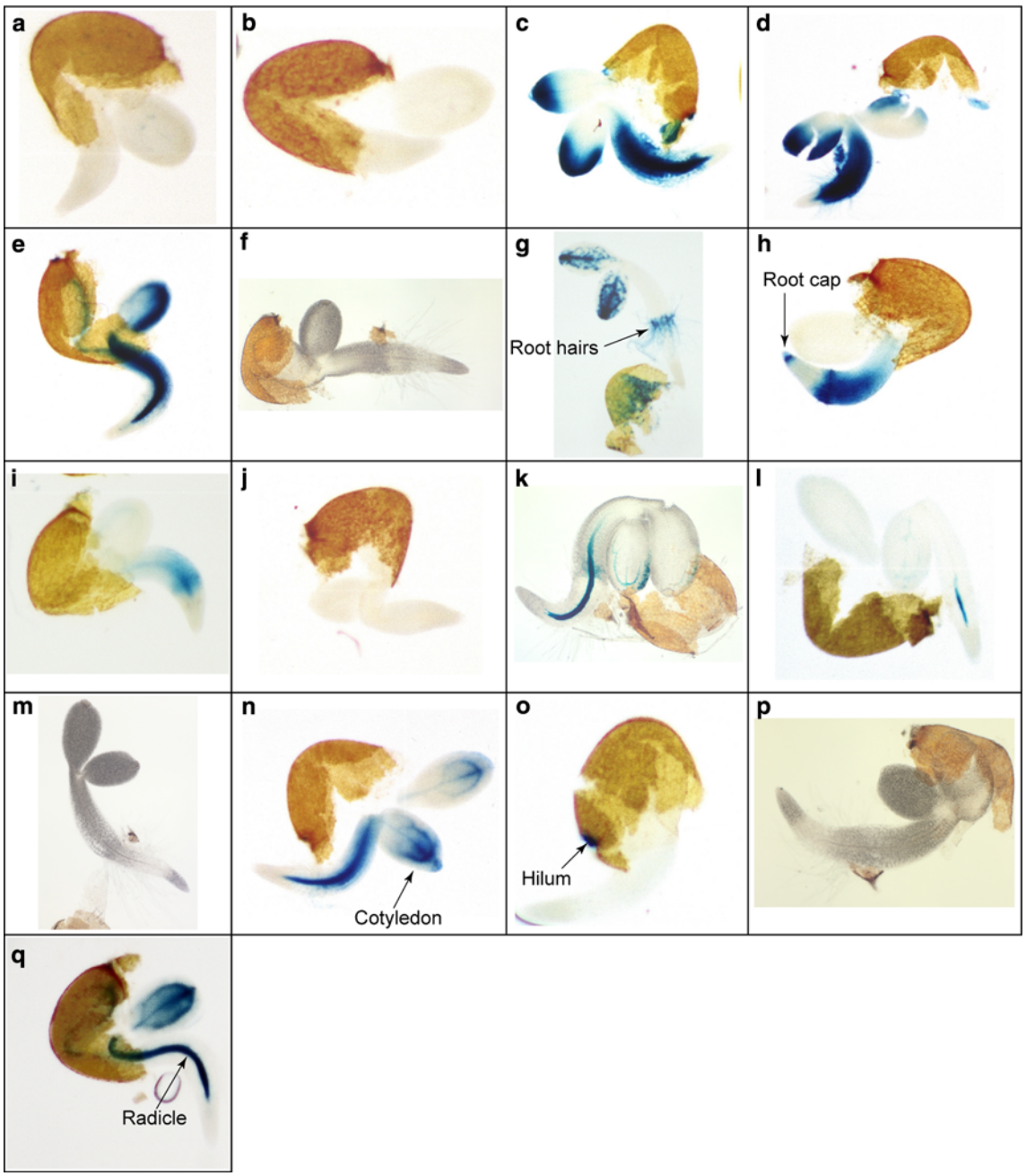

Fig. 6 Histochemical GUS localization of *LAC1p–LAC17p::GUS* in germinating seeds. **a** *LAC1p::GUS*; **b** *LAC2p::GUS*; **c** *LAC3p::GUS*; **d** *LAC4p::GUS*; **e** *LAC5p::GUS*; **f** *LAC6p::GUS*; **g** *LAC7p::GUS*; **h** *LAC8p::GUS*; **i** *LAC9p::GUS*; **j** *LAC10p::GUS*; **k** *LAC11p::GUS*; **l** *LAC12p::GUS*; **m** *LAC13p::GUS*; **n** *LAC14p::GUS*; **o** *LAC15p::GUS;* **p** *LAC16p::GUS*; **q** *LAC17p::GUS*

are unicellular structures derived from epidermis and have been proposed to serve several physiological functions such as prevention of water evaporation, detoxification, and protection against various environmental challenges. Their structures are characterized by thick cell walls, with basal supporting cells putatively lignified, and whose numbers vary with leaf growth/development (Hülskamp 2004). Also, at this stage, primary roots are elongated and a number of lateral roots have emerged.

During this stage, all but three laccases had readily detectable expression patterns, whose patterns in aerial versus underground organs are discussed separately (Figs. 7 and 8). For aerial organs, expression patterns for *LAC2p::GUS, LAC4p::GUS, LAC9p::GUS–LAC11p::-GUS, LAC14p::GUS*, *LAC15p::GUS* and *LAC17p::GUS* were similar with staining in the vasculature of cotyledons and rosette leaves, as well as in trichomes (Fig. 7b, d, i–k, n, o, q). By contrast, faint expression levels in leaf

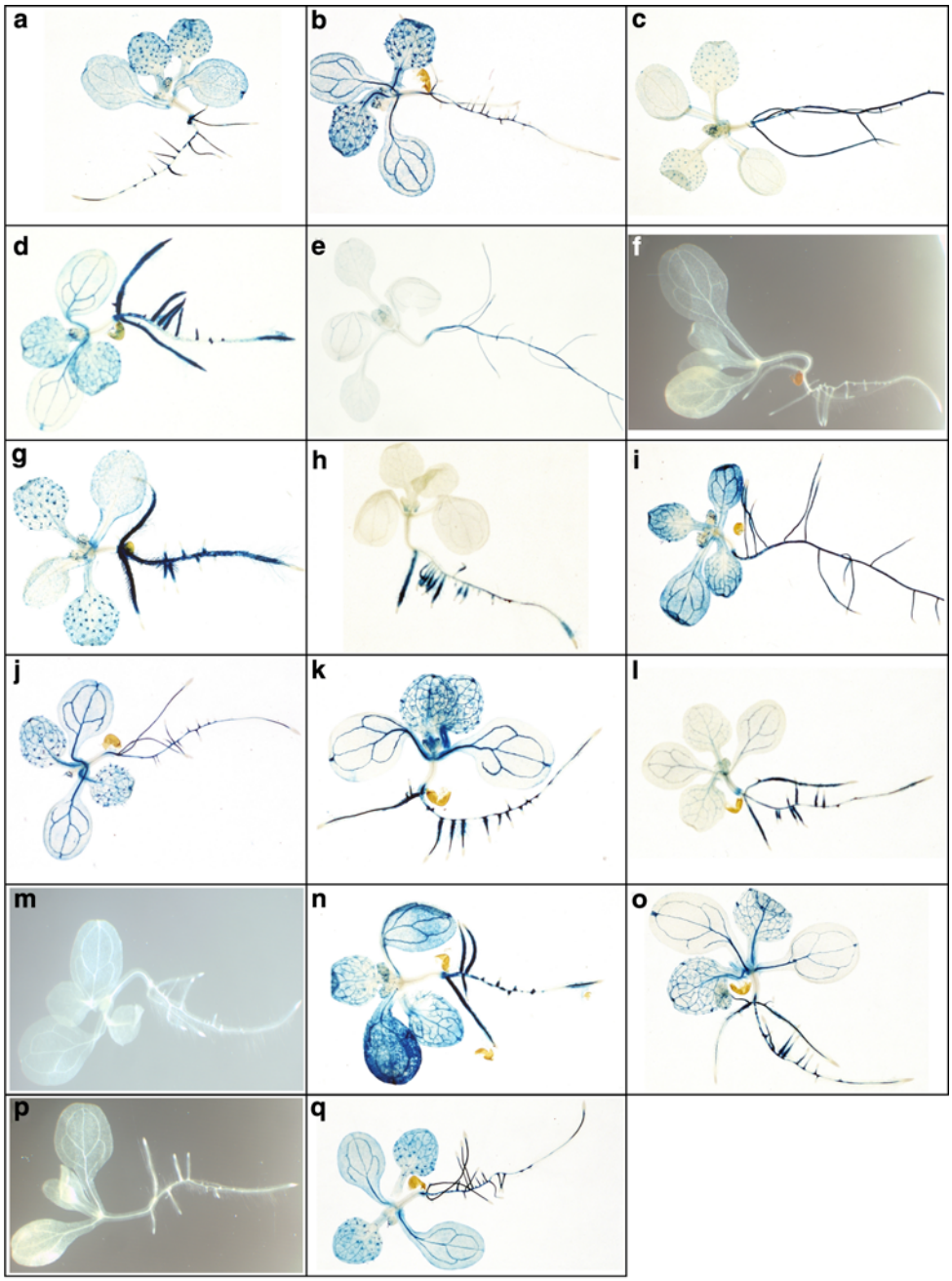

Fig. 7 GUS staining patterns of *LAC1p–LAC17p::GUS* in 14 days old seedlings. **a–q** Staining patterns for *LAC1p–LAC17p::GUS*, respectively, as in Fig. 6

vasculature were observed for *LAC5p::GUS* and *LAC12p::GUS*, with no staining detected in trichomes under the conditions employed (Fig. 7e, l). Expression patterns of *LAC1p::GUS*, *LAC3p::GUS* and *LAC7p::GUS* were, by contrast, mainly noted in leaf trichomes (Fig. 7a, c, g). For *LAC8p::GUS* very faint staining was detected only in leaf primordia (Fig. 7h). No expression of *LAC6p::GUS*, *LAC13p::GUS* and *LAC16p::GUS* was observed in aerial organs (Fig. 7f, m, p).

