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Invited Expert Review

Roles of lignin biosynthesis and regulatory genes in plant development

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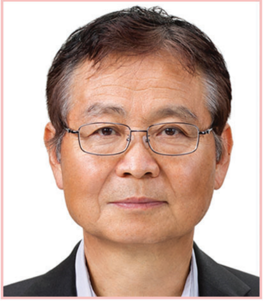

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Abstract Lignin is an important factor affecting agricultural traits, biofuel production, and the pulping industry. Most lignin biosynthesis genes and their regulatory genes are expressed mainly in the vascular bundles of stems and leaves, preferentially in tissues undergoing lignification. Other genes are poorly expressed during normal stages of development, but are strongly induced by abiotic or biotic stresses. Some are expressed in non-lignifying tissues such as the shoot apical meristem. Alterations in lignin levels affect plant development. Suppression of lignin biosynthesis genes causes abnormal phenotypes such as collapsed xylem, bending stems, and growth retardation. The loss of expression by genes that function early in the lignin biosynthesis pathway results in more severe developmental phenotypes when compared with plants that have mutations in later genes. Defective lignin deposition is also associated with phenotypes of seed shattering or brittle culm. MYB and NAC transcriptional factors function as switches, and some homeobox proteins negatively control lignin biosynthesis genes. Ectopic deposition caused by overexpression of lignin biosynthesis genes or master switch genes induces curly leaf formation and dwarfism.

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INTRODUCTION

The various types of plant cells differ in the composition and structure of their walls (Wang et al. 2013) to provide mechanical support to cells, tissue, and the entire body (Li et al. 2009). Along with cellulose and hemicellulose, lignin is a main component of secondary cell walls (Zhong et al. 2011). In grass species, lignin comprises approximately 20% of the secondary cell wall, filling pores between the polysaccharides (Leple et al. 2007; Vogel 2008). Lignin deposition begins when cell differentiation is completed and secondary thickening occurs in the walls (Rogers and Campbell 2004; Wang et al. 2013). The kind and amount of lignin units differ among plant species, tissues, and cell types, and are affected by developmental and environmental factors such as abiotic and biotic stresses (Li et al. 2009; Moura et al. 2010). In this review, we focus on the roles of lignin biosynthesis and regulatory genes in plant development.

LIGNIN COMPOSITION DIFFERS AMONG PLANT SPECIES

Lignin is a complex of aromatic polymers that consist of monomeric subunits, i.e., *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units (Boerjan et al. 2003; Rogers and Campbell 2004). These monolignols are produced in the cytoplasm and moved to the cell walls by an ATP-binding cassette transporter (Alejandro et al. 2012). Wall rigidity depends upon the ratio of those three monolignol units. In gymnosperms, lignin mostly contains G-units plus a small amount of H-units while angiosperm lignin is composed of G- and S-units (Figure 1) (Vanholme et al. 2010). When compared with G- or S-unit lignin, the levels of H-units are higher in softwood-compression wood and slightly higher in grasses (Vanholme et al. 2010). Woody plants have more S-units than do herbaceous plants (Zhang et al. 2006). In poplar (*Populus* spp.), it is easier to pulp wood from transgenic plants that overexpress ferulate 5-hydroxylase

