Laccases Direct Lignification in the Discrete Secondary Cell Wall Domains of Protoxylem1[W][OPEN]

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Plants precisely control lignin deposition in spiral or annular secondary cell wall domains during protoxylem tracheary element (TE) development. Because protoxylem TEs function to transport water within rapidly elongating tissues, it is important that lignin deposition is restricted to the secondary cell walls in order to preserve the plasticity of adjacent primary wall domains. The Arabidopsis (Arabidopsis thaliana) inducible VASCULAR NAC DOMAIN7 (VND7) protoxylem TE differentiation system permits the use of mutant backgrounds, fluorescent protein tagging, and high-resolution live-cell imaging of xylem cells during secondary cell wall development. Enzymes synthesizing monolignols, as well as putative monolignol transporters, showed a uniform distribution during protoxylem TE differentiation. By contrast, the oxidative enzymes LACCASE4 (LAC4) and LAC17 were spatially localized to secondary cell walls throughout protoxylem TE differentiation. These data support the hypothesis that precise delivery of oxidative enzymes determines the pattern of cell wall lignification. This view was supported by $lac4lac17$ mutant analysis demonstrating that laccases are necessary for protoxylem TE lignification. Overexpression studies showed that laccases are sufficient to catalyze ectopic lignin polymerization in primary cell walls when exogenous monolignols are supplied. Our data support a model of protoxylem TE lignification in which monolignols are highly mobile once exported to the cell wall, and in which precise targeting of laccases to secondary cell wall domains directs lignin deposition.

During all stages of a land plant’s life cycle, lignified secondary cell walls provide critical mechanical properties for water transport and the upright growth habit. Water conduction in xylem tissue occurs in tracheary elements (TEs), whose secondary cell walls are composed of cellulose, hemicelluloses, and the phenolic lignin polymer. Lignification of the secondary cell wall imparts strength, rigidity, and water impermeability to the polysaccharide components. The deposition of lignin in secondary cell walls is developmentally regulated, and different cell types generate distinctive secondary cell wall patterns. Protoxylem TEs, for example, form annular or helical secondary wall thickenings, whereas metaxylem TEs deposit secondary cell walls in a reticulated or pitted pattern (Esau, 1965). Protoxylem TEs form in young elongating plant tissues and therefore the restriction of lignin deposition to the annular or helical secondary cell wall thickenings, as opposed to the intervening primary cell walls, is crucial to allow continued axial elongation. The mechanisms restricting lignin deposition specifically to secondary cell wall thickenings have not been identified.

Monolignol (lignin monomer) biosynthesis occurs in the cytosol in close proximity to the endoplasmic reticulum (ER), because the pathway includes both cytosolic- and ER-localized enzymes (Bonawitz and Chapple, 2010). Multienzyme complexes, potentially anchored at specialized subdomains on the ER surface, have been postulated to channel phenolic metabolite production during lignification (Chen et al., 2011; Bassard et al., 2012). Both ATP-binding cassette (ABC) transporters of monolignols and proton-dependent transporters of monolignol glucosides have been proposed as monolignol export mechanisms (Ehlting et al., 2005; Miao and Liu, 2010). Genetic evidence does not support a role for monolignol glucosides as direct precursors of lignin in Arabidopsis (Arabidopsis thaliana; Chapple et al., 2012). Monolignol transport activity has been reported for heterologously expressed AtABCG29, which was capable...
of transporting p-coumaryl alcohol in yeast (Saccharomyces cerevisiae) cells and isolated plasma membrane vesicles (Alejandro et al., 2012). Localized monolignol biosynthesis and/or export are hypothesized to be mechanisms for concentrating monolignols in secondary, but not primary, cell wall domains in protoxylem TEs.