In roots, following 14 days post-germination, expression was evident for 14 laccases (Fig. 8). Staining in both primary and lateral roots was noted for *LAC2p::GUS–LAC5p::GUS*, *LAC9p::GUS*, *LAC10p::GUS*, *LAC12p::-GUS*, *LAC15p::GUS* and *LAC17p::GUS* (Fig. 8b–e, i, j, l, o, q), whereas staining of *LAC1p::GUS*, *LAC11p::GUS* and *LAC14p::GUS* was more specifically in lateral roots (Fig. 8a, k, n). For both *LAC7p::GUS* and *LAC8p::GUS* (Fig. 8g, h), diffuse staining occurred in primary and lateral roots with *LAC8p::GUS* staining in the root cap, whereas in root cap and meristem, expression of *LAC3p::GUS* and *LAC4p::GUS* was also detected (Fig. 8c, d). *LAC6p::GUS*, *LAC13p::GUS* and

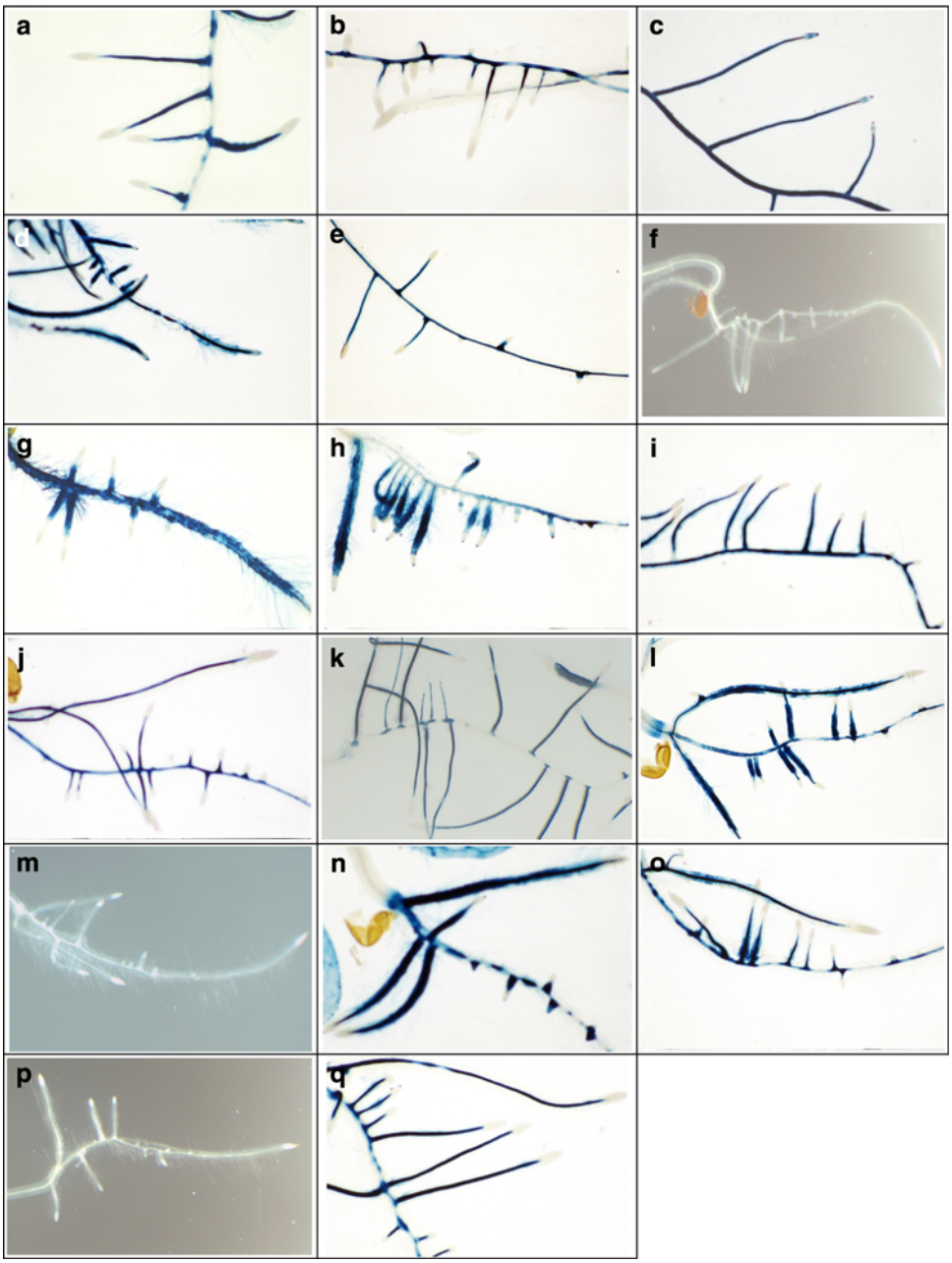

Fig. 8 Expression of promoter-GUS fusions of *LAC1p–LAC17p::GUS* in the roots of 14–21 days old seedlings. **a–q** Staining patterns for *LAC1p–LAC17p::GUS*, respectively, as in Fig. 6

LAC16p::GUS expression was not detected in roots (Fig. 8f, m, p).

GUS-staining in mature leaves

By 3–5 weeks growth (preanthesis to stage 1), the complete sets of rosette leaves are developed and fully expanded, and emergence of inflorescence stems begins indicative of transition from vegetative to reproductive growth. Then morphologically distinct new foliage, cauline leaves, develop at the inflorescence stem axillary meristem, with these having a complex venation pattern and being sessile (no petiole) (Dharmawardhana et al. 1992). In this regard, laccase staining patterns observed between rosette and cauline leaves are discussed separately below.

Rosette leaf expression was prominent in vasculature of *LAC2p::GUS*, *LAC4p::GUS*, *LAC5p::GUS*, *LAC9p::GUS–LAC12p::GUS*, *LAC14p::GUS*, *LAC15p::GUS* and *LAC17p::GUS* (Fig. 9b, d, e, i–l, n, o, q). However, expression of *LAC8p::GUS* was very faint, mostly being restricted to tertiary veins (Fig. 9h). For *LAC1p::GUS* and *LAC3p::GUS*, diffuse staining was observed in mesophyll tissue and supporting trichome epidermal cells (Fig. 9a, c). Interestingly, in the latter cell types, expression of *LAC2p::GUS* was also noted (Fig. 9b). For *LAC7p::GUS*, expression was mainly restricted to hydathodes (Fig. 9g),

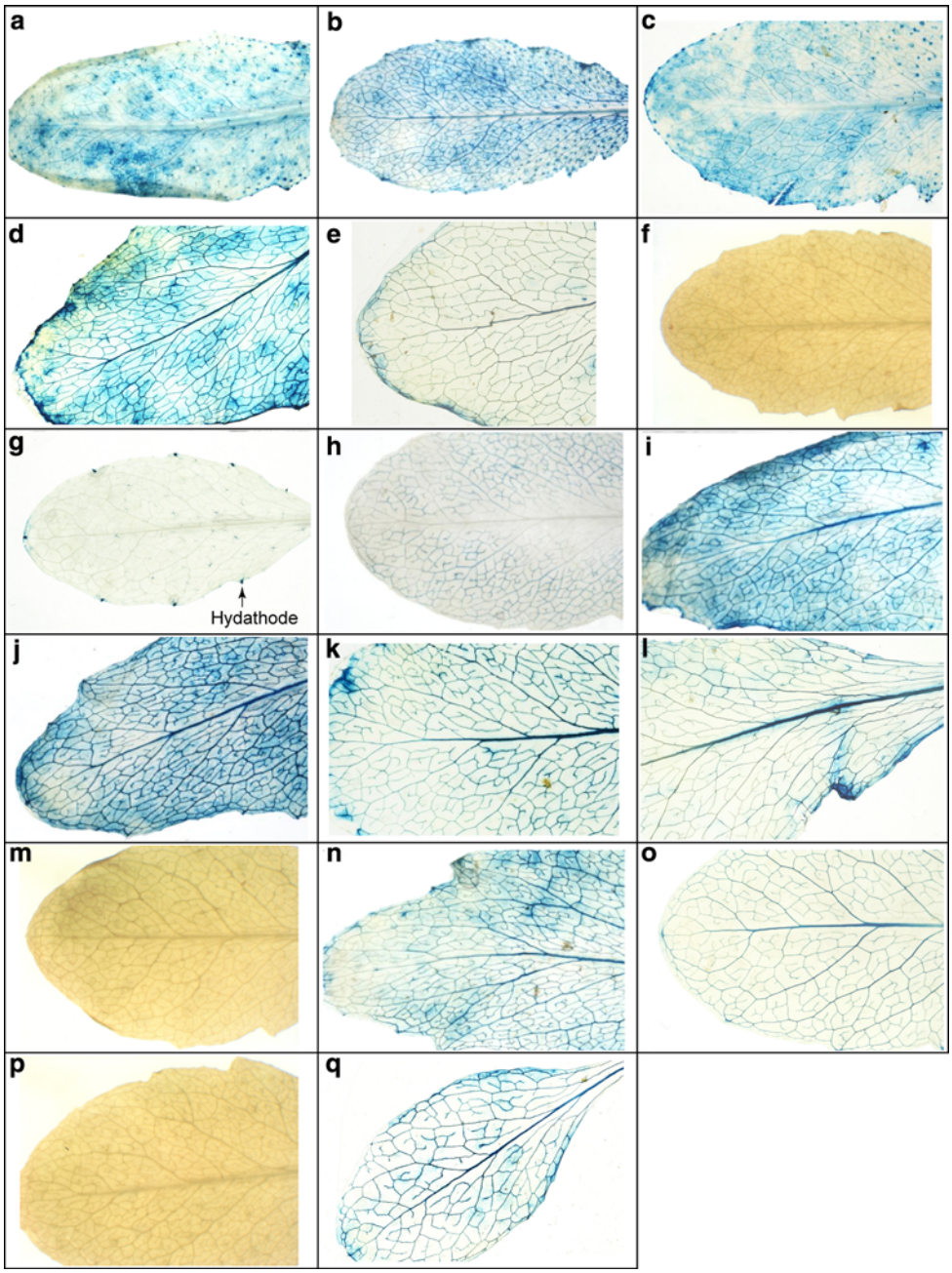

Fig. 9 Expression patterns in rosette leaves of *LAC1p–LAC17p::GUS* from preanthesis to stage 1 plant development. **a–q** Staining patterns for *LAC1p–LAC17p::GUS*, respectively, as in Fig. 6

these being water secreting points near leaf tips/margins. Xylem vessels usually exit in hydathodes, releasing their contents (ions, metabolites and proteins) under high root pressure, a process known as guttation (Pilot et al. 2004). Expression for *LAC6p::GUS, LAC13p::GUS* or *LAC16p::GUS* was, however, not evident in rosette leaves (Fig. 9f, m, p).

For cauline leaves, expression patterns of *LAC1p::GUS–LAC3p::GUS*, *LAC10p::GUS*, *LAC11p::GUS*, *LAC14p::-GUS*, *LAC15p::GUS* and *LAC17p::GUS* were somewhat similar staining mostly in the vasculature (Fig. 10a–c, j, k, n, o, q), with faint staining also noted for *LAC5p::GUS* and *LAC9p::GUS* (Fig. 10e, i). Expression of both *LAC4p::-GUS* and *LAC12p::GUS* was, by contrast, mainly in trichomes (Fig. 10d, l), whereas *LAC7p::GUS* was expressed in both trichomes and hydathodes (Fig. 10g). No expression of *LAC6p::GUS*, *LAC8p::GUS*, *LAC13p::GUS* or *LAC16p::GUS* was detected (Fig. 10f, h, m, p).