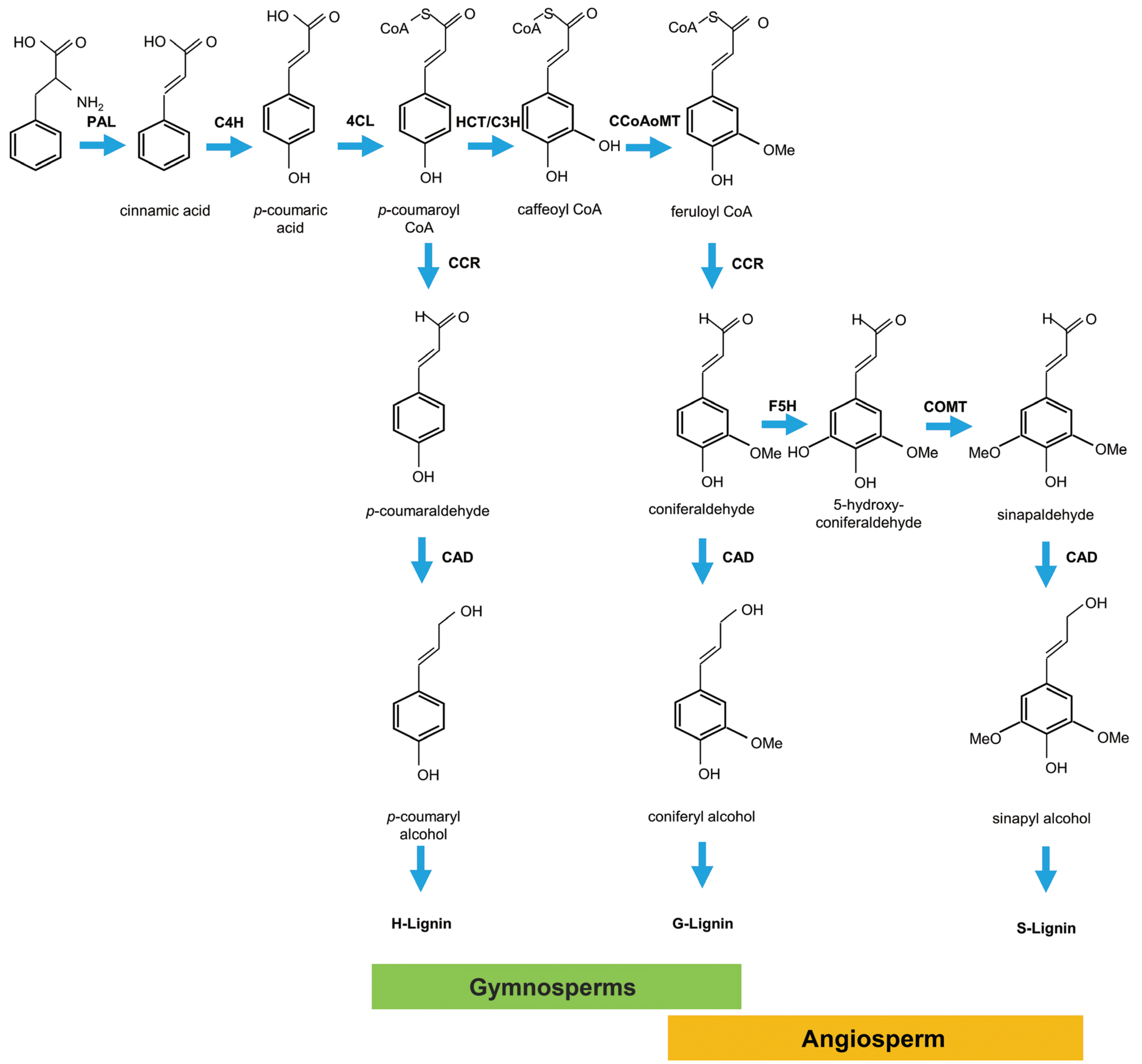

Figure 1. Lignin biosynthesis pathway

(F5H), an enzyme involved in S-unit biosynthesis (Stewart et al. 2009).

MUTATIONS IN LIGNIN BIOSYNTHESIS GENES CAUSE ABNORMAL GROWTH AND DEVELOPMENT

Lignin is involved in various plant processes. The proper lignin deposition in specialized cell types is essential for the appropriate plant development. The cell types for lignification exist to tracheary elements, sclerenchyma cells, endodermal cells, seed coat cells, and siliques cells in *Arabidopsis* (Barros et al. 2015). The lignin biosynthesis genes are mainly expressed in the xylem parenchyma cells surrounding the lignifying tracheary elements and fibers (Barros et al. 2015). Several genes for lignin biosynthesis have been identified and their roles in plant development have been investigated (Table 1).

Phenylalanine ammonia-lyase (PAL) functions at the first step in the phenylpropanoid pathway (Figure 1). *Arabidopsis* has four genes, *PAL1* through *PAL4*, that encode PAL (Raes et al. 2003). *Arabidopsis PAL3* is expressed at low levels in stems, while *PAL1*, *PAL2*, and *PAL4* are expressed at relatively high levels in stems during the later stages of development (Mizutani et al. 1997). At tissue level, they are detected in stem prexylems and surrounding mature vascular elements in the SAM and also at the inception of xylem differentiation in the early stages of vascular development (Liang et al. 1989).

Both PAL1 and PAL2 act as the primary isoforms; the lignin content in the *pal1 pal2* double mutant is reduced in parallel

Table 1. Mutant phenotypes of lignin biosynthesis genes

Gene	Species	Phenotype	Reference
PAL			
PAL1, *PAL2*, *PAL3*, *PAL4*	*Arabidopsis*	Stunted and less lignin	Huang et al. 2010
PAL2	Tobacco	Altered leaf shape, stunted growth, reduced pollen viability, and change flower morphology	Elkind et al. 1990
PAL1	*Salvia miltiorrhiza*	Stunted growth, delayed root formation, and altered leaves	Song and Wang 2011
C4H			
ref3	*Arabidopsis*	Dwarfism, male sterility, and the development of swellings at branch junctions	Schilmiller et al. 2009
4CL			
4CL	*Pinus radiate*	Dwarfism, absence of a straight	Wagner et al. 2009
Pv4CL1	*Panicum virgatum*	Browning in leaf midvein and brown patches in stem internodes	Xu et al. 2011
bmr2	Sorghum	Brown coloration of the midrib and sclerenchyma tissues.	Saballos et al. 2012
Os4CL3	Rice	Reduced plant growth	Gui et al. 2011
C3H			
ref8	*Arabidopsis*	Xylem collapse and dwarf phenotype	Franke et al. 2002
CCoAoMT			
ccomt1	*Arabidopsis*	Slightly reduced size in SD conditions	Do et al. 2007
CCR			
CCR	Tobacco	Reduced plant development, collapsed xylem and orange brown color	Piquemal et al. 1998
irx4	*Arabidopsis*	Growth retardation, altered leaf morphology, dark-green tissues, low fertility, collapsed xylem, and an inability to maintain an upright growth habit	Jones et al. 2001
CCR	Poplar	Orange-brown xylem	Leple et al. 2007
F5H			
fah1	*Arabidopsis*	Red color adaxial leaves in UV light	Rugger et al. 1999
COMT			
bm3	Maize	Reddish-brown pigmentation of midrib	Vignols et al. 1995
bmr12	Sorghum	Brown vascular tissue in the leaves and stem	Bout and Vermerris. 2003
CAD			
CAD2	Rice	Reddish-brown pigmentation, reduced culm stiffness	Zhang et al. 2006, Ookawa et al. 2014
CAD7	Rice	Flexible culm, semi-dwarfism, delayed flowering, and lower grain yields	Li et al. 2009

with an increase in the S to G ratio (Rohde et al. 2004). Furthermore, plants with the *pal1 pal2 pal3 pal4* quadruple mutant are stunted and accumulate less lignin (Huang et al. 2010). Downregulation of *PAL* leads to a significant change in lignin deposition as well as pleotropic phenotypes such as altered leaf shape, diminished plant growth, reduced pollen viability, and changes in flower morphology and pigmentation in tobacco (*Nicotiana tabacum*) (Elkind et al. 1990) and *Salvia miltiorrhiza* (Song and Wang 2011). Because the phenylpropanoid pathway has steps in common for producing various phenolic compounds that function in plant defenses, pollinator attraction, fruit dispersal, and mechanical support, the pleotropic phenotypes observed from the mutants in that pathway are expected. By contrast, mutations in the monolignol-specific branch of the lignin biosynthesis pathway show phenotypes that are related to the reduction of lignin in vascular tissues.

