Defining the Diverse Cell Populations Contributing to Lignification in Arabidopsis Stems

Rebecca A. Smith, Mathias Schuetz, Steven D. Karlen, David Bird, Naohito Tokunaga, Yasushi Sato, Shawn D. Mansfield, John Ralph, and A. Lacey Samuels

Department of Botany, University of British Columbia, Vancouver, British Columbia, V6T 1Z4 Canada (R.A.S., M.S., A.L.S.); Department of Biochemistry, and the US Department of Energy’s Great Lakes Bioenergy Research Center (GLBRC), Wisconsin Energy Institute, University of Wisconsin-Madison, Madison, Wisconsin 53726 (R.A.S., S.D.K., J.R.); Department of Biology, Mount Royal University, Calgary, Alberta T3E 6K6, Canada (D.B); Biology and Environmental Science, Graduate School of Science, Ehime University, Matsuyama, Ehime 790-8577, Japan (N.T., Y.S.); and Department of Wood Science, University of British Columbia, Vancouver, British Columbia, V6T 1Z4 Canada (S.D.M.)

ORCID IDs: 0000-0003-2363-2820 (R.A.S.); 0000-0001-5645-4501 (D.B.); 0000-0002-7435-7825 (N.T.); 0000-0002-0175-554X (S.D.M.); 0000-0002-6093-4521 (J.R.); 0000-0002-0606-8933 (A.L.S.).

Many land plants evolved tall and sturdy growth habits due to specialized cells with thick lignified cell walls: tracheary elements that function in water transport and fibers that function in structural support. The objective of this study was to define how and when diverse cell populations contribute lignin precursors, monolignols, to secondary cell walls during lignification of the Arabidopsis (Arabidopsis thaliana) inflorescence stem. Previous work demonstrated that, when lignin biosynthesis is suppressed in fiber and tracheary element cells with thickened walls, fibers become lignin-depleted while vascular bundles still lignify, suggesting that nonlignifying neighboring xylem cells are contributing to lignification. In this work, we dissect the contributions of different cell types, specifically xylary parenchyma and fiber cells, to lignification of the stem using cell-type-specific promoters to either knock down an essential monolignol biosynthetic gene or to introduce novel monolignol conjugates. Analysis of either reductions in lignin in knockdown lines, or the addition of novel monolignol conjugates, directly identifies the xylary parenchyma and fiber cell populations that contribute to the stem lignification and the developmental timing at which each contribution is most important.

Plant cells with thick secondary cell walls include some of the most essential cells in the plant body, tracheary elements (TEs) that act as water conduits, and fibers that provide structural support for the aerial shoot. To withstand the negative pressure of long distance water transport and to support the plant body, these cells deposit thick secondary cell walls composed largely of cellulose, hemicelluloses, and lignin. The lignin polymer is increasingly viewed as a major carbon sink and, concomitantly, both a potentially valuable renewable source of aromatic bioproducts and an impediment to the industrial processing of lignocellulosic biomass.

Lignin precursors, monolignols, are derived from Phe through an intricate series of reactions inside cells (Vanholme et al., 2010; Mottiar et al., 2016). The production and export of monolignols to the cell wall for lignin polymerization occurs late in xylem TE development, and many studies have shown that lignification of TEs begins prior to programmed cell death and continues after cell death (Tokunaga et al., 2005; Pesquet et al., 2013; Smith et al., 2013). This raises the question of where monolignols are produced for xylem cell lignification following programmed cell death. The “good neighbor” hypothesis postulates that TEs with lignified secondary cell walls receive monolignols from adjacent nonlignifying cells. The lignin precursors are exported into the cell wall where oxidative enzymes localized in the cell wall generate radicals that then facilitate the polymerization of lignin (Freudenberg, 1959; Fagerstedt et al., 2010; Zhao et al., 2013). The oxidative enzymes in the cell wall continue to function even after programmed cell death. Additionally, it has been demonstrated in cell cultures that TE-like cells can take

1 This study was supported by the Canadian Natural Sciences and Engineering Research Council CREATE and Discovery Grants held by A.L.S. and S.D.M.; the U.S. Department of Energy Great Lakes Bioenergy Research Center (DOE Office of Science BER DE-FC02-07ER64494) to S.D.K., R.S., S.D.M., and J.R.; and an NSERC Post Graduate Scholarship-Doctoral award (R.S.).