GUS-staining in inflorescence stems

The *Arabidopsis* stem has a collateral type of vascular bundle, with xylem developing inside and phloem outside of the procambium. Vascular bundles (*vb*) are arranged in a

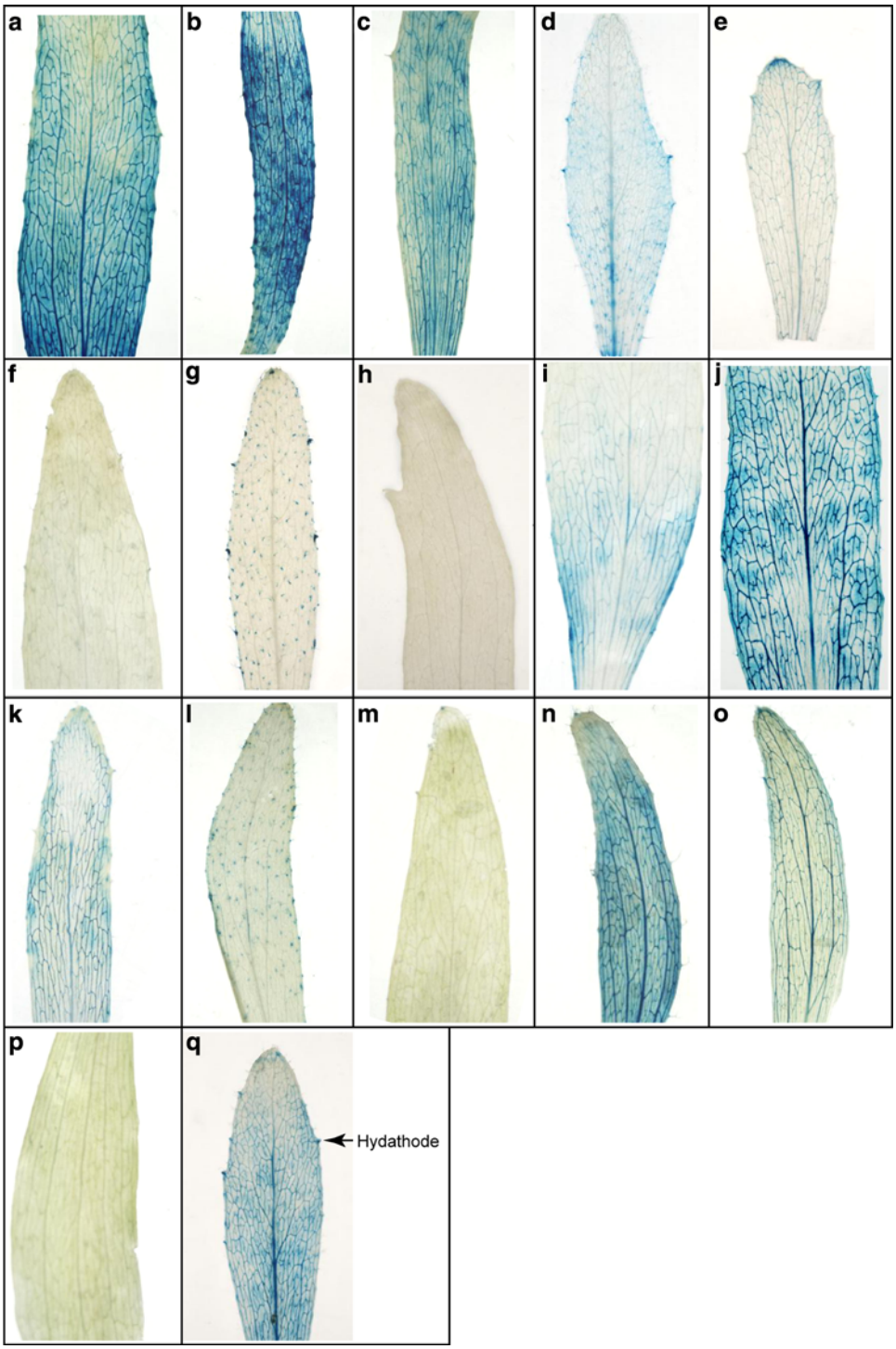

Fig. 10 GUS staining of *LAC1p–LAC17p::GUS* in cauline leaves from the onset of stage 1 to stage 2 plant development. **a–q** Staining patterns for *LAC1p–LAC17p::GUS*, respectively, as in Fig. 6

concentric ring, separated by the interfascicular cambium, which are differentiated and lignified during anthesis and onset of stage 1. Also in the interfascicular region, three to four layers of young interfascicular fibers (*if*) begin to develop with thick cell walls having modest levels of lignin. By stage 2, lignification is more conspicuous in the *if* and *vb* regions along the entire stem. Finally, by stage 3, the secondary vascular system is present throughout the stem (Altamura et al. 2001; Patten et al. 2010).

Staining of *LAC1p::GUS*, *LAC5p::GUS*, *LAC11p::GUS*, *LAC15p::GUS* and *LAC17p::GUS* was noted in stem xylem and both vascular/interfascicular cambia (Fig. 11a, e, k, o, q). However, for the latter cell types, expression of *LAC2p::GUS* and *LAC12p::GUS* was also evident

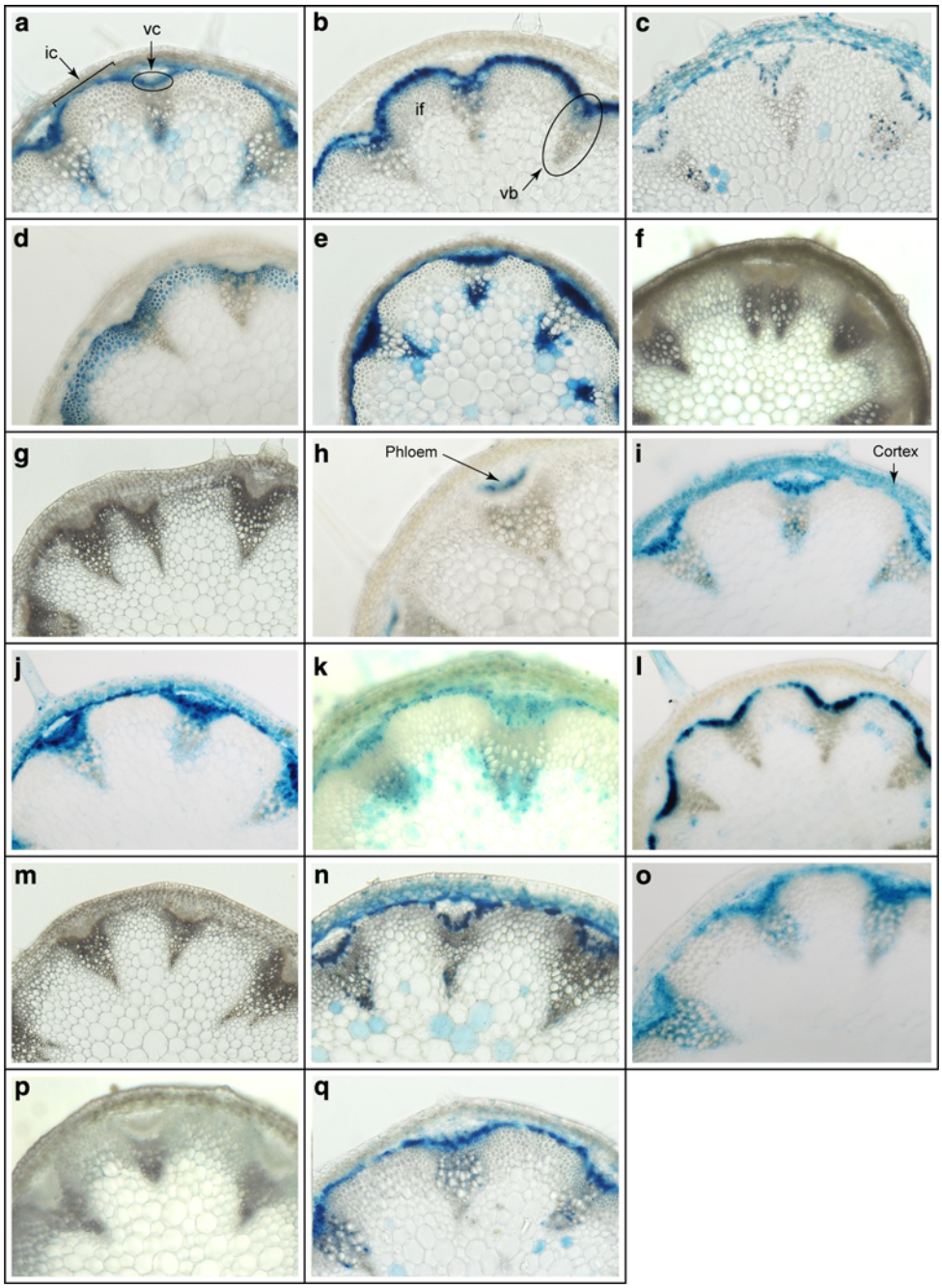

Fig. 11 GUS staining of *LAC1p–LAC17p::GUS* in the stem cross-sections from anthesis to stage 2. **a–q** Staining patterns for *LAC1p–LAC17p::GUS*, respectively, as in Fig. 6. *ic* Interfascicular cambium; *if* interfascicular fibers; *vc* vascular cambium; *vb* vascular bundle

(Fig. 11b, l). Prominent staining in *if* and xylem fibers (*xf*) was additionally observed for *LAC4p::GUS* (Fig. 11d), whereas *LAC9p::GUS*, *LAC10p::GUS* and *LAC14p::GUS* expression was observed in cambium, xylem and cortex (Fig. 11i, j, n). *LAC3p::GUS* staining was additionally observed in cortex, with sporadic vascular cambium staining (Fig. 11c). Expression of *LAC8p::GUS* was highly cell-specific, with staining mostly noted in the phloem region (Fig. 11h). No expression of *LAC6p::GUS*, *LAC7p::GUS*, *LAC13p::GUS* and *LAC16p::GUS* was observed (Fig. 11f, g, m, p).

GUS-staining in floral organs

In *Arabidopsis*, flowers usually develop from the floral meristem at the terminal position of the main inflorescence stem. The floral meristem produces four whorls of sepals, petals, stamens, and carpels (Smyth et al. 1990), where sepals and petals constitute vegetative organs and function in protecting inner reproductive organs during early floral development. The stamen constitutes the male reproductive organ and comprises a vascular filament and anther-bearing pollen grains, whereas the carpel is the female reproductive

organ containing three regions: an apical stigma, style, and a basal ovule bearing ovary. The stigma contains papillary cells with roles in pollen self-compatibility and aids in pollen tube penetration that eventually enters the ovary and fertilize the ovules. The ovary is an elongated cylinder that later in development forms silique valves, which are made of medial and lateral vascular bundles with heavily lignified xylem tissues.