Cinnamic acid 4-hydroxylase (C4H) functions at the second step in the pathway (Figure 2). *Arabidopsis* has one copy of *C4H*, with transcripts being most abundant in the stems and roots. Analyses of *Arabidopsis C4H* promoter-*GUS* plants have indicated that the gene is expressed highly in the vascular tissues and surrounding parenchyma in seedling roots and also in the veins of mature leaves. It is also expressed in reproductive tissues, especially immediately below the stigmatic surface (Bell-Lelong et al. 1997). Mutants in *C4H* gene show the phenotypes similar to the *PAL* quadruple mutant such as reduced lignin deposition, dwarfism, a loss of apical dominance, and male sterility in *Arabidopsis* (Schilmiller et al. 2009).

4-Coumarate-coenzyme A ligase (4CL) is the third enzyme in the general phenylpropanoid pathway, producing the monolignol precursor *p*-coumaroyl-CoA (Figure 1). Severe suppression of *4CL* in *Pinus radiata* results in a dwarf phenotype (Wagner et al. 2009). In addition, silencing of *4CL* in switchgrass (*Panicum virgatum*) causes browning in the leaf midvein and brown patches in stem internodes (Xu et al. 2011). In sorghum (*Sorghum bicolor*), the *brown midrib2* (*bmr2*) mutant shows brown coloration in the midrib sclerenchyma tissues (Saballos et al. 2012). All five *4CL* genes in rice (*Oryza sativa*) are stem-preferentially expressed (Gui et al. 2011). Their transcript levels generally decline as the plants age, but *Os4CL3* and *Os4CL4* show patterns of increased expression in stem tissues. *Os4CL3* exhibits the highest expression and is detected in thickening vascular cells and also around non-thickening parenchyma cells. *Os4CL3* antisense transgenic plants exhibit reduced growth. However, other agronomic traits, such as flowering time, tiller number, and blade length of the flag leaf, are unchanged in *Os4CL3*-suppressed plants (Gui et al. 2011).

Most of phenylpropanoid biosynthesis requires at least two hydroxylation steps. C4H introduces the first hydroxyl group at the 4-position of the aromatic ring of cinnamic acid (Franke et al. 2002). The next step in this pathway is mediated by *p*-coumarate 3-hydroxylase (C3H) (Figure 1). Defects in *C3H* cause *p*-coumarate esters to accumulate rather than *p*-coumaryl alcohol, and plants display developmental defects such as xylem collapse and a dwarf phenotype (Franke et al. 2002).

The caffeoyl coenzyme A 3-*O*-metyltransferase 1 (CCoAOMT) is responsible for methylation at the C3 position of the phenolic ring of caffeoyl CoA (Figure 1). Expression of *Arabidopsis AtCCoAOMT1* has been analyzed in research utilizing the *GUS* reporter gene (Do et al. 2007). There, GUS-