* Address correspondence to lsamuels@mail.ubc.ca.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: A. Lacey Samuels (lsamuels@mail.ubc.ca).

R.A.S., M.S., N.T., Y.S., and A.L.S. conceived the original research plans, designed the experiments, and analyzed the data; S.D.M., J.R., Y.S., and A.L.S. supervised the experiments; R.A.S., N.T., D.B., and M.S. performed the experiments; S.D.K. provided technical assistance; R.A.S. wrote the article with contributions from all authors.

OPEN Articles can be viewed without a subscription.

1028 Plant Physiology®, June 2017, Vol. 174, pp. 1028–1036, www.plantphysiol.org © 2017 American Society of Plant Biologists. All Rights Reserved. Downloaded from on July 30, 2017 - Published by www.plantphysiol.org Copyright © 2017 American Society of Plant Biologists. All rights reserved.
up and polymerize exogenously supplied monolignols (Hosokawa et al., 2001; Pesquet et al., 2013). Monolignols have some mobility within the cell wall (Tobimatsu et al., 2013) and therefore could migrate from neighboring cells into the cell wall of associated TEs.

Previously, we tested the good neighbor hypothesis in planta by silencing monolignol production in cells with thick secondary walls, but not in neighboring parenchyma cells. Monolignol production was silenced in cells developing secondary cell walls using an artificial microRNA targeting the CINNAMOYL-CoA REDUCTASE1 (CCR1) gene, driven by the CELLULOSE SYNTHASE7 (CESA7) promoter (Smith et al., 2013). In proCESA7::miRNA CCR1 plants, although TEs and fibers were incapable of contributing to their own lignification, cells within the xylem tissue still lignified normally. This suggests that good neighbors were able to rescue the lack of lignification of TEs and xylary fibers in these plants, and we hypothesized that long-lived xylary parenchyma cells were acting as good neighbors.

Fibers are an important structural component of primary and secondary xylem. The potential role of xylary fibers as good neighbors in xylem TE lignification could not be inferred from our previous work, where lignin precursor production was knocked down in all cells with thickened secondary cell walls. In vascular bundles, xylary fibers were still lignified, so the question of whether xylary fibers are required to produce monolignols for TE lignification could not be determined. These data raised the question of whether the contributions of monolignols from different cell populations in the xylem, for example, xylary parenchyma cells and xylary fibers, could be defined.

In contrast to the rescued lignin in vascular bundles, interfascicular fibers, found between the vascular bundles in Arabidopsis (Arabidopsis thaliana), had a severe loss of lignin when targeted by proCESA7::miRNA CCR1 (Smith et al., 2013), indicating that these interfascicular fibers do not have “good neighbors” and rely on monolignols produced within their protoplasts to fully lignify their secondary cell walls. In our previous study, the proCESA7::miRNA CCR1 was used as a relatively blunt tool to target down-regulation of monolignol production in both fibers and vessels. Given our earlier discovery that interfascicular fibers are cell-autonomous for monolignol production, they provide an attractive target for lignin manipulation; we therefore additionally sought to specifically target monolignol down-regulation in fibers without affecting vessels.

The objective of this work was to define which cell populations spatially contribute monolignols to lignification, and when each population temporally contributes in the Arabidopsis stem, as well as to provide strategies to manipulate lignin in diverse cell populations. The results demonstrate that xylary parenchyma cells function as good neighbors for lignification of nearby TEs during early stem developmental stages. As stem development proceeds, xylary fibers can contribute as good neighbors as well, although their contribution is redundant with the parenchyma. Fibers, both xylary and interfascicular, become the dominant lignified cells of the stem, and using a fiber-specific promoter, we demonstrate strong decreases in lignification and increased saccharification in plants with reduced lignin in their fibers.