GUS-staining in flowers was analyzed from anthesis until the end of stage 2, with expression of 14 laccases detected (Fig. 12). Staining in the style and vasculature of sepals, petals and stamen filaments was noted for *LAC1p::GUS*, *LAC2p::GUS*, *LAC4p::GUS*, *LAC5p::GUS*, *LAC9p::GUS*, *LAC10p::GUS*, *LAC14p::GUS*, *LAC15p::GUS* and *LAC17p::GUS* (Fig. 12a, b, d, e, i, l, n, o, q). Additionally, *LAC3p::GUS–LAC5p::GUS* expression was noted in the stigmatic papillae (Fig. 12c–e), whereas *LAC7p::GUS*, *LAC11p::GUS* and *LAC12p::GUS* were faintly expressed in style and stamen filaments (Fig. 12g, k, l). By contrast, prominent *LAC8p::GUS* expression was restricted to pollen

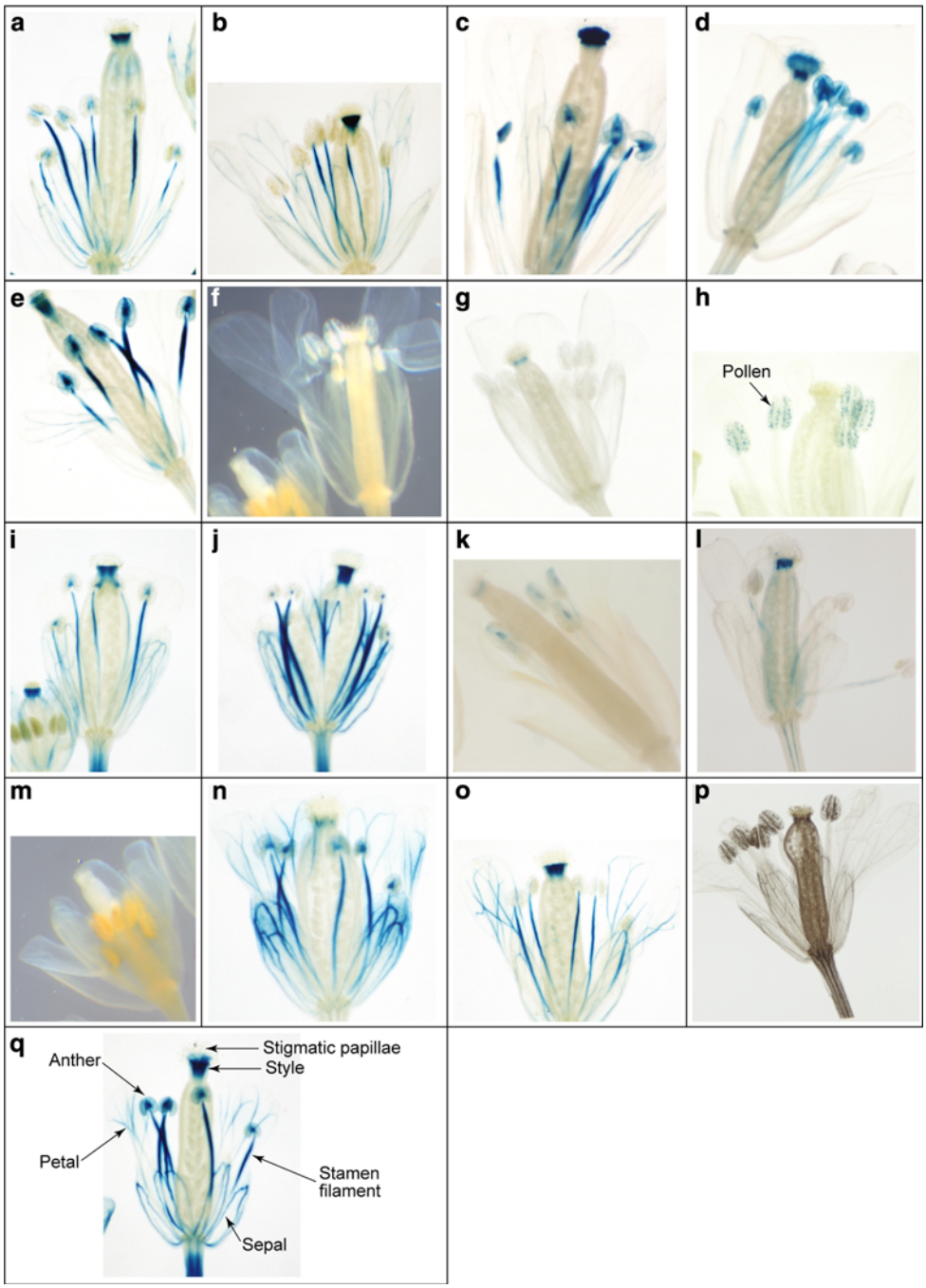

Fig. 12 GUS staining patterns of *LAC1p–LAC17p::GUS* in floral organs at anthesis to stage 2. **a–q** Staining patterns for *LAC1p–LAC17p::GUS*, respectively, as in Fig. 6

grains (Fig. 12h). For *LAC6p::GUS*, *LAC13p::GUS* and *LAC16p::GUS* staining was not observed (Fig. 12f, m, p).

GUS-staining in siliques and seeds

Soon after fertilization, at about 4–5 weeks growth corresponding to stage 1, development of fruit, the fertilized gynoecium, occurs. For *Arabidopsis*, this produces dry dehiscent fruits called "siliques". Morphologically, each has two carpels joined by a central replum with fertilized ovules (seeds) attached. Carpel walls are made up of three layers collectively known as valves, with lignification in the inner en*b* layer considered to contribute to silique dehiscence as fruits dry by stage 3. Seeds are attached to the replum through funiculi that act as a channel in providing nutrients during early stages of seed development (Haughn and Chaudhury 2005).

In siliques, staining was observed for 14 laccases (Fig. 13). Specifically, for *LAC1p::GUS–LAC5p::GUS*, *LAC8p::GUS–LAC12pGUS*, *LAC15p::GUS* and *LAC17p::GUS* expression was noted in the replum and abscission zone (Fig. 13a–e, h–l, o, q). Staining was also conspicuous in the embryo for *LAC7p::GUS* and *LAC14p::GUS* (Fig. 13g, n), whereas staining of *LAC2p::GUS*, *LAC4p::GUS*, *LAC12p::GUS* and *LAC15p::GUS* was evident in valves (Fig. 13b, d, l, o). Again, no *LAC6p::GUS*, *LAC13p::GUS* and *LAC16p::GUS* expression was noted (Fig. 13f, m, p).

Staining in seed coats (testa) was observed for *LAC4p::GUS* and *LAC15p::GUS* (Fig. 14). Specifically, for *LAC4p::GUS*, staining was observed in cell wall seed coats, especially the columella (Fig. 14a), an intracellular volcano-shaped central dome-like structure present in the outer integument, and mainly consisting of mucilage and a reinforced thickened secondary cell wall (Western et al. 2000). In the seed coat, *LAC15p::GUS* staining was also very prominent (Fig. 14b), and spatially distinct from *LAC4p::GUS* (Fig. 14a).

Comparison of expression data with *in silico* databases

Laccase gene expression profiles were evaluated from publicly available microarray databases (Hruz et al. 2008; Fig. 15), and compared with both RT-PCR and promoter GUS-fusion expression data. Detectable expression patterns for *LAC2*, *LAC4*, *LAC5*, *LAC10–LAC12* and *LAC17* were noted using all three approaches, with the others showing some inconsistencies. In terms of inconsistent expression patterns, *LAC1* expression was evident in seedlings, roots, leaves and flowers, while by RT-PCR its expression was noted in similar tissues except leaves

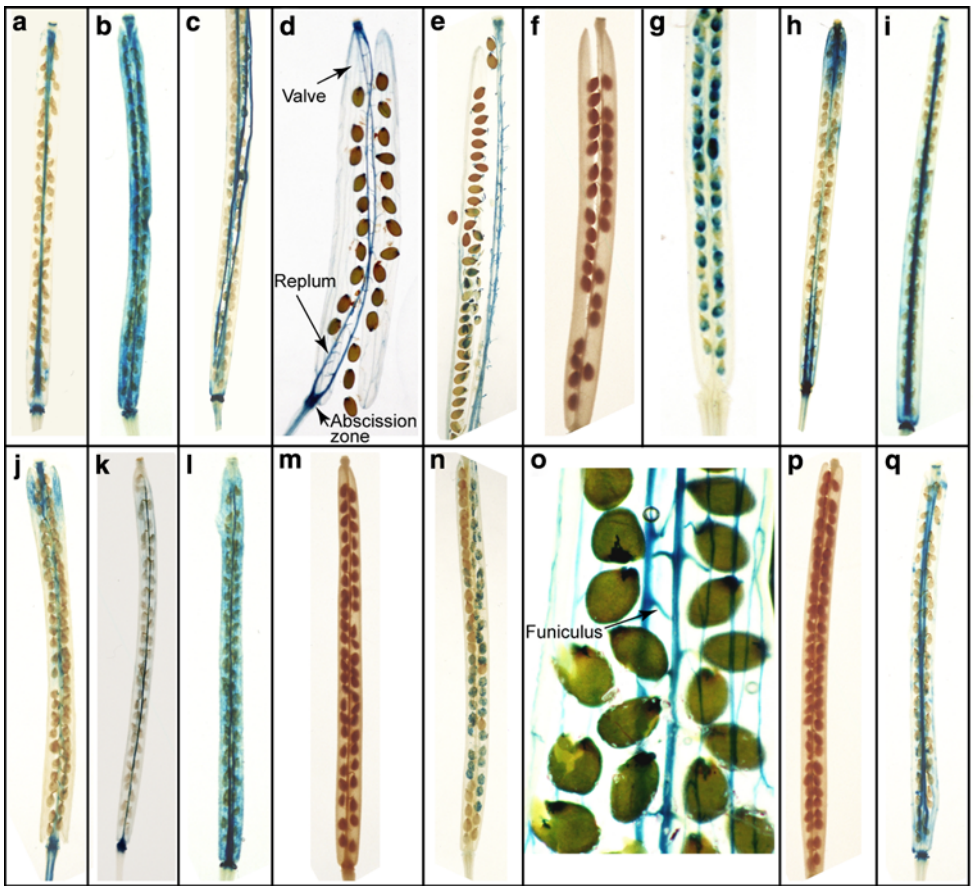

Fig. 13 Expression of *LAC1p–LAC17p::GUS* in the siliques from the onset of stage 1 to stage 2. **a–q** Staining patterns for *LAC1p–LAC17p::GUS*, respectively, as in Fig. 6