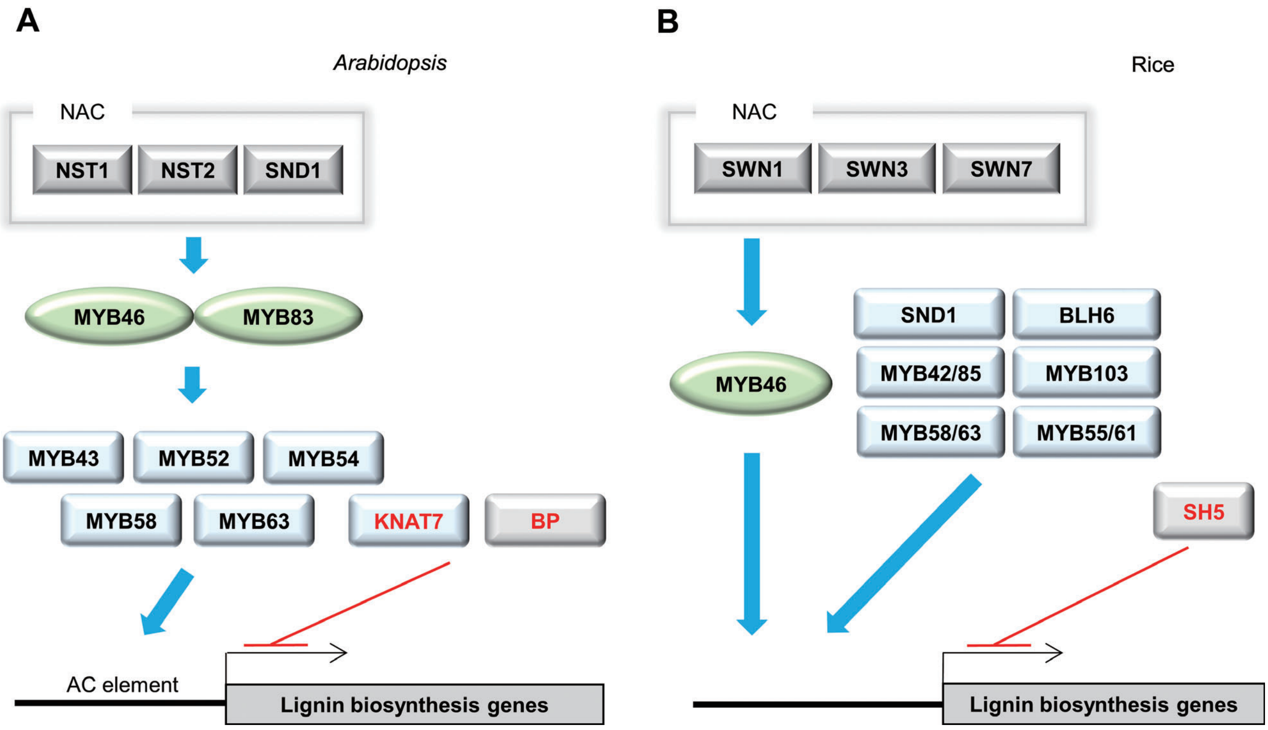

Figure 2. Transcriptional network for lignin biosynthesis in *Arabidopsis* (A) and rice (B)

staining was constitutive in plantlets, but preferential in the vascular tissues of leaves and flowers from mature plants. Signals were found in xylem and interfascicular fibers of stems and in the replum of siliques. Mutants in *CCoAoMT1* from *Arabidopsis* shows slightly reduced growth under short-day conditions, but no visual phenotype under long days (Do et al. 2007).

Cinnamoyl-CoA reductase (CCR) is the first enzyme in the monolignol-specific branch of the lignin biosynthesis pathway, where it converts feruloyl-CoA to coniferaldehyde (Leple et al. 2007) (Figure 1). Downregulation of *CCR* in tobacco significantly reduces lignin contents and the rate of plant development, as characterized by xylem that collapses and has an orange-brown color (Piquemal et al. 1998).

Arabidopsis AtCCR1 is expressed in the flowers and leaves and highly expressed in the stems, especially in tissues undergoing lignification (Lauvergeat et al. 2001). In contrast, *AtCCR2* is poorly expressed during normal developmental stages but strongly and transiently induced during incompatible interactions with *Xanthomonas campestris* pv. *campestris* (Lauvergeat et al. 2001). These data suggest that *AtCCR1* is involved in constitutive lignifications, whereas *AtCCR2* confers pathogen resistance. In *Arabidopsis*, a loss-of-function *CCR* mutant has significantly less lignin while its levels of cellulose and hemicellulose remain unchanged (Jones et al. 2001; Goujon et al. 2003a). This mutant shows pleiotropic phenotypes such as growth retardation, altered leaf morphology, dark-green tissues, low fertility, collapsed xylem, and an inability to maintain an upright growth habit (Jones et al. 2001).

In poplar, *PoptrCCR12* is preferentially expressed in the xylem while other CRR genes present divergent profiles that include preferential expression in the leaves, bark, or both. Some *CCR* genes are also induced or repressed under various abiotic stresses (Barakat et al. 2011). Downregulation of *CCR* expression in poplar also results in reduced lignin contents and orange-brown xylem (Leple et al. 2007). Analyses of transgenic poplars with decreased *CCR* expression have shown that ethanol yields are 161% higher from trees that are most severely affected (Van et al. 2014). However, although this downregulation of *CCR* improves pulping characteristics, it has negative effects on the growth characteristics of transgenic poplar plants (Leple et al. 2007).

In maize (*Zea mays*), *ZmCCR1* mRNA is present at high levels in adventitious roots, seminal roots, leaves, and stalks, where active lignification occurs. Transcripts of *ZmCCR2* are barely detectable in vegetative tissues, but strongly induced in roots by water deficits, suggesting that this gene is involved in root acclimation (Fan et al. 2006). Downregulation of maize *CCR1* leads to plants with enhanced digestibility but no compromise in their growth and development (Tamasloukht et al. 2011). Although the lignin content is only slightly reduced, the lignin structure is significantly changed in the mutant, i.e., the number of H-units is sharply decreased and the S-to-G ratio is slightly increased. Alterations in lignin deposition are mainly observed in the sclerenchymatic fiber cells that surround the vascular bundles. Genes specifically associated with fiber wall formation are downregulated in those mutants. In transgenic perennial ryegrass (*Lolium perenne*), reduced expression of *CCR1* also causes significant changes in lignin levels and composition (Tu et al. 2010). Those plants show enhanced digestibility but no obviously detrimental alterations in their morphology.

Coniferaldehyde 5-hydroxylase (F5H), a cytochrome P450-dependent monooxygenase, has a key function in the formation of S units. The *Arabidopsis fah1* mutants defective in F5H do not produce lignin S units or an intermediate sinapoylmalate, a UV-protective phenylpropanoid compound (Garcia et al. 2014). In contrast to the wild type, which emits blue fluorescence due to the presence of sinapoylmalate, the *fah1* mutants do not show this fluorescence.

In the later steps of lignin biosynthesis, mutations of genes such as *caffeic acid O-methyltransferase* (*COMT*) and *cinnamyl alcohol dehydrase* (*CAD*) have a milder influence on lignin deposition and plant development compared with mutations observed in the early steps of the phenylpropanoid pathway (Jones et al. 2001). In the *comt1* mutant of *Arabidopsis*, S units are absent and are replaced by 5-hydroxyguaiacyl S unit precursors (Goujon et al. 2003b). Analysis of a promoter trap line has indicated that activity of the *Arabidopsis COMT1* promoter is mainly restricted to vascular tissues, especially in the xylem, mature phloem, and differentiating fibers (Goujon et al. 2003b).