RESULTS

Xylary Parenchyma Cells Contribute to Lignification, Especially in Young Stems

To test the hypothesis that xylary parenchyma cells contribute monolignols to TE lignification in planta, we studied early stem development in Arabidopsis. The vascular bundles of the uppermost portion of the stem (i.e. the top 9 cm) are dominated by the first-formed xylem cells, TEs and xylary parenchyma cells (Fig. 1A). The A1PRX47 promoter was previously identified to be expressed mainly in xylary parenchyma cells (Tokunaga et al., 2009). To verify the xylary parenchyma...
expression in transverse sections of Arabidopsis stems, in addition to the proAtPRX47::GUS lines, wild-type plants were transformed with proAtPRX47::GFP reporters. Transverse paraffin sections of the stems of proAtPRX47::GUS lines showed blue GUS substrate staining in xylary parenchyma (Fig. 2, A and B). It was not possible to capture the GFP-containing cytoplasm in transverse sections of the proAtPRX47::GFP lines. However, in longitudinal sections, green fluorescence was observed adjacent to TEs (Supplemental Fig. S1A).

We previously demonstrated that an artificial microRNA (miRNA CCR1), specifically targeted to the CCR1 gene that encodes one of the first monolignol-specific biosynthetic enzymes, is effective at silencing monolignol biosynthesis and does not migrate away from the cells in which it is expressed (Smith et al., 2013). Monolignol production was therefore repressed in xylary parenchyma cells using a proAtPRX47::miRNA CCR1 construct, and these plants had wild-type growth habit. In the 9-cm-tall wild-type stems, the lignin in the secondary walls of round, open TEs was stained red with phloroglucinol-HCl (Fig. 3A). In contrast, in phloroglucinol-stained sections of stems of proAtPRX47::miRNA CCR1 plants, the TEs displayed irregular xylem phenotypes (Fig. 3B, arrows), typically seen in cells with impaired cell wall lignification (Jones et al., 2001; Goujon et al., 2003a; Mir Derikvand et al., 2008; Ruel et al., 2009; Thévenin et al., 2011). Furthermore, proAtPRX47::miRNA CCR1 stem sections stained slightly with phloroglucinol-HCl, indicating that these TEs still lignify to some extent and that some degree of cell-autonomous lignification exists. As the proAtPRX47::miRNA CCR1 stems matured (~30 cm tall) and abundant xylary and interfascicular fibers differentiated, the lignification in vascular bundles was similar to that of wild-type stems (Supplemental Fig. S2A). This suggests that xylary parenchyma cells play an important role in the lignification of TEs early in the primary stem development, prior to xylary fiber development.

In addition to knocking down monolignol production in xylary parenchyma during lignification, an alternative approach of introducing exotic monolignol conjugates using the AtIPRX47 promoter was devised. Arabidopsis does not naturally produce monolignol conjugates, so the appearance of conjugates can be used to evaluate the contribution of xylary parenchyma cells. Figure 3. Xylary parenchyma cells play a role in lignification of TEs early in stem development. A and B, Arabidopsis stem cross sections were taken 9 cm from the shoot apical meristem. proAtPRX47::miRNA CCR1 stems (B) had less staining than the wild type (A) at this stage of development and the proAtPRX47::miRNA CCR1 displayed some collapsed TEs (arrows, inset) unlike wild-type TEs, which were round (asterisks). Stem sections are representative of four to six biological replicates. Scale bars = 15 μm. DFRC followed by GC-MS/MS analysis was used to identify coniferyl ferulate (CA-FA) (C) and coniferyl p-coumarate (CA-pCA) conjugates (D) in proAtPRX47::FMT and proAtPRX47::PMT plants, respectively. The highlighted peaks represent the major fragment (m/z 131) released from the conjugate’s parent ion (m/z 400 or 370). In proAtPRX47::FMT and proAtPRX47::PMT plants, ferulates and p-coumarate conjugates, respectively, are produced by xylary parenchyma cells and are incorporated into the lignin polymer of lignifying neighboring cells. None of the novel conjugates were detected in wild-type plants.
to lignification in plants with normal lignin levels. Novel monolignol ferulate conjugates were introduced into poplar lignin by expressing a FERULOYL-CoA MONOLIGNOL TRANSFERASE (FMT) gene from Chinese angelica (Angelica sinensis) (Wilkerson et al., 2014). Similarly, the introduction of another acyltransferase gene from rice (Oryza sativa), p-COUMAROYL-CoA MONOLIGNOL TRANSFERASE (PMT), into poplar and Arabidopsis led to the production of monolignol p-coumarates that are novel to the lignin of both plants (Smith et al., 2015). Using the proAtPRX47 promoter, the production of monolignol ferulate and monolignol p-coumarate conjugates was targeted to xylary parenchyma cells (proAtPRX47::FMT; proAtPRX47::PMT, respectively). If the xylary parenchyma cells are contributing monolignols to the neighboring cell walls, the novel conjugates should be detected in lignin despite their being synthesized by cells without lignified cell walls. To determine if these novel conjugates were present in the lignin of proAtPRX47::FMT- and proAtPRX47::PMT-expressing plants, the degradative lignin analysis technique derivation followed by reductive cleavage (DFRC), coupled with gas chromatography-tandem mass spectrometry, was performed. This degradative method releases signature fragments that can only be derived from the novel monolignols when they are incorporated into lignin polymer via radical coupling reactions that produce β-ether linkages (Wilkerson et al., 2014). Detecting these diagnostic products provides evidence for integration of the novel monolignol conjugates into the lignin polymer of Arabidopsis stems. When either FMT or PMT was expressed in xylary parenchyma cells, both of the corresponding novel conjugates could be detected in the lignin of transgenic, but not wild-type plants (Fig. 3, C and D). Together, both the knockdown of lignin production in xylary parenchyma resulting in the irregular xylem phenotype and the incorporation of novel monolignols originating from xylary parenchyma cells to lignification, with the knockdown phenotype additionally revealing that the contribution is most significant in the early stages of xylem differentiation.