An alternative mechanism for controlling the pattern of lignin deposition could be the site-specific localization of the enzymes required for monolignol polymerization within the cell wall. Monolignol polymerization occurs by oxidative combinatorial coupling of monolignols, catalyzed by cell wall-localized laccases and/or peroxidases (Vanholme et al., 2012). A peroxidase, originally isolated from xylogenic Zinnia elegans cell cultures, has been localized to secondary cell walls, which demonstrates the spatial association of oxidative enzymes with lignin deposition (Sato et al., 2006), but the function of this peroxidase in vivo has not been demonstrated. Recent analysis of the Arabidopsis LACCASE4 (LAC4), LAC11, and LAC17 genes showed that loss of function of these genes had a dramatic effect on lignification of metaxylem and fiber cells in inflorescence stems (Berthet et al., 2011; Zhao et al., 2013). However, the roles of either laccases or peroxidases in controlling the spatial pattern of cell wall lignification in protoxylem TEs have not been investigated.

Studying protoxylem TE development is challenging because they are located deep within root or shoot tissues, but the identification of key transcriptional regulators that activate differentiation of protoxylem TEs has led to the development of a genetically tractable experimental system. In this system, the activity of VASCULAR NAC DOMAIN 7 (VND7), a NAC domain transcription factor that regulates protoxylem TE cell fate (Kubo et al., 2005), is paired with an inducible transcriptional activator, viral protein16 (VP16) coupled to a glucocorticoid receptor (GR; Yamaguchi et al., 2010). Upon induction with dexamethasone (DEX), nearly all cells in Arabidopsis seedlings transdifferentiate into protoxylem TE-like cells (Yamaguchi et al., 2010). This system allows protoxylem development to be studied in diverse mutant backgrounds, and permits the expression and high-resolution imaging of proteins tagged with GFP during xylogenesis.

This study examines the cellular mechanisms underlying the discrete lignification of spiral or annular secondary cell walls in protoxylem TEs. With GFP tagging and live-cell monitoring in the VND7-VP16-GR lines, we tested whether monolignol biosynthetic enzymes, putative monolignol transporters, or laccases were specifically localized in lignifying cell wall domains. Fluorescently tagged monolignols were used to assay whether monolignols would polymerize in secondary cell wall domains in laccase loss-of-function mutants, as well as in primary cell walls of overexpression lines. Whereas earlier studies identified which members of the laccase gene family were active in lignification of metaxylem vessel and fiber cell types in the Arabidopsis stem (Berthet et al., 2011; Zhao et al., 2013), this work directly addresses the role of these oxidative enzymes in directing lignin polymerization in lignified secondary cell wall domains adjacent to un lignified primary cell walls of the protoxylem TE.

RESULTS

Live-Cell Imaging of Lignification of Induced Protoxylem TEs

Although the genes encoding lignin biosynthetic enzymes are up-regulated in plant lines expressing the
master transcription factor VND7, the presence of lignin in the induced protoxylem TEs has not been established. To examine lignification in induced protoxylem TEs, cell wall phenolic auto-fluorescence was profiled using two-photon excitation microscopy, with UV excitation (350–370 nm) and emission (420–540 nm; Fig. 1). In hypocotyls from uninduced control seedlings, the only intrinsic fluorescence from lignified secondary cell walls came from endogenous protoxylem TEs in the central vascular cylinder (Fig. 1A). However, within 48 h of VND7 induction, nearly 100% of hypocotyl epidermal cells of VND7-VP16-GR seedlings transdifferentiated into protoxylem TEs with highly fluorescent secondary cell walls (Fig. 1, B and C).

In order to verify that the UV auto-fluorescence observed in VND7-induced TEs is dependent on monolignol biosynthesis, the expression of two key monolignol biosynthetic genes, CINNAMATE-4-HYDROXYLASE (C4H) or CINNAMYL-CoA REDUCTASE1 (CCR1), was suppressed through the use of targeted artificial microRNAs (Schwab et al., 2006). Plants constitutively overexpressing the C4H or CCR1 microRNA recapitulated the range of phenotypes previously described in c4h and ccr1 mutants (Jones et al., 2001; Ruegger and Chapple, 2001; Schilmiller et al., 2009; Thévenin et al., 2011), including irregular xylem and a marked reduction in lignin deposition (Pro-35S:miRNA C4H lines, Supplemental Fig. S1; and Pro-35S:miRNA CCR lines, Smith et al., 2013). Constitutive expression of the C4H or CCR1 microRNA during VND7-induced TE formation did not inhibit secondary cell wall formation (Fig. 1, D and E, insets) but two-photon microscopy revealed severely reduced UV autofluorescence in these cell wall domains (Fig. 1, D and E), compared with the wild type (Fig. 1C). Together, these results indicate that the autofluorescence in secondary cell walls of VND7-induced protoxylem TEs is from lignin, because it is dependent not only on the function of the general phenylpropanoid pathway enzyme C4H but also on the function of the...
monolignol-specific pathway enzyme CCR1. This establishes the VND7-inducible protxylem system as a valid model for analysis of cell wall lignification.