In transgenic *Arabidopsis* plants, the maize *ZmCOMT* promoter is also expressed preferentially in vascular tissues that undergo lignification (Fornalé et al. 2006). This promoter is also induced by wounding, but only in the vascular tissues. The naturally occurring *brown midrib* (*bm*) mutants in members of the grass family are defective in lignin biosynthesis genes (Rogers and Campbell 2004). For example, maize *bm3* and sorghum *bmr12* mutants, in which the leaf midrib is reddish-brown, are defective in *COMT* (Vignols et al. 1995; Bout and Vermerris 2003). Because COMT converts 5-hydroxyguaiacyl (5-OH-G) to S units, *bm3* mutants have fewer S units and a concomitant accumulation of 5-OH-G units, along with increased cell wall digestibility (Li et al. 2008). In perennial ryegrass, lignin levels are reduced when *COMT1* is downregulated (Tu et al. 2010).

Cinnamyl-alcohol dehydrogenase functions in the last step of monolignol biosynthesis, converting hydroxyl-cinnamaldehydes into their corresponding alcohols (Hirano et al. 2012). Among the nine CAD-like genes in *Arabidopsis*, two operate in lignin biosynthesis (Sibout et al. 2005). Two of the nine putative CADs in *Arabidopsis* – *AtCAD4* (*AtCAD-D*) and *AtCAD5* (*AtCAD-C*) – are the most active (Kim et al. 2004). Promoter activities for both are high in the fibers and xylem bundles (Sibout et al. 2003). A double mutant that lacks both of those genes exhibits a 94% reduction in traditional G and S subunits, as well as an overall decline in the amounts of lignin in xylem and fiber tissues (Sibout et al. 2005).

Of the 12 CAD genes present in the rice genome, *OsCAD2* is the most abundantly expressed. *OsCAD1* is also expressed in the culm while the others are either not expressed or are expressed only at very low levels (Hirano et al. 2012). RNA *in situ* hybridization has shown that rice *CAD* is expressed mainly in highly lignified cells, e.g., vascular bundles and sclerenchyma cells below the stem epidermis (Li et al. 2009). Mutants in *OsCAD2* display reddish-brown pigmentations in the panicle, internode, and basal leaf sheath at the heading stage (Zhang et al. 2006). Although those trait alterations do not significantly affect plant development, culm stiffness is reduced (Ookawa et al. 2014). Culm stiffness is an important agronomic trait because it is related to lodging resistance. *OsCAD7* is expressed in sclerenchyma cells of the secondary cell wall and vascular bundle region (Li et al. 2009). A knockout mutant in *OsCAD7* causes a flexible culm phenotype as well as semi-dwarfism, delayed flowering, and lower grain yields (Li et al. 2009).

Among the four *bm* mutants that occur naturally in maize, CAD activity and *CAD* transcript levels are significantly reduced in *bm1* (Halpin et al. 1998). Although the gross anatomy of stem tissues is not altered, the total lignin content is 20% lower in the mutant. Mapping of the *bm1* locus has led to suggestions that it is an allele of *CAD*. Transgenic *CAD*-RNAi maize plants present a reduction in the total lignin content but no significant changes in their growth and development (Fornalé et al. 2011). In sorghum, *Bmr6* encodes the major CAD and all three main lignin subunit levels are significantly reduced in the *bmr6* null mutant (Sattler et al. 2010). Downregulation of *CAD* expression by RNA-silencing in switchgrass causes a decline in lignification and enhanced saccharification characteristics (Saathoff et al. 2011). A similar phenotype is observed when *CAD* expression is suppressed in *Festuca arundinacea* Schreb., a widely grown monocotyledonous forage crop (Chen et al. 2003).

Lignin has an important role in transporting water in vascular tissues. The cross-linking of polysaccharide compounds of cell walls by lignin intensifies plant structural support and resistance to cell collapse under the tension of water transport. Lignin biosynthesis genes are expressed mainly in vascular tissues at various developmental stages as well as in shoot apical meristem (SAM), epidermis cells, and floral organs. Most mutation in lignin biosynthesis genes causes collapsed xylem element with growth retardation.

ALTERING EXPRESSION OF TRANSCRIPTION FACTORS FOR LIGNIN BIOSYNTHESIS GENES AFFECTS PLANT DEVELOPMENT

Secondary cell wall related genes for cellulose, xylan, lignin biosynthesis must be coordinately expressed during secondary cell wall development. The most abundant cell types for secondary cell wall deposition are fibers and tracheary elements. Secondary cell wall biosynthesis is positively regulated by NAC and MYB transcription factors in both dicot and monocot species (Figure 2A) (Zhong and Ye 2014).