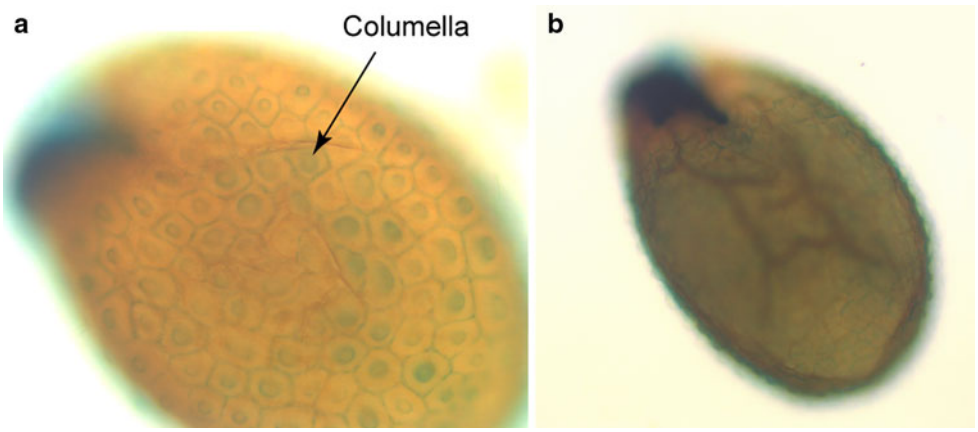

Fig. 14 Staining of *LAC4p::GUS* and *LAC15p::GUS* in the mature seeds. **a** *LAC4p::GUS* staining in the columella of the seed coat. **b** *LAC15p::GUS* staining in cell walls of the seed coat. In the hilum region, staining was observed for both *LAC4p::GUS* and *LAC15p::GUS*

(Fig. 15). *LAC1* expression was, however, observed in all tissues by promoter-GUS fusions. While *LAC3* expression was documented in seedlings, roots, leaves and reproductive organs using the Genevestigator database (Fig. 15), by RT-PCR, its expression was, however, detected in seedlings, roots, stems and reproductive organs, whereas with GUS staining *LAC3* was found in all tissues. Both *LAC6* and *LAC8* expression levels were noted in seedlings and reproductive organs, with *LAC8* expression also evident in rosette leaves according to microarray data (Fig. 15). By contrast, *LAC8* expression was additionally observed in cauline leaves and siliques using RT-PCR, with *LAC8p::GUS* staining noted in all tissues except for the cauline leaves. Using both RT-PCR and promoter GUS-fusions, *LAC6* expression was not detected in any tissues. Furthermore, microarray data indicated that *LAC7* and *LAC13* expression profiles were somewhat similar to *LAC3*. On the other hand, by RT-PCR, *LAC7* expression was evident only in roots and reproductive organs, with *LAC13* noted only in the former. The *LAC7p::GUS* expression pattern was also similar to that reported in databases, whereas *LAC13p::GUS* staining was not noted in any tissues examined. Microarray data also indicated that *LAC14* expression was not detectable in any tissues (Fig. 15), whereas using RT-PCR, its expression was evident mainly in reproductive organs and by GUS-fusions in all tissues. Both databases and RT-PCR also indicated expression of *LAC15* in roots and reproductive organs, while *LAC15p::GUS* staining was again evident in all tissues. Additionally, although microarray data indicated very low signal values for *LAC16* in roots (Fig. 15), its expression was not detected in any tissues by RT-PCR and GUS-fusions.

Changes in laccase gene expression (>4×) in response to stress stimuli using microarray databases

Laccase transcript expression patterns in response to various biotic/abiotic stimuli were assessed using publicly available microarray expression datasets to gain insight into potential physiological roles (Table 3; Toufighi et al. 2005). While several laccases responded to various stimuli, only those with at least fourfold change are discussed herein. Interestingly, *LAC6*, *LAC13* and *LAC16,* where no expression was detected in the present study, responded to a range of stimuli. *LAC6* was down-regulated in response to *Heterodera schachtii* (cyst nematode). It was also the only laccase gene up-regulated under turnip mosaic virus (TuMV) treatment indicative of a potential role in plant-pathogen interactions, although by contrast only *LAC17* was down-regulated to TuMV and *Botrytis cinerea* treatments, suggesting its potential role in pathogenesis of these organisms. *LAC16* responded to various abiotic stimuli with expression being either up-regulated (genotoxicity, UV-B, nutrient and hormone) or down-regulated (salt, drought, UV-B, wounding and heat). Besides *LAC16, LAC12* was the only other laccase up-regulated under high glucose (3%) treatment. Of the remaining laccase genes expressed, several responded to various stimuli. For example, *LAC14* was up-regulated at early stages in response to cold, osmotic and salt stresses, and down-regulated under drought and oxidative treatments. Under both oxidative and heat stress, only *LAC1* was up-regulated, however, and it was also one of the up-regulated genes under osmotic, drought and UV-B treatments. *LAC2* was the only laccase down-regulated by various hormone treatments. On the other hand, several laccases were up-regulated under nutrient [iron deficiency (*LAC4*, *LAC7*, *LAC11* and *LAC17*) and nitrate starvation (*LAC2* and *LAC5*)] as well as ABA (*LAC5* and *LAC12*) treatments. By contrast, *LAC13* expression was observed only when plants were subjected to nitrate starvation and ABA treatment. *LAC17* had a peculiar regulation pattern under iron deficiency with expression up-regulated near root hair regions and down-regulated near root tip zones, whereas *LAC5* expression was down-regulated near root hair regions.

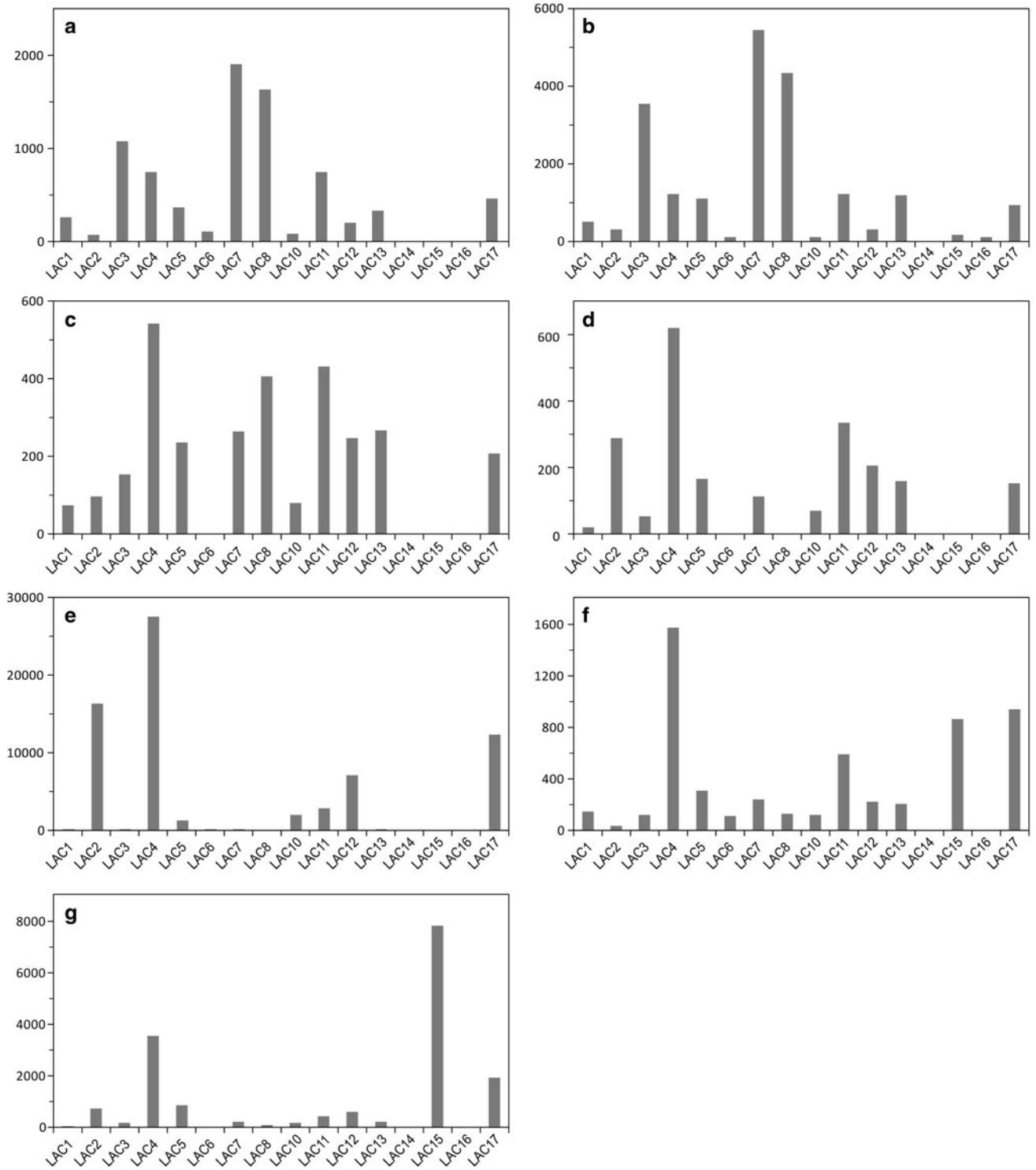

Fig. 15 Putative gene expression profiles of the laccase multigene family across different organs as inferred from Genevestigator. *Y*-axis represents linear signal values after normalization and the *X*-axis represents the gene numbers of *Arabidopsis* laccases. **a** Seedlings; **b** roots; **c** rosette leaves; **d** cauline leaves; **e** stems; **f** flowers; and **g** siliques

Putative DNA regulatory elements in laccase promoters

Laccase promoter sequences were analyzed for potential DNA regulatory *cis*-elements using bioinformatics tools available in PLACE (Higo et al. 1999) and Athena (O'Connor et al. 2005) databases. This resulted in detection of various putative *cis*-elements, including the TATA box for basal transcription initiation (Fig. 16). All 17 laccase

Table 3 *In silico* expression profiling of laccases in response to biotic/abiotic stimuli