Dynamic Rearrangement of Monolignol Biosynthetic Enzymes during Protopxyelem TE Differentiation

One hypothesis to explain specific deposition of lignin only in spiral cell wall thickenings of protxylem TEs is that monolignol biosynthetic enzymes in these cells may be preferentially organized around sites of secondary cell wall deposition. To directly image the subcellular distribution of C4H during protxylem TE differentiation, a C4H-GFP translational fusion was stably transformed into VND7-VP16-GR plants. Similar C4H-GFP fusions were shown to be functional in plants and localized to the ER when transiently expressed in tobacco (Nicotiana tabacum) leaf cells (Ro et al., 2001; Bassard et al., 2012). In epidermal cells of uninduced C4H-GFP-transformed VND7-VP16-GR plants, C4H-GFP was clearly localized to the reticulate network of tubular ER, as confirmed by colocalization with an ER-localized red fluorescent protein (RFP)-HDEL marker (Supplemental Fig. S2). Induction of VND7-mediated protxylem TE differentiation had a profound impact on the pattern of C4H-GFP distribution. Live-cell imaging was used to visualize the C4H-GFP-labeled ER pattern, as it changed from an open tubular ER network early in development (Supplemental Fig. S2, A–C) to a denser lamellar pattern concentrated between developing secondary cell wall domains (Fig. 2B; Supplemental Fig. S2, D–G). Continuous ER strands were parallel to, and generally excluded from, domains directly below developing secondary cell wall thickenings, which were observed in bright-field microscopy (Fig. 2A). Evidence of ER exclusion below secondary cell wall domains was also observed in endogenous root protxylem TEs using transmission electron microscopy (TEM) of high-pressure frozen/freeze-substituted samples (Fig. 2C). The changes of C4H-GFP distribution during VND7-induced TE differentiation are therefore associated with overall changes in ER distribution and morphology, but do not appear to be attributable to relocalization of C4H-GFP to specific regions of the ER.

Because CCR1 is specific to monolignol biosynthesis, its subcellular distribution was tracked during VND7-mediated TE lignification. First, the function of the CCR1-GFP fusion protein was tested by complementing ccr1−/− loss-of-function mutants, in which CCR1-GFP rescued the irregular xylem phenotype of ccr1−/− mutants (Supplemental Fig. S3). CCR1 is predicted to be a cytosolic enzyme and CCR1-GFP localization exactly matched cytosolic-localized (cyt) GFP (Fig. 2, D and E). During all stages of VND7-mediated TE differentiation, both CCR1-GFP and cytGFP signals were observed throughout the cytoplasm, with no bias toward secondary cell wall domains (Fig. 2, F–I). Thus, spiral secondary cell wall lignification is not correlated with the spatial distribution of the C4H and CCR1 monolignol biosynthetic enzymes.

ABC Transporters Localize to All Plasma Membrane Domains during Protopxyelem TE Differentiation

ABC11, ABCG29, and ABCG33 transporters were previously identified as candidate monolignol exporters (Ehlting et al., 2005; Kaneda et al., 2011; Alejandro et al., 2012). To test the hypothesis that these putative monolignol transporters could have a restricted plasma membrane localization adjacent to secondary cell wall domains, GFP translational fusions of ABCB11, ABCG29, and ABCG33 were transformed into VND7-VP16-GR plants. When VND7-mediated protxylem TE differentiation was induced, all fluorescently tagged ABC transporters were localized to plasma membranes (Fig. 3, Supplemental Fig. S4). Yellow Fluorescent Protein (YFP)-tagged ABCG11, which was previously shown to function as a plasma membrane-localized cuticular wax and cutin exporter (Bird et al., 2007), was included as a positive control for plasma membrane localization (Supplemental Fig. S4). Secondary cell wall domains were counterstained with the cellulose-specific Pontamine Fast Scarlet 4B (S4B) dye (Fig. 3, B and C; Anderson et al., 2010). When the GFP channel signal was merged with that of the cellulose counterstain, it was clear that transporters were equally distributed along both secondary and adjacent primary wall regions (Fig. 3, A and C). This uniform plasma membrane distribution of candidate monolignol ABC transporters persisted until the initiation of programmed lignification.