In *Arabidopsis*, all three factors—NST1, NST2, and SND1—belong to the secondary wall NAC (SWN) group and serve as transcriptional switches that activate genes for secondary cell wall biosynthesis (Zhong and Ye 2014). SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1 (SND1) is a key transcriptional activator of secondary wall synthesis. An investigation of transgenic *Arabidopsis* plants expressing *SND1:GUS* has revealed that *SND1* is expressed in fiber cells that are lengthening in their elongating internodes, as well as in interfascicular fibers and the metaxylem of mature internodes (Zhong et al. 2006). Close examination of that metaxylem has indicated that the gene is active in xylary fiber cells but absent in developing vessels. *Arabidopsis* has 11 *SND1*-related genes: *SND2*, *SND3*, *MYB20*, *MYB42*, *MYB43*, *MYB52*, *MYB54*, *MYB69*, *MYB85*, *MYB103*, and *KNAT7*. All are highly expressed in interfascicular fibers and xylem cells undergoing secondary wall thickening, but not in parenchymatous pith cells (Zhong et al. 2008). They are also preferentially expressed in inflorescence stems and the developing protoxylem in internodes. The GUS reporter system and *in situ* mRNA hybridization experiments with *SND2*, *SND3*, *MYB52*, *MYB54*, *MYB69*, *MYB85*, *MYB103*, and *KNAT7* have shown that all eight are expressed in the developing protoxylem, the only secondary wall-containing cell type present (Zhong et al. 2008). In addition, *SND2*, *SND3*, *MYB52*, and *MYB54* are expressed in the elongating interfascicular fiber cells. Within non-elongating internodes, all eight transcription factors are highly expressed in the developing metaxylem cells and interfascicular fibers. In the roots, they are predominantly expressed in the developing secondary xylem. Such results provide evidence that these SND1-regulated genes are developmentally associated with secondary wall biosynthesis in vessels and fibers. Repression of *SND1* prevents plants from standing erect because the walls are not as thick in the fiber cells (Zhong et al. 2006, 2007a).

Secondary cell wall development is essential for various biological processes, such as the dehiscence of anther, the shattering of silique pod as well the formation of tracheary elements and fibers. The secondary walls of anther endothecium have striated patterns similar to tracheary elements and it is necessary for anther dehiscence and generation. In *Arabidopsis*, analyses of promoter activities using the *GUS* reporter gene have demonstrated that *NAC SECONDARY WALL THICKENING PROMOTING FACTOR1* (*NST1*) is expressed in the stamens and carpels of spikelets, inflorescence stems, the basal portion of the siliques, and the midribs of leaf veins (Mitsuda et al. 2005). At the tissue level, *NST1* promoter activity is found on the phloem side of vascular bundles in the midrib. For inflorescence stems, that gene is active on the adaxial side of the cambium, which undergoes thickening of the lignified secondary walls. By contrast, promoter activity for *NST2* is primarily observed in the anther walls and pollen grains (Mitsuda et al. 2005). This suggests that the *NST* genes play major roles during reproductive development especially in the anther. Double null mutants of *NST1* and *NST2* have severely indehiscent anthers, where secondary cell wall plays important roles for dehiscence and affect the tensile force for the rupture of the stomium. Ectopic expression of those genes under the CaMV *35S* promoter causes growth retardation and curling rosette leaves with ectopic lignification (Mitsuda et al. 2005).

NAC and MYB transcription factors have been demonstrated to positively regulate secondary cell wall biosynthesis in vessels and fibers. Secondary wall NACs (SWNs) function as the top master switches for activating proper secondary cell wall biosynthesis. SWNs directly activate downstream transcription factors as well a number of genes involved in secondary cell wall biosynthesis binding to the 19-bp SNBE (Secondary Wall NAC Binding Element) (Zhou et al. 2014). SND1 controls *MYB46* and *MYB83* by binding to the promoters at the SWN binding elements (Figure 2A) (McCarthy et al. 2009).

MYB46 is expressed in fibers and xylem cells undergoing secondary wall thickening (Zhong et al. 2007b). In transgenic *Arabidopsis* plants, the promoter is active in developing vessels within the stem protoxylem. Activity is intensive in both the interfascicular fibers and developing metaxylem of the internodes. Close examination of the metaxylem has revealed that the promoter functions in both developing vessels and xylary fibers. Therefore, these results suggest that *MYB46* expression is associated with secondary wall thickening in the interfascicular fibers, xylary fibers, and vessels. The MYB83 transcription factor is another molecular switch within the SND1-mediated transcriptional network. *MYB83* is directly activated by SND1 and its close homologs, including NST1, NST2, VND6, and VND7. In stem tissues, MYB83 promoter-driven *GUS* activity is found specifically in fiber and vessel cells that are undergoing secondary wall thickening (McCarthy et al. 2009). The promoter is also expressed in vessels but not in xylary fibers in the developing secondary xylem of roots.

Overexpression of those MYB transcription factors induces ectopic deposition of secondary cell walls, reduces plant growth rates, and stimulates the formation of curly leaves, as observed from *SND1*-overexpressing plants (McCarthy et al. 2009). The *myb46 myb83* double mutants exhibit severe growth retardation following seed germination because of defects in functional vessels (McCarthy et al. 2009). These results indicate that the MYB proteins act as master switches by directly activating cell wall biosynthesis genes.

Using the estrogen-inducible system, Zhong and Ye (2012) have identified *MYB43*, *MYB52*, *MYB54*, *MYB58*, *MYB63*, and *KNAT7* as downstream transcription factors of MYB46 (Figure 2A). They are also preferentially expressed in the xylem and interfascicular fibers. For example, *MYB58* is predominantly expressed in protoxylem vessels while *MYB63* is preferentially expressed in interfascicular fibers and xylem cells of non-elongating internodes in which both xylem and interfascicular fibers are undergoing secondary wall thickening (Zhou et al. 2009).