Fibers Produce Their Own Monolignols and Are Not Required for TE Lignification

As stems matured, xylary fibers were the most abundant cell type within the xylem, and interfascicular fibers were also prominent between vascular bundles (Fig. 1B). Xylary and interfascicular fibers start differentiating ~10 cm from the shoot apical meristem and rapidly develop lignified secondary cell walls. Within the vascular bundle, xylary fibers are potential candidates as “good neighbors” for TE lignification, because of their unique development compared to TEs. TEs produce their secondary cell walls and very rapidly undergo programmed cell death following their differentiation, while fibers have a longer developmental timeline compared to TEs, based on the expression of genes associated with programmed cell death (Ohashi-Ito et al., 2010). Therefore, xylary fibers could supply monolignols to the cell walls of neighboring TEs. To determine if fibers can function as good neighbors, lignin biosynthesis was knocked down in all fibers using the fiber-specific AtPRX64 promoter (Tokunaga et al., 2009) driving the miRNA CCR1. The activity of the promoter in fibers was observed in GUS and GFP reporter lines (Fig. 2, C and D; Supplemental Fig. S1). In contrast to the irregular xylem phenotype of the young stems of proAtPRX47::miRNA CCR1 stems stained with chloroglucinol-HCl appear similar to wild type in stain intensity and cell morphology (Supplemental Fig. S2).

As fibers differentiated in the 15- to 21-cm stems, the wild-type stem sections stained deep red with chloroglucinol in TEs, xylary fibers, and interfascicular fibers (Fig. 4B). In contrast, there was a strong reduction in the staining of developing xylary fibers and interfascicular fibers in the proAtPRX64::miRNA CCR1 stems (Fig. 4A). This was expected, as the fiber-specific promoter driving the miRNA CCR1 would lead to loss of monolignol biosynthesis in these cells. At maturity (~30 cm), the wild-type stem sections (Fig. 4D) and proAtPRX64::miRNA CCR1 stem sections (Fig. 4C) displayed similar levels of lignification in the vascular bundles, while proAtPRX64::miRNA CCR1 stem sections continued to have strongly reduced interfascicular fiber lignin staining (Fig. 4C). Thus, in just a narrow developmental window, decreased lignin in vessels suggests that xylary fibers act as good neighbors for TEs. However, because lignification in vascular bundles is wild type at maturity, we hypothesized that monolignol contributions from xylary fibers are not strictly required for TE lignification.