Figure 3. ABC transporters are evenly localized in plasma membranes during protxylem TE differentiation in VND7-VP16-GR seedlings. A, Maximum projection image of cotyledon epidermal cells undergoing protxylem TE differentiation, expressing GFP-ABCG33. B, Cellulose stain, Pontamine S4B, counterstained image of cells shown in A, highlighting secondary cell wall domains. C, Median optical slice of induced protxylem TE, showing invaginations of GFP-ABCG33 (green), counterstained with Pontamine S4B (red) demonstrating secondary cell wall domains, with inset image of the optical cross section of one secondary wall thickening. D, Induced protxylem TE from plants expressing GFP-ABCG29, with inset showing plasma membrane localization over primary and secondary wall domains. Bars = 15 μm.
cell death, when fragmentation of the GFP-labeled plasma membranes was observed (Supplemental Fig. S5; Supplemental Movie S1). These results demonstrate that the candidate monolignol ABC transporters, including ABCG29 (Fig. 3D), are not preferentially excluded from, nor localized to, the plasma membrane domains facing developing secondary cell walls.

**Laccase-Dependent Monolignol Polymerization in Secondary Cell Wall Domains**

The results of the preceding experiments suggest that monolignols are likely produced and exported into the cell wall in a diffuse, rather than targeted, manner. If this model is correct, then exogenously supplied monolignols are predicted to be highly mobile throughout the cell wall matrix, and should only become polymerized within the secondary cell wall domains. The tools to assess this prediction were recently developed, in the form of fluorescently tagged monolignol analogs, such as γ-nitrobenzofuran (NBD)-tagged coniferyl alcohol (CA), that become polymerized into lignin via oxidase-mediated radical coupling (Tobimatsu et al., 2011). When applied in planta, these NBD-tagged probes were incorporated into the lignifying tissues in Arabidopsis and *Pinus radiata* plants (Tobimatsu et al., 2013).

The availability of NBD-tagged monolignols together with the VND7-induced protoxylem system provided the opportunity to document the lignification of discrete cell wall domains in individual protoxylem TEs. NBD-CA and untagged CA were applied together to induced VND7-VP16-GR seedlings and labeling of the differentiating TEs was monitored. To ensure that free monolignols were not simply being trapped in the polymer matrix after treatment with fluorescent monolignols, and that only the insoluble lignin polymer was being imaged, treated seedlings were thoroughly washed in 49% methanol:1% acetic acid at 45°C for up to 72 h to remove excess NBD-CA, prior to imaging.

Secondary cell wall domains, as identified by S4B staining for cellulose, were strongly labeled by NBD-CA treatment, whereas adjacent primary cell walls were not (Fig. 4, A–C, arrowheads and arrows, respectively). Similarly, in leaf tissue in which only a single leaf mesophyll cell had transdifferentiated into a protoxylem TE, incorporation of NBD-CA was restricted to the secondary cell walls of the transdifferentiated cell (Fig. 4, D–F). Collectively, these results demonstrate that monolignols can diffuse freely throughout the apoplast and become polymerized into lignin only in the secondary cell walls of differentiating protoxylem TEs.