Secondary cell walls in grass family are mainly composed of cellulose, hemicellulose, and lignin, a composite structure similar to dicot species. In grass species, secondary cell walls containing sclerenchymatous fibers play important roles in major mechanical tissues, especially stem. Understanding of the macular mechanism for secondary cell wall development in grass species is important, because secondary cell wall thickness leads to an enhancement in the mechanical strength of stem and improves agronomic traits for getting high grain yield without lodging. In grass species, molecular mechanisms for transcriptional activation of secondary cell wall biosynthesis are evolutionally conserved in *Arabidopsis* (Zhong et al. 2011)

Three rice OsSWN genes and four maize ZmSWN genes are known to be expressed in secondary wall-forming cells with high similarity to the *Arabidopsis* secondary wall master switch, SND1 (Zhong et al. 2011; Chai et al. 2015). Analyses of transgenic rice plants expressing the promoter-*GUS* have shown that the *OsSWN2* promoter is preferentially expressed in the bundle sheath, including vascular vessels in the xylem, whereas the *OsSWN1* promoter is active in both the bundle sheath and the sclerenchymatous cells beneath the epidermis (Yoshida et al. 2013). *In situ* hybridization analysis of maize stems has indicated that four ZmSWN genes are strongly expressed in bundle sheath fibers, which comprise the bulk of the secondary wall-containing cells (Zhong et al. 2011). Transgenic *Arabidopsis* plants overexpressing *OsSWN* or *ZmSWN* via the CaMV *35S* promoter exhibit phenotypes of curly leaves and reduced rosette sizes due to a massive ectopic lignin deposition in mesophyll cells, a phenomenon that contrasts with that in the wild type, where lignin is deposited mainly in vascular tissues (Zhong et al. 2011). These overexpressors also accumulate cellulose and xylan ectopically. Expression of secondary wall biosynthesis genes, such as the lignin-biosynthesis *4CL1* and genes involved in cellulose synthase and xylan synthesis, can also be induced in transgenic plants. The monocot SWN proteins also stimulate expression of *SND1*-regulated transcription factors such as *SND3*, *MYB46*, *MYB83*, *MYB85*, and *MYB103* (Figure 2B). These findings indicate that the regulatory roles of *SND1* have been highly conserved during evolution. OsSWNs and ZmSWNs activate *OsMYB46* and *ZmMYB46* by binding to the secondary wall NAC-binding elements (SNBEs) in the *MYB46* promoters (Zhong et al. 2011) (Figure 2B).

Rice *OsMYB46* and maize *ZmMYB46* are the most homologous to *Arabidopsis MYB46*. All of these monocot genes induce the production of curly leaves and ectopic deposition of lignin, cellulose, and xylan when overexpressed in *Arabidopsis* (Zhong et al. 2011). In the *OsMYB46*-overexpressing plants, epidermal cell walls become thickened and ectopic lignin deposition affects normal epidermal cell development and leaf morphology (Zhong et al. 2011).

In maize, five MYB transcription factors (*ZmMYB2*, *ZmMYB8*, *ZmMYB31*, *ZmMYB39*, and *ZmMYB42*) control expression of *ZmCOMT* (Fornalé et al. 2006). Whereas *ZmMYB8* and *ZmMYB42* are mainly expressed in the aerial portions, *ZmMYB31* is expressed in both the roots and the aerial parts. Analysis of maize databases has indicated that *ZmMYB39* is highly expressed during early growth stages (Fornalé et al. 2006). When maize *ZmMYB42* is overexpressed in *Arabidopsis*, expression of several lignin biosynthesis genes is repressed and the S-to-G ratio is altered (Sonbol et al. 2009). Similarly, overexpression of *ZmMYB31* in *Arabidopsis* downregulates several genes involved in the synthesis of monolignols (Fornalé et al. 2010). These transgenic plants are dwarf and have significantly less lignin. Chromatin immunoprecipitation (ChIP) experiments have shown that *ZmMYB31* interacts with the promoter regions of maize *COMT* and *A1* during early sheath development (Fornalé et al. 2010). In addition, those experiments have demonstrated enrichment of the promoter region for maize *F5H*.

Whereas most NAC and MYB transcription factors positively regulate lignin biosynthesis genes, some homeobox genes negatively control lignin biosynthesis.

Mutants in *Brevipedicellus* (*BP*) that encodes a KNOTTED1-like homeobox (KNOX) protein, increased transcript levels of *PAL1*, *C4H*, *4CL*, *CAD1*, *COMT*, and *CCoAOMT*, leading to pleiotropic phenotypes such as short internodes, downward-facing siliques, and irregular epidermis cells (Mele et al. 2003). This protein forms a heterodimer with a BEL1-like (BELL) protein, PENNYWISE (PNY; also called BELLRINGER, REPLUMLESS, and VAMAANA), and the complex suppresses lignin deposition by inhibiting the lignin biosynthesis genes (Smith and Hake 2003; Rutjens et al. 2009) (Figure 2A). These proteins also promote meristem maintenance (Mele et al. 2003; Khan et al. 2012). These suggest that suppression of lignin biosynthesis is necessary for meristem maintenance. *In situ* hybridization experiments have demonstrated that *PNY* and *BP* show a similar expression pattern on the flanks of the inflorescence meristem as well as at the bases of floral meristems and in the cortex cells (Smith and Hake 2003).

In rice, *Oryza sativa homoeobox1* (*OSH1*) and *OSH15* are highly homologous to *Arabidopsis BP* (Sakamoto et al. 2006). Loss-of-function mutants of *OSH1* lack the SAM and >90% of the mutants have arrested development at the three-leaf stage (Tsuda et al. 2011). A loss-of-function mutation in *OSH15* exhibits dwarfism due to a defect in internode elongation in the upper region (Sato et al. 1999). Three maize genes, *Knotted1*, *Gnarley1*, and *Roughsheath1*, are putative orthologs of *Arabidopsis BP* (Townsley et al. 2013). In maize and tobacco plants that overexpress *Knotted1*, lignin deposition is decreased, suggesting that this homeobox protein inhibits lignin biosynthesis. Those plants are less rigid and show a dwarf phenotype (Townsley et al. 2013).