Treatments[a]	Up-regulated[b]	Down-regulated[b]
Cold (4°C)	Roots and shoots: ***LAC14*** (30 min)[c]	Shoots: *LAC6* (24 h)[c]
Osmotic (300 mM mannitol)	Roots: ***LAC14*** (30 min) and *LAC12* (3 h)	Roots: *LAC2* (6 h)
	Shoots: *LAC1* (3 h) and *LAC2* (24 h)	Shoots: *LAC6* (12 h) and *LAC10* (24 h)
Salt (150 mM NaCl)	Roots: ***LAC14*** (30 min) and *LAC8/9* (3 and 6 h)	Roots: *LAC10* (3 h) and *LAC16* (6 h)
Drought (16 day seedlings to 15 min dry air stream)	Shoots: *LAC1* (3 h) and *LAC15* (24 h)	Shoots: ***LAC16*** (15 min) and ***LAC14*** (30 min)
		Roots: *LAC2* and *LAC6* (6 h)
Genotoxic (1.5 μg/ml bleomycin + 22 μg/ml mitomycin)	Shoots: ***LAC15*** (30 min)	Roots: *LAC2* (24 h)
	Roots: *LAC2* and *LAC16* (3 h)	
Oxidative (10 μM methyl viologen)	Shoots: *LAC1* (3 h)	Shoots: ***LAC14*** (30 min) and *LAC12* (6 h)
UV-B	Shoots: *LAC1* and *LAC16* (3 h)	Shoots: ***LAC16*** (30 min)
Wounding (punctured with pins)	Shoots: ***LAC8/9*** (30 min) and *LAC1* (3 h)	Shoots: ***LAC16*** (15 min) and *LAC17* (12 h)
Heat (38°C)	Shoots: *LAC1* (3 h)	Shoots: ***LAC16*** (15 min) and *LAC10* (3 h)
		Roots: *LAC2* (4 h)
Nutrient		
3% glucose for 4 h	Whole plant: *LAC12* and *LAC16*	
Iron deficiency for 24 h	Roots (near root hair zone): *LAC4*, *LAC7*, *LAC11* and *LAC17*	Roots (near root hair zone): *LAC5*
		Roots (~150 to 350 μM above root tip): *LAC17*
Nitrate starvation	Leaf: *LAC2*, *LAC5*, *LAC13* and *LAC16*	
Hormone	Whole plant	Whole plant
10 μM ABA	*LAC5*, *LAC12*, *LAC13* and *LAC16* (3 h)	*LAC15* (3 h)
10 μM ACC	***LAC16*** (30 min)	
10 nM Brassinolide		*LAC2* (3 h)
1 μM IAA		*LAC2* (3 h)
10 μM Salicylic acid		*LAC2* (3 h)
1 μM Zeatin	*LAC16* (3 h)	*LAC2* (3 h)
Biotic		
Botrytis cinerea		Leaf: *LAC17*
Heterodera schachtii		Roots: *LAC6*
Turnip mosaic virus		
Stage 1		Leaf: *LAC17*
Stage 3	Leaf: *LAC16*	

Expression values were compiled using the BAR Expression Browser tool available at http://bar.utoronto.ca and Genevestigator databases

ABA abscisic acid; *ACC* 1-aminocyclopropane-1-carboxylic acid; *IAA* indole-3-acetic acid

[a] All treatments except hormone were performed on 16 day seedlings (Col-0). Hormone treatments were performed on 7-day old seedlings

[b] Only genes with ≥fourfold changes in expression were considered significant

[c] Time points at which tissues were harvested: 15, 30 min, 1, 3, 4, 6, 12 and 24 h. Early (15 and 30 min) response genes indicated in bold

promoter sequences had putative binding sites for MYB and/or MYC families of transcription factors (TFs), as well as ARF binding site and T-box promoter motifs and W-Box elements, although relative positions of transcription start site varied (Fig. 16). Of these, MYB and MYC TFs are commonly found in promoters of dehydration-responsive and phenylpropanoid pathway genes (Abe et al. 2003), and specifically laccase promoter regions indicated enriched binding sites (*P* value: $<10^{-4}$; statistical significance of over-represented TF binding sites) for MYB4 and MYB1AT TFs. The ARF binding site is also mostly present in promoters of early auxin responsive genes (Ulmasov et al. 1999), whereas the T-box promoter motif is one of the *cis*-elements found in promoters of light-regulated genes (Chan et al. 2001). The W-Box elements, by contrast, are considered to interact with salicylic

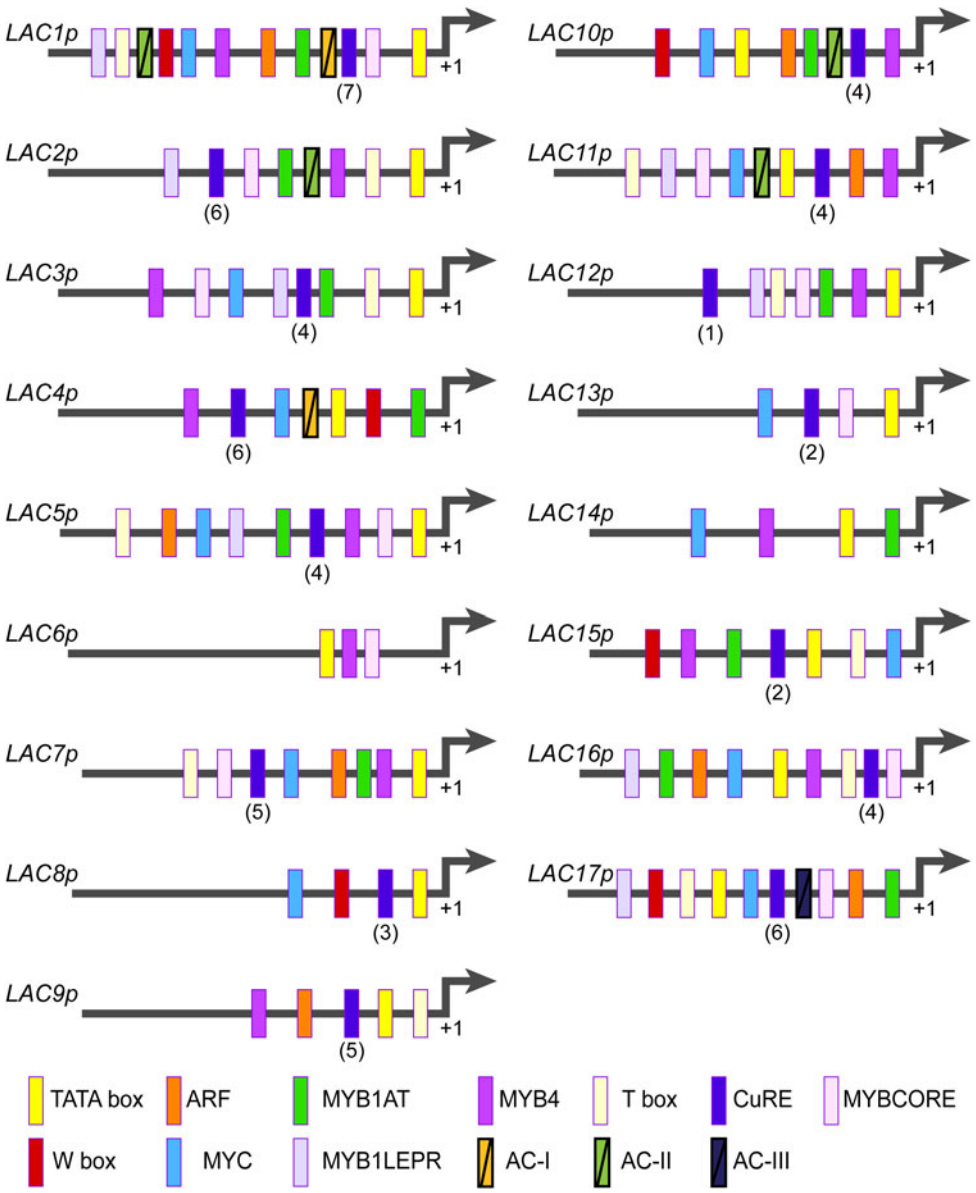

Fig. 16 Schematic representation of putative *cis* elements in laccase promoter sequences. The *cis* elements were analyzed using Athena and copper response elements (CuRE) using PLACE. For CuRE, the number of instances the GTAC motif is present is indicated in *parenthesis*. The transcription start site was shown by an *arrow*. The figure is not drawn to scale and the positions of the elements are relative to the transcription start site (+1)

acid-induced WRKY DNA binding proteins in mediating pathogen defense or wounding (Chen and Chen 2002). Additionally, promoter sequences of 15 laccases, except *LAC6*/*LAC14*, contained GTAC motifs when analyzed by Signal Scan at PLACE (shown in parenthesis in Fig. 16). These elements are thought to be recognized by copper response regulators (CRR1) in mediating target gene expression in responses to Cu levels, and are thus considered responsible for copper homeostasis in plants (Cardon et al. 1999).

Promoter analysis also indicated presence of potential *cis* AC elements, these being common regulatory elements in promoters of genes preferentially expressed in vascular tissues (Hatton et al. 1995). These AC elements, which are of three classes (AC-I, ACCTACC; AC-II, ACCAACC; and AC-III, ACCTAAC), are binding sites for the MYB transcription factor family and preferentially regulate various genes in phenylpropanoid metabolism expressed in stem vasculature (Raes et al. 2003). A search for such elements in *Arabidopsis* laccase promoters indicated that six laccases (*LAC1*, *LAC2*, *LAC4*, *LAC10*, *LAC11*, and *LAC17*) have at least one AC element, and expression was noted for these laccases in the vasculature. Specifically, *LAC1* and *LAC4* have motifs indicative of AC-I, whereas *LAC1*, *LAC2*, *LAC10* and *LAC11* have AC-II and *LAC17* has an AC-III element. However, other laccases (*LAC3*, *LAC5*, *LAC8*, *LAC9*, *LAC12*, *LAC14*, and *LAC15*) expressed in vascular bundles do not have such elements in their corresponding promoter sequences, suggesting involvement of additional regulatory factors. Besides *cis*-elements, promoter sequences of a few laccases (*LAC1*, *LAC5*, *LAC10*, *LAC13*, and *LAC15*) suggest

presence of non-coding regions for small RNAs, thereby reflecting additional complexity in defining a given gene's promoter region (Lu et al. 2005).

Evidence of epigenetic modifications in laccase promoter and coding sequences

Epigenetic modifications, such as DNA methylation and histone modifications, regulate gene expression during normal growth/development by influencing chromatin structure/organization (Zilberman et al. 2007). It was thus instructive to compile and analyze microarray data to further understand regulation of *Arabidopsis* laccases, as several sequences can have one or some form of epigenetic modification. In particular, histone modifications, such as H3K27me3, were found in promoter and/or coding regions of 11 laccases (*LAC1–LAC5*, *LAC7*, *LAC9*, *LAC10*, and *LAC14–LAC16*) and H3K9ac in four laccases (*LAC1*, *LAC3*, *LAC14*, and *LAC15*). Additionally, evidence for DNA methylation in coding sequences of eight laccases (*LAC1–LAC3*, *LAC6*, *LAC11*, *LAC13*, *LAC15*, and *LAC17*) was observed.