If monolignols are exported to, and are mobile in, all cell wall domains, but become polymerized only in discrete secondary cell wall thickenings, then the presence of laccases and/or peroxidases in those spiral wall thickenings could determine the sites of lignin polymerization. The *LAC4* and *LAC17* genes are two of the most strongly differentially expressed genes in plant cells responding to increased VND7 or VND6 activity (Ohashi-Ito et al., 2010; Yamaguchi et al., 2010, 2011). In order to directly test the contribution of these laccases to the lignification patterns of protoxylem TE cell walls, the inducible VND7 construct was transformed into *lac4 lac17* double mutants. In contrast with strong NBD-CA incorporation in the secondary cell walls of induced protoxylem in the wild-type VND7-VP16-GR seedlings (Fig. 5A, arrowheads), *lac4*...
lac17 double mutants did not incorporate the fluorescent monolignols after induction of VND7 (Fig. 5C, arrowheads). At the same time, VND7-mediated secondary cell wall formation was not affected in lac4 lac17 mutants because secondary wall thickenings in both wild-type (Fig. 5B) and lac4 lac17 double mutants (Fig. 5D) were identical, as assessed by cellulose staining with Pontamine S4B, and immunofluorescent labeling of xylan using LM10 antibodies. These results demonstrate that although other secondary cell wall components such as glucuronoxylan or dense deposits of cellulose were present in the lac4 lac17 mutants, the polysaccharides were not sufficient to trap free monolignols in secondary cell walls in the absence of the required laccases.

The loss-of-function mutant analysis demonstrated that LAC4 and LAC17 were necessary for protoxylem lignification. In order to evaluate whether these laccase proteins are sufficient for lignin polymerization, we generated plant lines constitutively overexpressing LAC4 (Pro-35S: LAC4) or LAC17 (Pro-35S: LAC17) and tested whether primary cell walled cells in Arabidopsis seedlings would lignify. We predicted that the presence of LAC4 or LAC17 in a cell wall, even a primary cell wall, would be sufficient to enable cell wall lignification when monolignols were exogenously applied. When Pro-35S: LAC4 or Pro-35S: LAC17 seedlings were treated with exogenously supplied, fluorescently tagged monolignols, primary cell walls in cotyledon epidermal cells had strong fluorescence, thus indicating cell wall lignification (Fig. 5, E and F). By contrast, wild-type cotyledons did not incorporate the fluorescently tagged monolignols (Fig. 5, G and H). Taken together, it is evident that laccases are both necessary and capable of directing lignification in cell walls when monolignols are available.

LAC4 and LAC17 Localize to Secondary Cell Walls in Protoxylem TEs

If the presence of the enzymes responsible for oxidative coupling of monolignols is the key factor determining where lignin is deposited, then the spatial organization of these apoplastic proteins is predicted to be tightly controlled. To test this hypothesis, LAC4 and LAC17 were tagged with mCherry (ProLAC4: LAC4-mCherry and ProLAC17: LAC17-mCherry), a pH-stable monomeric RFP that can function in the acidic environment of the apoplast. The irregular xylem phenotype of lac4 lac17 double-mutant fluorescence stems was complemented by expression of the LAC4-mCherry construct, thus demonstrating that the LAC4-mCherry protein is functional in planta (Supplemental Fig. S3). In primary roots from lac4 lac17 seedlings expressing ProLAC4: LAC4-mCherry, the fluorescent signal was specifically detected.

Figure 5. LACs are necessary and sufficient to direct lignin polymerization in Arabidopsis cell wall domains. A, Maximum projection image of hypocotyl epidermal cells induced to transdifferentiate into protoxylem TEs in VND7-VP16-GR seedlings, showing NBD-CA fluorescence in the secondary cell walls (arrowheads). B, VND7-induced protoxylem TE showing the following: i, cellulose deposition (Pontamine S4B stain); ii, whole mount immunolocalization of xylan (LM10 antibody); and iii, merged images. C, Maximum projection image of epidermal cells induced to transdifferentiate into protoxylem TE, in VND7-VP16-GR seedlings in lac4 lac17 double-mutant backgrounds showing that NBD-CA was not incorporated into the secondary cell walls (arrowheads). D, VND7-VP16-GR induced protoxylem TE in lac4 lac17 double mutants showing identical deposition patterns for the following: i, cellulose (Pontamine S4B stain); ii, xylan (LM10 immunolocalization); and iii, merged images, as observed in VND7-VP16-GR alone shown in B. E and F, Constitutive expression of LAC4

(©) or LAC17 (©) catalyzes lignin polymerization of NBD-CA in primary cell walls of cotyledon epidermal cells. G, Control wild-type cotyledon epidermal cells do not incorporate NBD-CA into their cell walls. H, Corresponding bright-field image of control wild-type cotyledons with one cell outlined. Bars = 25 μm.
in endogenous TEs and was localized to the helical and pitted secondary cell wall thickenings of protoxylem and metaxylem TEs, respectively (Fig. 6, A and B). The expression and subcellular localization of LAC17-mCherry was identical to those observed for LAC4-mCherry, and is shown in Supplemental Figure S6.