To test this hypothesis and investigate the role of fibers in vessel lignification, vascular bundles were examined in nst1 nst3 mutants, as this loss of function mutant is missing both NAC transcription factors controlling fiber cell fate (Mitsuda et al., 2007; Zhong et al., 2007). The lignification of nst1 nst3 stems at 21 cm was investigated by embedding stems in Technovit resin, cutting sections at 3 μm, and staining with crystal violet solution (Drnovsek and Perdih, 2005). In the mutants, the only lignified tissues that showed the crystal violet fluorescent red staining were the vessel elements (Fig. 4E), while in wild type (Fig. 4F), both the vessels and fibers were brightly stained. While the range of corrected fluorescence was smaller in the nst1 nst3 mutants compared to wild type, the mean cell wall fluorescence was not affected by the mutations (Fig. 4G). As in the mature stems in the CCR knockdown lines, the lignin fluorescence in mutant and wild-type xylem was not significantly different (Fig. 4G). The continued lignification of the vessels when fiber lignification is reduced or absent suggests that a large enough pool of monolignols can be assembled from among the xylary parenchyma and/or TEs.
Suppressing Monolignol Biosynthesis in Fibers Results in Reduced Recalcitrance of Stem Biomass toward Saccharification

Down-regulating lignin using a promoter active in all fibers (proAtPRX64::miRNA CCR1) led to the loss of lignin in interfascicular fibers, whereas in vascular bundles, xylary fiber lignification was delayed (Fig. 4A) but not abolished at maturity (Fig. 4C). The vessel lignification appears to be resilient to monolignol losses from fiber good neighbor populations. In contrast, fiber cells are easily directly manipulated, because they have cell-autonomous lignification. We hypothesized that fibers offer an attractive target for lignin engineering, for example, by reducing lignin just in fibers. To test the effect of loss of the fibers’ contribution to whole stem lignification, lignin deposition of wild-type and proAtPRX64::miRNA CCR1 stems was assessed using Klasson lignin analysis (Coleman et al., 2008). Lignin content significantly decreased to 63-83% of the wild-type values in 15- to 21-cm tall proAtPRX64::miRNA CCR1 stems (Fig. 5A). Thus, specifically targeting lignin down-regulation to fibers can decrease lignin content in the stems of Arabidopsis while the vessels still lignify normally (Fig. 4).

Plants expressing miRNA CCR1 in fibers with decreased lignin content were tested for changes in recalcitrance, using a digestibility assay with dilute base pretreatment (Santoro et al., 2010). This analysis, which uses incomplete saccharification, demonstrates relative accessibility to cell wall polysaccharides by various treatments in different plant lines. The proAtPRX64::miRNA CCR1 plants produced greater Glc yields relative to wild-type plants (Fig. 5B). Thus, down-regulation of lignin biosynthesis in fiber cell types leads to reduced lignin without affecting the integrity of TEs (Fig. 4) or an associated yield penalty (Supplemental Fig. S3) and ultimately improves the access to secondary cell wall polysaccharides.

In addition to exploiting the degree of cell autonomy of fiber lignification for decreasing lignin, we also predicted that driving the FMT and PMT with the fiber-specific proAtPRX64 promoter could be used to incorporate novel monolignol conjugates into lignin. Indeed, proAtPRX64::PMT and proAtPRX64::FMT plant lines produced lignins that released the diagnostic DFRC conjugates (Fig. 5C). This provides further evidence that targeted lignin manipulation can be achieved by manipulating gene expression and resulting monolignol contributions in subpopulations of lignifying cells.

DISCUSSION

The results here underscore that lignification of the Arabidopsis stem is a collective effort of the community of cell populations. The ability of neighboring nonlignifying cells to contribute to lignification has been proposed by many researchers (Hosokawa et al., 2001; Tokunaga et al., 2005; Pesquet et al., 2013) and was unequivocally demonstrated when the loss of
monolignol biosynthetic capacity in lignifying cells did not abolish lignification in the xylem (Smith et al., 2013). In this study, the good neighbor populations responsible for contributing monolignols to TE lignification were defined by targeted suppression of cell wall lignification using artificial microRNAs and by the production of novel monolignol conjugates in different good neighbor populations. The role of xylary parenchyma cells is most prominent during early stages of xylem development in the inflorescence stem, prior to the development of substantial fiber cell populations (Fig. 3). As the entire xylem tissue could be rescued when fiber-specific monolignol production was reduced, the results show that xylary parenchyma can also contribute to xyler fiber, but not interfascicular fiber, lignification (Fig. 4). As development proceeds, xylary fibers function as good neighbors, producing monolignols for TE lignification, but also to lignify their own secondary cell walls (Fig. 4). Therefore, xylary fibers are a uniquely flexible cell type in the xylem in that they can act as good neighbors for adjacent TEs, autonomously lignify their own cell walls, and accept monolignols from neighboring cells.