Discussion

Of the 17 *Arabidopsis* laccases none have a known physiological function, except for *TT10*/*LAC15*, and then only in the seed coat (Pourcel et al. 2005). We thus considered it useful to establish actual patterns of gene expression for each laccase using RT-PCR and promoter-GUS analyses, in order to gauge unique and/or potentially redundant gene expression patterns, particularly since the former is valuable in establishing future approaches to identify precise biochemical/physiological processes. This study is thus the first report in addressing cell-specific expression patterns of the *Arabidopsis* laccase multigene family at both mRNA and promoter levels.

Inducible expression patterns of genes not detected by GUS staining

From GUS expression analyses, three *Arabidopsis* laccases (*LAC6*, *LAC13*, and *LAC16*) were not detected under "normal" growth/development conditions, although expression was detected under various stress conditions (Table 3). In particular, given the response of *LAC16* gene expression to various stress conditions, this might be a potential target for exploring in future its precise physiological role(s), e.g., its up-regulation during TuMV infestation suggests a potential role in host-pathogen interactions. On the other hand, *LAC6* expression was down-regulated under biotic (*H. schachtii*, a cyst nematode) treatment, implying a potential role in nematode pathogenesis. Interestingly, *LAC13* expression was one of several laccases up-regulated under nitrate starvation and ABA treatments, albeit with physiological roles proper presently unknown.

Unique expression patterns

Unique cell type-specific expressions were observed for a few laccases, such as *LAC4*, *LAC7*, and *LAC8* (Table 4). The latter has a unique cell-type expression pattern in germinating seeds, with expression evident in root cap and meristem potentially suggesting a role in root protection/penetration during early stages of root growth/development. *LAC8* expression was also uniquely noted in leaf primordia, suggesting a potential role in very early stages of leaf development. Interestingly, it was additionally uniquely expressed in phloem of inflorescence stem tissues and in pollen grains of floral organs. In this context, a loblolly pine laccase (*Lac 7*) was previously reported as uniquely expressed in immature pollen cones with putative roles in pollen cone growth or pollen formation proposed (Sato et al. 2001). *LAC8,* as well as *LAC1* and *LAC9*, were also up-regulated very early upon wounding as noted from microarray databases (Table 3), suggesting potential roles in wound responses.

LAC7, by contrast, was uniquely expressed in root hairs of early germinating seeds and hydathodes, where expression of other members was not evident. Although not essential for plant growth and development, root hairs are considered to have nutrient sensing roles (Shin et al. 2005). Provisionally, such a role might be envisioned for *LAC7* given recent reports of its regulation by miRNA in response to Cu-deficiency and Cu-excess (Abdel-Ghany and Pilon 2008), but this needs to be established. Additionally, its expression was up-regulated besides three other laccases (*LAC4*, *LAC11*, and *LAC17*) near the root hair zone under iron-deficiency conditions.

Arabidopsis LAC4 was uniquely expressed in stem tissue *if*, anther walls, and seed coat columella. These expression patterns are apparently consistent with other studies reporting *LAC4* gene expression in *Arabidopsis* stems being highly correlated with cellulose biosynthesis genes during secondary cell wall synthesis (Persson et al. 2005). Furthermore, its expression was up-regulated in *Arabidopsis* transgenic plants over-expressing NST1, a transcription factor required for anther dehiscence by regulating secondary cell wall thickenings (Mitsuda et al. 2005). In addition, MYB58, a potential transcriptional factor regulating genes involved in secondary cell wall formation in stem *xf*, was reported to directly bind AC *cis*-elements in the *LAC4* promoter region in vitro (Zhou et al. 2009), suggesting it may be required for LAC4 expression. This observation is also consistent with *LAC4p::GUS*

expression in stem fibers noted in this study. On the other hand, LAC4 has highest sequence identity (64.2%) to the poplar lac3 putatively involved in maintaining *xf* structural integrity in stem tissues (Ranocha et al. 2002). While expression patterns of *LAC4p::GUS* observed in our study, and the close homology to poplar lac3, might suggest a putative role for LAC4 in secondary cell wall synthesis, once again the biochemical/physiological roles are currently unknown. An *Arabidopsis lac4* T-DNA insertional knockout mutant had, however, little to no effect on secondary cell wall formation in stem fibers (Brown et al. 2005), perhaps indicative of other functions in these cell types. *LAC4p::GUS* expression in thickened secondary cell walls of columella may also suggest a potential role in seed coat cell wall thickening and integrity. However, its expression was also up-regulated near root hair zones in response to iron deficiency as inferred from microarray databases. Accordingly, detailed biochemical/genetic studies are necessary to clearly define function(s) of LAC4 in these cell types.

Moderate redundant expression patterns

In certain cell types, such as mature root cap and meristem (*LAC3*, *LAC4* and *LAC8*), cortex of inflorescence stems (*LAC3*, *LAC9*, *LAC10* and *LAC14*), stigmatic papillae (*LAC3*, *LAC4* and *LAC5*), anther walls (*LAC4*, *LAC5* and *LAC17*), and embryos (*LAC5*, *LAC7* and *LAC14*), a limited level of apparent redundancy of laccase expression was observed (Table 4). Accordingly, genetic analyses of individual and multiple knockout mutant lines for specific laccases may potentially find utility to probe *in planta* physiological/biochemical roles in these cell types, e.g., expression of laccases in stigmatic papillae potentially have roles in pollen penetration and pathogen defense as several genes involved in secondary metabolism have been identified in these tissues (Edlund et al. 2004). It would also be of interest to establish physiological role(s) laccases play in embryogenesis, given there are no reports of these being expressed in this non-lignified cell type in other plants.

Expression patterns of TT10/LAC15

Pourcel et al. (2005) reported that the gene locus corresponding to a transparent testa mutant (*tt10*) is a laccase (*LAC15*) highly expressed in seed coats. A putative role in oxidative polymerization of flavonoids in the seed coat was thus proposed, although no "true" biochemical substrate for TT10/LAC15 was reported. The brown coloration of the *Arabidopsis* seed coat is due to oxidized proanthocyanidins in the first layer of the inner integument (ii1) and the first layer of outer integument (oi1) during seed maturation (Pourcel et al. 2005). Interestingly, the pale yellow seed coat color of the *tt10* mutant apparently changes to a brown color upon standing, suggesting oxidative polymerization can also result, albeit at a slower process (Fig. 17). Indeed, in our study, *LAC15p* driven GUS expression was evident in seed coats, specifically in cell walls where oxidized proanthocyanidins accumulate. However, its expression was detected in other tissues suggesting different physiological/biochemical roles, e.g., expression was noted in the hilum of early germinating seeds and vasculature of aerial/underground organs where proanthocyanidin accumulation has not been reported. This observation was consistent with other studies that reported its expression through mRNA profiling in various tissues besides seeds (McCaig et al. 2005; Pourcel et al. 2005; Abdel-Ghany and Pilon 2008). Regulation of *LAC15* very early in response to genotoxic treatment might perhaps suggest a potential role in DNA damage/repair. Also, only *LAC15* was down-regulated in response to ABA, a plant hormone involved in dormancy.

Table 4 Unique to putatively redundant expression patterns in different organs and tissues types as inferred from GUS data

Organ	No redundancy (only 1 laccase expressed)	Moderately redundant tissue types (2–4 laccases expressed)	Highly redundant tissue types (>4 laccases expressed)
Roots	Root hairs (*LAC7*)	Cortex (*LAC7* and *8*)	Vascular bundles
		Root tip meristem and root cap (*LAC3*, *4* and *8*)	
Leaves	Hydathodes (*LAC7*)		Vasculature and trichomes
Stems	Interfascicular fibers (*LAC4*)	Cortex (*LAC3, 9, 10* and *14*)	Xylem, vascular and interfascicular cambium
	Phloem (*LAC8*)		
Flowers	Pollen grains (*LAC8*)	Anther walls (*LAC4*, *5* and *17*)	Filaments, vasculature of sepals and petals, xylary bundles of style
		Stigmatic papillae (*LAC3*, *4* and *5*)	
Siliques			Replum, valves, funiculi
Seeds	Seed coat cell walls (*LAC15*)	Embryo (*LAC5*, *7* and *14*)	
	Seed coat columella (*LAC4*)		

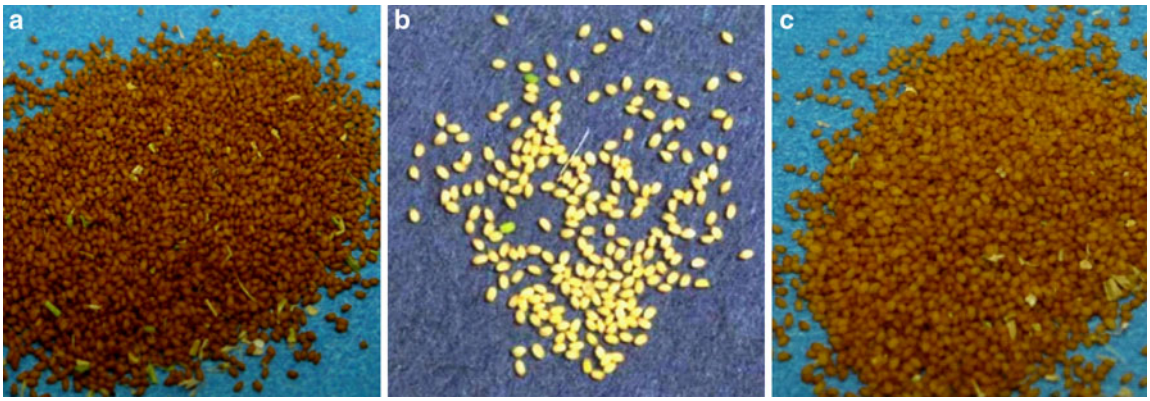

Fig. 17 Effect of storage on seed coat color in *lac15/tt10* mutant seeds. The wild type seed (**a**) is typically *dark brown color* with mutant seeds showing pale *yellow seed color* (**b**) from freshly harvested plants. However, upon long-term storage, the seed coat color of these mutants slowly changes to near wild type *color* (**c**)

Highly overlapping expression patterns

Multiple expressions of several laccases in a given organ/tissue was previously reported in various plant species using either cDNA cloning, Northern hybridization, or protein isolation approaches (LaFayette et al. 1999; Ranocha et al. 1999; Sato et al. 2001; Gavnholt et al. 2002; McCaig et al. 2005; Caparrós-Ruiz et al. 2006). In this study, multiple *Arabidopsis* laccase expression was also noted in all tissues examined, e.g., in roots and the vasculature (*LAC1–LAC5*, *LAC9–LAC12*, *LAC14*, *LAC15*, and *LAC17*), this being consistent with Birnbaum et al. (2003) studies on genome-wide expression profiles from these organs. Expression of multiple laccases has also been reported in maize roots, albeit with no defined physiological/biochemical roles proposed (Caparrós-Ruiz et al. 2006). Additionally, recent reports of lignans accumulating in *Arabidopsis* roots (Nakatsubo et al. 2008) suggest putative roles of "root expressed" laccases in lignan biosynthesis. This provisional conclusion results from isolation of laccases along with the (auxiliary) dirigent proteins (DPs) in *F. intermedia* and *Arabidopsis* (Davin et al. 1997; Pickel et al. 2010; Vassão et al. 2010), where the DPs mediate stereoselective bimolecular phenoxy coupling in lignan biosynthesis in presence of oxidases such as laccases (Davin et al. 1997). Another possible role can be envisioned in metal homeostasis given recent identification of several laccases being regulated by microRNAs (miRNAs) in response to Cu levels (Abdel-Ghany and Pilon 2008). Although a potential role in *ex planta* phytoremediation (Wang et al. 2004) has also been envisaged for a root-specific laccase from cotton plant when expressed in *Arabidopsis*, its "true" physiological role was not identified. Thus, until sufficient biochemical/genetic evidence are obtained, the physiological significance of multiple laccases being expressed in roots presently remains unclear.