To visualize the LAC4-mCherry pattern at high resolution, protoxylem was induced on the plant surface using VND7-VP16-GR plants transformed with the ProLAC4:LAC4-mCherry construct. The LAC4-mCherry signal was specifically localized to the developing secondary cell wall domains during the early stages of VND7-induced TE differentiation, as shown in a single optical section (Fig. 6, C and D). The concentration of LAC4-mCherry within developing secondary cell walls continued throughout TE differentiation and persisted after programmed cell death (Fig. 6E, maximum projection image). The pattern of LAC4-mCherry distribution was identical to the patterns of LM10 immunofluorescence labeling of glucuronoxylan in secondary cell walls (Fig. 6, F and G, immunolabeled sections and inset). The localization of LAC4 and LAC17 exclusively to secondary cell wall domains, and loss of monolignol incorporation in the lac4 lac17 double mutants, indicate that apoplastic targeting of these laccases plays a key role in the precise pattern of lignin deposition in protoxylem TEs.

**DISCUSSION**

The goal of this study was to identify mechanisms that allow protoxylem TEs to specifically deposit lignin in spiral cell wall thickenings, while the intervening primary cell walls remain lignin free. This pattern of secondary cell wall lignification is crucial for the ability of protoxylem TEs to facilitate water transport to young and rapidly expanding plant organs. The combination of two technologies (fluorescently tagged monolignols and the VND7-inducible protoxylem system in Arabidopsis) provided a platform to directly visualize the lignification process in an experimental system with rich transcriptomic and molecular genetic resources. The precise spatial association of fluorescent protein-tagged LAC (LAC4-mCherry and LAC17-mCherry) with the spiral wall domains demonstrated that these oxidative enzymes are localized to secondary cell walls. The loss of lignification in lac4lac17 mutants, and ectopic lignification in overexpression lines of LAC4 and LAC17, demonstrated that laccases are necessary and sufficient to direct lignin polymerization. By contrast, localization of GFP-tagged monolignol biosynthetic enzymes and putative monolignol transporter proteins in developing protoxylem TEs demonstrated that the spatial distribution of these components was not correlated with the lignified cell wall pattern.

The relevant laccases in this protoxylem system were predicted from transcriptomic studies that identified LAC4 and LAC17 as genes that were strongly differentially expressed in response to the master regulator transcription factor VND7 (Yamaguchi et al., 2010, 2011).
LAC11 is a third redundant component required for lignification in the fiber-rich stem, and this is consistent with the preferential activation of LAC11 expression by the fiber-specific transcription factor SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1 (Ohashi-Ito et al., 2010; Zhao et al., 2013). Thus, analyses of both gene expression and mutant phenotypes indicate that although the combination of LAC4, LAC17, and LAC11 support lignification in stems, LAC4 and LAC17 are the major players in protoxylem TE lignification.

Although clarification of the functional members of the lac gene family is interesting, it is the precise localization of these laccases to the spiral secondary cell walls of protoxylem TEs that is most relevant for the targeted lignification of these secondary cell wall domains. During protoxylem development, there are many Golgi bodies that pause at the microtubule-lined plasma membrane domain underlying the secondary cell wall (Wightman and Turner, 2008), presumably to deliver xylose, cellulose synthases, and cell wall proteins. LAC4-mCherry fluorescence was detected in nascent secondary cell wall thickenings from the earliest stages of secondary cell wall formation, suggesting a testable hypothesis that laccases are secreted simultaneously with secondary cell matrix polysaccharides. The molecular mechanisms underlying the specific targeting of secondary cell wall cellulose synthases, xylos, and laccases to the plasma membrane domains below developing secondary cell walls are still unknown. The high flux of vesicle delivery to the microtubule-lined plasma membrane domains may represent a transient reorientation of secretory vesicle flow during this stage of differentiation. A similar phenomenon was hypothesized to account for the directed delivery of plasma membrane proteins to the periadjacent cell wall during arbuscular mycorrhizal symbiosis in Medicago truncatula (Pumplin et al., 2012). Although a transient reorientation of secretory vesicle traffic is the simplest explanation, an alternative hypothesis of uniform secretion of laccases coupled to rapid remobilization toward secondary cell walls, resulting in the observed pattern of LAC4 and LAC17 localization, cannot be ruled out. The development of LAC4-mCherry and LAC17-mCherry proteins provides important tools to study these hypothesized targeting mechanisms.