When the contribution of xylary parenchyma cells to lignification was repressed, some residual TE lignification was observed in young stems of proAtPRX64::miRNA CCR1 lines, as it is expected that TEs contribute to their own lignification. Evolutionarily, TE cell-autonomous lignification may play a more important role, as many plant taxa that evolved earlier than gymnosperms and angiosperms (e.g., lycophytes) do not have parenchyma cells within the xylem tissue. It is therefore possible that the ability of xylary parenchyma cells to contribute to lignification is a more recent trait, adopted to ensure that short-lived water-conducting TEs develop the extent of cell wall lignification required to facilitate efficient water transport without risking vascular collapse.

The good neighbor effect of xylary fibers on lignification is more difficult to define in Arabidopsis plants than is the contribution from xylary parenchyma cells. In Arabidopsis, xylary parenchyma cells do not develop a lignified cell wall and, as a result, the production of monolignols must be solely for the purpose of non-cell-autonomous lignification. Xylary fibers, on the other hand, are lignifying their own cell walls as well as contributing to TE lignification. This has potential implications for the lignification of woody tissue, as many trees inherently have an abundance of fibers surrounding the TEs throughout the xylem tissue. Studies on the lignin composition of poplar fiber cell walls suggest that fibers contribute monolignols to secondary cell walls of vessels in trees (Gorzsás et al., 2011), consistent with the data presented here.

The conjugates produced by the FMT and PMT enzymes represent an innovative tool to demonstrate the active contribution of xylary parenchyma cells to lignification in plants. They also demonstrate the plasticity of plants to permit the production and incorporation of alternative or unique monomers into their lignins, as hypothesized nearly two decades ago (Ralph et al., 1997, 1998; Sederoff et al., 1999; Ralph, 2006), and as reviewed (Vanholme et al., 2012; Wagner et al., 2012; Mottiar et al., 2016). In Arabidopsis, this phenomenon often occurs as a result of disrupting key enzymatic reactions in the lignin biosynthetic pathway (Vanholme et al., 2012). For example, the cell wall lignins of CAFFEIC ACID 3-O-METHYL TRANSFERASE loss-of-function mutants incorporate 5-hydroxyconiferyl
alcohol, a monomer that is typically not a product of the monolignol biosynthetic pathway (Marita et al., 2001; Ralph et al., 2001; Goujon et al., 2003b). Other plant species incorporate alternative phenolic monomers into their cell walls, even without genetic manipulation of the lignin biosynthetic pathway. Grasses, for example, incorporate acylated monolignols, such as monolignol p-coumarate conjugates, into their cell wall lignins (Ralph, 2010). The monolignol biosynthetic and conjugating enzymes are in the cytoplasm of the good neighbor and lignifying cells. The long-lived nature of some of these cell populations implies that monomers must be exported across the cell membrane for incorporation into the lignin polymer.

Defining the roles of the different cell populations in lignification has implications for the targeted manipulation of lignin biosynthesis to allow plants to grow as normally as possible, while simultaneously reducing the recalcitrance of the cell wall (Fig. 5; Supplemental Fig. S3). Given that they represent a large proportion of biomass, fibers represent interesting cell types to target. The next step will be defining the proportional contributions of xylary parenchyma and fiber cell populations in different lignifying systems such as grasses and woody eudicots. The proAtPRX64::GUS and proAtPRX64::GFP assays and the CCR1 knockdown phenotype indicate that the promoter of AtPRX64 was active in both xylary and interfascicular fibers. However, there is potential to tailor the recalcitrance of different feedstocks (e.g. grasses) by taking advantage of the different developmental origins of xylary fibers and interfascicular fibers that arise from different sources (procambium and ground meristem, respectively (Essau, 1943). In Arabidopsis primary xylem, targeting changes in lignin quality or quantity to fibers did not compromise growth or vascular integrity, as has also been reported for Arabidopsis interfascicular fibers (Yang et al., 2013) and other lignified tissues (Eudes et al., 2012). It will be interesting to learn whether this is also the case in grasses and woody secondary xylem in which fibers compose a large part of the xylary tissue.