Global transcript profiling of *Arabidopsis* primary stems previously indicated that six laccases (*LAC2*, *LAC4*, *LAC5*, *LAC11*, *LAC12*, and *LAC17*) were expressed in vascular tissues (Ehlting et al. 2005; Persson et al. 2005; Sibout et al. 2005). In our study, in addition to these, expression of other laccases (*LAC1*, *LAC3*, *LAC8–LAC10*, *LAC14*, and *LAC15*) was observed. While the physiological/biochemical roles of "stem expressed" *Arabidopsis* laccases remain undefined, our study is consistent with several reports of multiple laccases being expressed in stem tissue xylem from various plant species (LaFayette et al. 1999; Ranocha et al. 1999; Sato et al. 2001; Gavnholt et al. 2002). Additionally, in rosette leaves, overlapping expression patterns in vasculature were observed for several laccases (*LAC2*, *LAC4*, *LAC5*, *LAC9–LAC12*, *LAC14*, *LAC15*, and *LAC17*). Expression of laccases in leaves of other plant species, mainly gymnosperms and monocots, was previously reported with no definitive roles proposed other than an unproven role in lignification (Sato et al. 2001; Caparrós-Ruiz et al. 2006). Furthermore, response of several laccases to various stimuli as inferred from publicly available microarray databases suggest a few have potential roles under stress conditions. For instance, down-regulation of *LAC17* in response to biotic treatments might imply a role in pathogen defense and the very early response of *LAC14* to various abiotic stimuli might suggest a potential role under such conditions.

Overlapping expression patterns of most laccases in flowers was also observed mainly in sepal vasculature (*LAC1–LAC3*, *LAC5*, *LAC9*, *LAC10*, *LAC14*, *LAC15*, and *LAC17*), petals (*LAC2*, *LAC10*, *LAC14*, *LAC15*, and *LAC17*), filaments and style (*LAC1–LAC5*, *LAC9–LAC12*, *LAC14*, *LAC15*, and *LAC17*). Additionally, in siliques, most laccases were expressed in the replum and abscission zones, regions where lignification occurs (i.e., *LAC1–LAC5*, *LAC8–LAC12*, *LAC14*, *LAC15*, and *LAC17*). Expression of other laccases in reproductive organs was

previously reported in other plant species, mainly monocots (Gavnholt et al. 2002) and gymnosperms (Sato et al. 2001) but with no precise physiological roles defined. Hence, the physiological significance of expression of multiple laccases in these tissues/organs remains a mystery.

Approaches for analyzing gene expression patterns

RT-PCR and promoter-GUS analyses combined with *in silico* gene profiling were also used to more comprehensively understand expression patterns of laccases. However, each approach has its pros and cons. Analysis of promoter-GUS fusions helps provide insights into spatial and temporal expression patterns at the cell/tissue level, but possible involvement of downstream sequences from the transcription start site, such as introns and 5′- and 3′-untranslated regions in regulating gene expression, were excluded in this analysis (Sieburth and Meyerowitz 1997). On the other hand, while RT-PCR and microarray profiling report actual presence of gene transcript, genes expressed at very low levels in a few cell types may not be detected.

While results obtained using these approaches generally agreed with each other, various inconsistencies were noted. Correlation in gene expression profiles across different studies showed some variations which might be due to biological, technical and technological differences as noted earlier for some multigene families and genes that are expressed at low levels including laccases (McCaig et al. 2005; Becnel et al. 2006; Abdel-Ghany and Pilon 2008). Such variations can partly arise due to regulation of gene expression either at transcriptional, post-transcriptional levels, or a combination of same (Zhang et al. 2007). Also the possibility of cell type-specific *trans*-acting factors mediating tissue-specific expression of laccases cannot be excluded. Further, false-positive and false-negative rates were recently observed across a number of genes between their promoter activities and in vivo RNA transcript levels (Cooper et al. 2006). Such discrepancies necessitate multiple approaches to clearly delineate expression profile of any gene.

Regulation of *Arabidopsis* laccases

Promoter analysis indicated presence of *cis* copper response elements in promoter regions of 15 laccases (except *LAC6* and *LAC14*) suggesting responsiveness to Cu levels. Cu is an essential micronutrient required in various redox-mediated physiological processes in higher plants with both high and low levels of Cu affecting plant growth/development (Burkhead et al. 2009). Cu is also a cofactor for several enzymes such as plastocyanin (PC), cytochrome *c* oxidases (CCO), Cu, Zn superoxide dismutases (SOD), polyphenol oxidases, ethylene receptors, laccases, phytocyanins, ascorbate oxidases and Cu diamine oxidases involved in diverse physiological processes. Some of these, such as PC and CCO, are essential for plant growth/development, and plants have evolved mechanisms of regulating Cu levels. In general, Cu levels *in planta* tend to be higher in roots followed (in order) by flowers, siliques/seeds, leaves (mostly trichomes) and shoots (Burkhead et al. 2009). It is interesting that laccase redundancy, as observed by our expression analysis, closely correlates with Cu levels, although the precise physiological significance remains unclear. Furthermore, several laccases (*LAC2–LAC4*, *LAC7*, *LAC12*, *LAC13* and *LAC17*) are negatively regulated under Cu-deficient conditions by miRNAs (Abdel-Ghany and Pilon 2008), with the latter being 21–24 ribonucleotides acting at the post-transcriptional level in mediating target gene expression during plant growth and development (Jones-Rhoades et al. 2006). In plants, miRNA either act as negative regulators by degrading target mRNA in a tissue and development specific manner or in response to environmental stimuli. The presence of both Cu response elements and target sites for miRNA in several laccases might suggest lacasses are tightly regulated in response to Cu levels; however, as before, a precise functional significance is presently unclear. In future, specific miRNAs targeting laccases can be utilized as a tool to knock-down several laccases in order to gain insight into their putative functional roles *in planta*.

Bioinformatics analysis also indicated *cis*-natural antisense transcripts (NAT) at the loci of two laccase genes (*LAC6* and *LAC17*). NATs are endogenous antisense transcripts transcribed from the same gene loci as a sense coding transcript but on a complementary strand, and are regulatory RNA molecules known to participate in several steps of gene regulation including RNA editing, splicing, DNA methylation and RNA interference; however, their biological functions are yet to be explored (Wang et al. 2005). Furthermore, epigenetic modifications such as DNA methylation (*LAC1–LAC3*, *LAC6*, *LAC11*, *LAC13*, *LAC15* and *LAC17*) and histone modification were found in promoter or transcribed regions of several *Arabidopsis* laccases. In particular, histone modifications such as H3K27me3 (*LAC1–LAC5*, *LAC7*, *LAC10* and *LAC14–LAC17*) and H3K9ac (*LAC1*, *LAC3*, *LAC14* and *LAC15*) reportedly regulating gene expression by influencing chromatin architecture (Zhang et al. 2007). H3K27me3 histone modification and DNA methylation were also reported as a major silencing mechanism in regulating gene expression during normal plant growth/development (Zhang et al. 2007; Zilberman et al. 2007), with H3K9ac modification positively effecting gene expression. Interestingly, a combination of these modifications at any given development stage can influence gene expression (Zhang et al. 2007; Zilberman et al. 2007).

Arabidopsis laccase sequence characteristics

When deduced amino acid sequences of *Arabidopsis* laccases were analyzed for post-translational modifications, all laccases indicated potential sites for *N*-glycosylation and phosphorylation with several also containing potential *O*-glycosylation sites. Numerous functions have been proposed for protein glycosylation, such as ensuring protein folding, stability (Ceriotti et al. 1998), cell-wall formation (Kang et al. 2008) and also essential in maintaining activity (Graziani et al. 1990). Phosphorylation is a reversible post-translation modification mostly involved in protein–protein interactions and signaling cascades, but can also regulate gene expression in response to various stimuli, protein activity and localization (Hunter 2000). Accordingly, further studies are required to delineate the functional significance and variability of glycosylation and phosphorylation sites among the different laccases.

Phylogeny and expression patterns

Molecular phylogeny indicated that *Arabidopsis* laccases cluster into six arbitrary clades; however, expression patterns did not show similar clustering. Interestingly, *Arabidopsis* laccases of group 5 (LAC7 and LAC8) exhibited very unique cell-type expression patterns perhaps suggesting diverse physiological roles. Additionally, LAC8 and LAC9 of group 5 are the only two putative high redox potential plant laccases that have Ile as an axial ligand instead of Leu that is most common. As their biochemical characteristics are lacking, the significance of this residue variation is not known. Based on sequence features, phylogeny indicated low redox potential plant laccases cluster together, i.e., in group 4. In addition, *Rhus* and to a certain extent *Acer,* also of group 4, are the only two plant laccases that were extensively investigated in terms of biochemical and spectroscopic properties (Reinhammar 1970; Weymouth et al. 1993). Three low redox potential *Arabidopsis* laccases (LAC6, 14 and 15) also cluster in this group although their expression patterns differed markedly. Diverse physiological roles, such as lignification (*Acer*) (Sterjiades et al. 1992), wound healing (*Rhus*) (Yoshida 1883), oxidative polymerization of flavonoids (*Arabidopsis*, LAC15) (Pourcel et al. 2005) and *ex planta* phytoremediation (cotton) (Wang et al. 2004) have also been proposed for laccases of group 4. Laccases from other clusters were mainly proposed to have putative roles in lignification and maintenance of cell wall integrity, but genetic evidence thus far gives no indication of such roles and hence their biochemical functions remain unclear. It was evident from phylogeny that laccases are encoded as multigene families across most plant species; however, their functions are still enigmatic and unresolved. Until sufficient biochemical data is available for plant laccases, the significance of such clustering is unclear.

In conclusion, this study showed laccases are expressed beyond zones of lignification and a few exhibit either unique cell type-specific expression patterns and/or are inducible. These findings do, however, provide new clues into determining precise physiological roles of laccases which are still a mystery in terms of roles *in planta*.

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