The model that monolignols are exported out of the cell into all areas of the apoplast, rather than being selectively exported into secondary versus primary cell walls, is in agreement with the recent characterization of Caspary strip lignification in root endodermal cells. Lee et al. (2013) demonstrated that although plasma membrane-bound Caspary strip membrane domain proteins (CASP) function to organize the extracellular oxidative peroxidases specifically to lignify Caspary strips, other plasma membrane proteins, including the monolignol transporter ABCG29, are excluded from Caspary strip domains by the CASP complexes (Roppolo et al., 2011; Alejandro et al., 2012). Given the exclusion of ABCG29 from the lignifying domain of the Caspary strip, we hypothesized that a similar, or inverse, pattern might be observed in induced protoxylem TE expressing a variety of GFP-tagged putative monolignol transporters (Alejandro et al., 2012). However, the uniform localization of several different ABC transporters during protoxylem differentiation was not consistent with the hypothesis that localized monolignol export facilitates specific lignification patterns. A careful examination of the transcriptome of the VND7-induced seedlings (Yamaguchi et al., 2010, 2011) did not reveal increased expression of CASP, CASP-like proteins, or peroxidases. Instead, which cell domains lignify appears to be determined by the oxidative machinery and is independent of the source of monolignols, an observation compatible with the good neighbor hypothesis that cells surrounding lignifying TEs may actively contribute to TE lignification (Hosokawa et al., 2001; Tokunaga et al., 2005; Pesquet et al., 2013; Smith et al., 2013). However, future research may identify other components that may be limiting for the lignification process during protoxylem TE differentiation in addition to LAC4 and LAC17.

In lac4 lac17 mutants, the secondary cell wall polysaccharide environment remained intact, but this was not sufficient to create the secondary cell wall lignification pattern. These data indicate that differences in nanoscale cell wall polysaccharide environments between the pectin/xyloglucan-rich primary cell wall and the xylan-rich secondary cell wall do not contribute to trapping monolignols in protoxylem secondary cell wall domains. Instead, the presence of laccases in secondary cell wall domains appears to be a key feature, because LAC4 and LAC17 were both necessary for, and capable of, directing cell wall lignification.

### MATERIALS AND METHODS

#### Plant Growth

All Arabidopsis (Arabidopsis thaliana) plants were grown under 16-h-light/21°C and 8-h-dark/16°C conditions. Seeds were surface sterilized using chlorine gas generated in a closed container using 100 mL of bleach and 3 mL of concentrated HCl for 3 to 6 h. Seeds were subsequently plated on one-half-strength Murashige and Skoog (MS) medium (Sigma), kept in the dark at 4°C for 2 to 3 d, and subsequently moved to growth conditions. Pro-35S:RFP-HDEL plants and plastidid (Nelson et al., 2007) were obtained from the Arabidopsis Biological Resource Center (stock no. CD3 960). The lac4-2 lac17 lines were obtained from Richard Sibout and Lise Jouanin and were originally described in Berthet et al. (2011). Pro-35S:VND7-VP16-GR seedlings and plastidic were originally described in Yamaguchi et al. (2010). VND7 activity in 7- to 10-d-old seedlings was induced as described in Yamaguchi et al. (2010).