MATERIALS AND METHODS

Plant Growth Conditions and Staining

Arabidopsis (Arabidopsis thaliana) Col-0 seeds were grown on 0.5× Murashige and Skoog media (Sigma-Aldrich) with appropriate antibiotics (25 μg/mL hygromycin for proAtPRX64::FMT/PMT lines and 50 μg/mL kanamycin for proCESA7::proAtPRX7::FMT/PMT lines) under continuous light for 2 weeks. Antibiotic-resistant seedlings were transferred to soil (Sunshine mix 4; Sungrow Horticulture) and grown in a growth chamber at 21°C under 16-h-light/8-h-dark cycles. Plant inflorescence stems were destructively sampled when 9 cm tall, or 15 to 21 cm tall or after 2 months (~30-cm-tall mature stems), and thin hand sections were taken from the base of the stem for histochemical staining. Sections from four to six independent stems were stained with phloroglucinol-HCl (10% phloroglucinol in 95% ethanol [v/v] with five drops of 1 M HCl) for 5 min, mounted in water, and analyzed using bright-field microscopy (Leica DMR microscope). Mature (~2-month-old) stems were also destructively sampled for chemical analysis.

Inflorescence stems were harvested and fixed in 4% paraformaldehyde, 50 mM PIPES (pH 7.5), 5 mM MgSO4, and 5 mM EGTA solution at room temperature overnight. Samples were then dehydrated with an ethanol series (10, 30, 50, 70, 90, 99.5, and 100%), for 30 min each, followed by 100% ethanol overnight. Technovit 8100 (Kulzer) was prepared according to the manufacturer’s specifications. Samples were infiltrated with 1:1 (v/v) Technovit:ethanol solution at room temperature for 4 h, then rinsed in 100% Technovit and stored at 4°C for 2 d. Samples were washed two times with fresh Technovit solution (30 min each) and were embedded in Technovit polymerization solution using polypropylene embedding molds as per the manufacturer’s instructions. Technovit blocks of nst1 nst3 and wild-type 21-cm stems were sectioned to produce 3-μm specimen sections on a Leica UCT6 and placed upon distilled water droplets on glass slides prefarmed to ~2°C on a slide warming block. Slides were left on the block until dry. Lignified cell walls were stained with a 5 μM crystal violet solution (Dnmovsek and Perdih, 2005) and imaged using a rhodamine filter and a 63× oil immersion lens. Images were captured using a digital camera, controlling the exposure and F-stop for all images. From both genotypes, four to five bundles were analyzed using ImageJ. Images were converted to 32-bit grayscale and the xylem selected by thresholding. Mean fluorescence and area (μm²) were measured from both the xylem region and background (nonxylem regions). Corrected fluorescence was calculated as per (McCloy et al., 2014). Briefly, total fluorescence from xylem was measured and corrected by the formula:

\[ \text{ID} = \frac{\text{SA}_{\text{x}} - \text{SA}_{\text{b}}}{\text{FA}_{\text{x}}} \]

where ID is integrated density of xylem fluorescence, SA is area of the xylem, and FA is background fluorescence of all nonxylem regions within the micrograph. Welch’s two-sample t test for unequal variances was performed using R (t = −0.6385, df = 3.92, P = 0.5589).

GUS Staining

GUS staining and observation of the paraffin sections with or without phloroglucinol-HCl staining were performed using 4-week-old proAtPRX64::GUS lines and proAtPRX64::GUS lines as described previously (Tokunaga et al., 2009).

Confocal Imaging

Imaging of proAtPRX64::proAtPRX47::GFP stem longitudinal sections was performed on an Olympus FV1000 Multiphoton Laser Scanning Microscope using conventional GFP settings for eGFP (488/509). Stem longitudinal sections were mounted in water on a glass slide and imaged using an Olympus water dipping 25× objective. Longitudinal sections 5 cm from the top of the mature stems were mounted on slides in water and imaged using Olympus 10× or 20× air objectives. The images were captured using Fluoview FV1000 software (Olympus) and processed using Velocity image analysis software (Improvision).

Transgenic Lines

Gateway cloning technology (Invitrogen) was used to generate the following constructs related to Peroxidasease64 (AtPRX64; AT4G2180), Peroxidasease47 (AtPRX47; AT4G33420), and Cinnaomyol Coa Reductasease1 (CCR1; AT1G19590). The proAtPRX64::GFP and proAtPRX47::GFP constructs (pMDC107; Curtis and Grossniklaus, 2003) were generated using 900 and 1,393 bp, respectively, upstream of the translational start sites for the AtPRX64 and AtPRX47 genes. The other Gateway constructs generated were proAtPRX64::mRNA CCR1, and proAtPRX47::mRNA CCR1 (pKGW; Karimi et al., 2002) expression vectors (primers in Supplemental Table S1). T-DNA insertional mutants were obtained from the Arabidopsis Biological Resource Center for NAC Secondary Wall Thickening Promoting Factor1 (NST1; AT2G6770; SALK_102057) and NST3 (AT1G07950; SALK_120069; Alonso et al., 2003). Artificial microRNA s were designed using the Web microRNA Designer (http://wmd2.weigelworld.org) and amplified using primers listed in Supplemental Table S1, as described previously (Schwab et al., 2006). pDONR::FMT and pDONR::FMT lines were generated as previously described (Withers et al., 2012; Wilkerson et al., 2014). Gateway cloning technology (Invitrogen) was used to generate the following constructs: proAtPRX47::FMT, proAtPRX47::FMT (pKGW; Karimi et al., 2002), proAtPRX47::FMT, and proAtPRX47::FMT (pMDC99; Curtis and Grossniklaus, 2003). Plant expression constructs were introduced into Agrobacterium tumefaciens strain GV3101 and transformed into Arabidopsis Col-0 using the floral dip method (Clough and Bent, 1998) to generate transgenic plants.
Chemical Analysis

Dry Arabidopsis stems (WT; proAtPRX47::miRNA CCR1; proAtPRX64::miRNA CCR1) at 9 or 21 cm height were ground to a fine powder using a mortar and pestle, or Wiley milled using a 40 mesh screen. Stem tissue was acetone-extracted in a Soxhlet apparatus for 7 to 16 h by boiling with dry acetone overnight. Acid-insoluble lignin was determined using Klason lignin analysis of 100 mg extractive-free tissue, as previously described (Coleman et al., 2008). Acid-soluble lignin was determined by measuring the absorbance of the filtrate at 205 nm and calculated using an extinction coefficient of 100 L/g·cm. For DFRC analysis, mature stems cut into small pieces and then milled (Retsch MM400 mill) to a coarse powder were employed. The ground samples were then solvent-extracted in water three times and 80% ethanol 3 to 4 times and freeze-dried for 2 d. Approximately 20 to 500 mg, accurately weighted, of freeze-dried stem tissue from each line was used. DFRC was performed as described previously (Wilkerson et al., 2014).

Digestibility Analysis

Digestibility analysis of Glc and pentose yield was performed as described (Santoro et al., 2010), using ~2 mg of mature stem sample for each of three technical replicates.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AIPRX47, AT1G33420; AIPRX64, AT1G2180; AICCR1, AT1G9590; NST1, AT2G46770; NST3, ATIG23770; AsfM GenBank JAT75320.1; OsPMT, LOC_Os09g18744.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Cell-type-specific expression is conferred by AIPRX47 and AIPRx64 promoters.

Supplemental Figure S2. proAtPRX47::miRNA CCR1 stems appeared similar to wild-type stems (Fig. 3, A and C) at 21 cm and mature stages of development.

Supplemental Figure S3. Wild-type and miRNA plant growth habits.

Supplemental Table S1. Primer sequences.

ACKNOWLEDGMENTS

We thank the Arabidopsis Biological Resource Center for distributing Arabidopsis mutant seeds, the UBC Bioimaging Facility, and Shane Cantu and Nick Santoro at the Great Lakes Bioenergy Research Center’s Cell Wall Digestibility Facility.

Received April 5, 2017; accepted April 12, 2017; published April 17, 2017.

LITERATURE CITED


Smith et al.