All transgenic plant lines were generated using ecotype Columbia-0 of Arabidopsis plants, Agrobacterium tumefaciens (strain GV3101), and the floral dip method. Primary transformants were plated on one-half-strength MS medium and selected for survival on 50 μg mL⁻¹ kanamycin, 25 μg mL⁻¹ hygromycin, and 25 μg mL⁻¹ Basta (DL-phosphinothricin; Bayer), or by spraying 10-d-old seedlings grown on soil with 120 μg mL⁻¹ Basta.

#### Cell Wall Labeling

Cellulose staining using Pontamine S4B (Sigma) was performed by incubating seedlings in 10 mg mL⁻¹ S4B solution in one-half-strength MS medium for 10 to 20 min and washing once in one-half-strength MS medium prior to imaging. Labeling cell wall lignin using NBD-CA was carried out as follows: a mixture of 0.5 mM NBD-CA (Tobimatsu et al., 2011, 2013) and 5 mM CA (Sigma) was added to liquid one-half-strength MS medium to a final concentration of
1 and 10 μM, respectively, together with 10 μM desmethasone (Sigma) for VND7 induction. VND7-VP16-GR seedlings were incubated in this induction solution for 4 to 48 h, and they were gently shaken under regular growth conditions (listed above). No differences in NBD-CA incorporation were observed when preinducing VND7 activity for 24 h prior to adding the NBD-CA mix. Seedlings were subsequently rinsed twice for 15 min in one-half-strength MS medium and fixed in 4% paraformaldehyde, 50 mM PIPES, 5 mM MgSO4, and 0.5 M EDTA solution overnight. Seedlings were subsequently washed twice in TBS plus Tween 20 (TBST; 10 mM Tris, pH 7.2, 0.5% NaN3, 0.2% Tween 20) solution. Seedlings were heated to 45°C for 4 to 72 h in 49% (v/v) methanol:1% (v/v) acetic acid solution with gentle shaking to wash away unincorporated NBD-CA. Seedlings were subsequently washed and rehydrated in a graded methanol series, mounted in one-half-strength MS medium, and imaged. For whole-mount immunolabeling, VND7-induced seedlings were fixed as described above and stored in TBST buffer at 4°C until hybridization. Fixed seedlings were incubated in 5% bovine serum albumin (Sigma) in TBST for 1 to 2 h at room temperature and were subsequently incubated with LM10 primary antibody (McCartney et al., 2005) at 1:50 dilution overnight at 4°C. Seedlings were rinsed three times in TBST solution for 5 min and were subsequently incubated in a 1:100 dilution of secondary anti-rat Alexa 488 antibody (Invitrogen) at room temperature for 1 h. After washing twice with TBST, samples were mounted onto slides and imaged. For immunolocalization of thick sections, the above procedure was performed on sectioned material from LR white resin infiltrated seedlings after high-pressure freezing and freeze substitution in glutaraldehyde and uranyl acetate. Sections were generated using a Leica Ultracut UC Ultramicrotome and were mounted on Teflon-coated glass slides (Electron Microscopy Sciences).

Microscopy and Image Analysis
For live-cell imaging, a Perkin-Elmer UltraView VoX spinning disk confocal mounted on a Leica DMi6000 inverted microscope and a Hamamatsu 9100-02 CCD camera were used with the following excitation and emission filters: GFP (488 and 525), YFP (514 and 540), and RFP (561 and 595). Samples were mounted in water and imaged using a Leica oil immersion 63× objective (1.4 numerical aperture, Plan-Apo). All images were processed using Volocity image analysis software (Improvision) and Photoshop software (Adobe Systems). For Supplemental Figure S3.

Phenotypic characterization of Pro35S:miRNA C4H plants.

Supplemental Figure S2. C4H-GFP localization and ER structure during VND7-induced protoplasty TE differentiation.

Supplemental Figure S3. Complementation of irregular xylem phenotypes of ccr1-134 and lac4 lac17 mutants with GFP or mCherry fusion proteins.

Supplemental Figure S4. ABC transporters are evenly localized in plasma membranes during protoplasty TE differentiation in VND7-VP16-GR seedlings.

Supplemental Figure S5. C4H-YFP and YFP-ABCG11 localization during protoplasty programmed cell death of transdifferentiated Arabidopsis epidermal cells.

Supplemental Figure S6. LAC17 localizes specifically to protoplasty TE secondary cell walls.


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