A BEL1-type homeobox gene *SH5* in rice is preferentially expressed in the lamina joint, leaf sheath pulvinus region, and young inflorescences, and especially in the abscission zone (Yoon et al. 2014). Lignin-staining has revealed a gradient of deposition from the lower to the uppermost part of the rice internodes, producing a pattern correlated with that of expression of lignin biosynthesis genes (Hirano et al. 2012). In particular, the zones of basal cell division have a low level of expression. These observations suggest that lignification must be repressed in order for cell division and elongation to occur in the internodal meristem regions. Overexpression of *SH5* decreases lignin deposition in the culm and spikelet junction region, especially within the abscission zone (Yoon et al. 2014). Expression of lignin biosynthesis genes such as *COMT1*, *PAL1*, *COA1*, *CCR19*, and *CAD2* is lower in the abscission zone of those overexpressing plants.

Using co-expression network analysis of rice plants, Hirano et al. (2013) have identified several transcription factors, including *OsMYB58/63*, *OsMYB42/85*, *OsMYB55/61*, *OsMYB103*, *OsSND1*, and *BELL1-LIKE HOMEODOMAIN 6* (*BLH6*), as being regulators of secondary cell wall formation. Overexpression of those genes in rice is manifested by a common morphology, such as dwarfism and narrow, dark-colored leaves. The plants also have increased secondary cell wall development and altered lignin contents. In particular, *BLH6* overexpression causes ectopic deposition of lignin and cellulose in the parenchyma cells. By contrast, downregulation of those genes decreases linin deposition and causes a severe dwarf phenotype.

LIGNIFICATION AFFECTS AGRONOMIC TRAITS AND INDUSTRIAL APPLICATIONS

Lignin is important for enhancing rigidity to protect plants against pathogen attacks and mechanical stress (Zhang et al. 2006). It increases the strength of cell walls by cross-linking with cellulose and hemicellulose (Li et al. 2009). Culm mechanical strength is an important agronomic trait in crop breeding because lodging causes significant losses in yield (Li et al. 2009; Ookawa et al. 2014). Reduced lignin contents often affect lodging resistance (Ookawa et al. 2014).

In rice, most brittle culm mutants have decreased amounts of cellulose. The mutant in *BC6* that encodes CesA, a cellulose synthesis enzyme, exhibits phenotypes with significantly less cellulose and thinner walls in sclerenchyma cells (Kotake et al. 2011). Mutants in *BC11*, which encodes CESA4, have reduced culm mechanical strength and irregular growth, e.g., dwarfism and abnormal leaf apices at the seedling stage; plus drooping leaves, small panicles, and partial sterility at the mature stage (Zhang et al. 2009). In addition to lower cellulose contents, those culm mutants have a smaller amount of lignin (Oh et al. 2013).

When attempting to increase yields, reducing the occurrence of "shattering" is an important step in the domestication process for many crops. Seed dispersal involves abscission zone formation and lignification. In *Arabidopsis*, a seedpod has three tissues: Two laterally positioned valves that protect seeds; a thin ridge of cells, or "replum", where seeds are attached; and two valve margins that connect the replum and valves (Lewis et al. 2006). The valve margin consists of a lignified layer and a separation layer. When that margin separates, the fruit opens for seed dispersal (Liljegren et al. 2004; Lewis et al. 2006). Functionally redundant *SHP1* and *SHP2* MADS box genes modulate differentiation of the dehiscence zone and promote lignification (Liljegren et al. 2000). The *REPLUMPESS* (*RPL*) and *BP* homeobox genes are key regulators for replum development and control the preferential expression of genes in the valve margin (Venglat et al. 2002; Roeder et al. 2003). Whereas the abscission zone in *Arabidopsis* is located at that margin, the zone in cereal crops is at the base of the pedicel (Tang et al. 2013). In *Sorghum propinquum*, a wild sorghum, shattering occurs when SpWRKY is expressed during the development of floral organs and seeds (Tang et al. 2013). *SpWRKY* is an ortholog of *Medicago MtSTP* and *Arabidopsis AtWRKY12*, both of which regulate cell wall biosynthesis and lignin deposition (Wang et al. 2010; Tang et al. 2013). In *Glycine max*, a NAC gene, *SHATTERING1-5*, activates the biosynthesis of secondary walls and promotes their thickening in fiber cap cells (Dong et al. 2014).

Lignin is a major concern in the pulp and paper industry because harsh chemical treatments are required in order to remove it from wood so that one can obtain pure cellulose (Peter et al. 2007; Bonawitz and Chapple 2013). The ability to alter lignin contents could also help in improving feed digestibility and lignocellulose saccharification for biofuel production (Ookawa et al. 2014). Genetic engineering techniques used to achieve such modifications usually exploit the regulation of lignin biosynthesis genes (Leple et al. 2007; Bonawitz and Chapple 2013). In transgenic poplar, downregulation of *CCR* reduces the lignin content and coloration of the outer xylem (Leple et al. 2007). Although their growth is negatively affected, those plants show improved pulping characteristics. Abnormal lignin deposition influences plant development primarily because of dwarfing, collapsed xylem tissue, and problems with the vascular system (Leple et al. 2007; Vanholme et al. 2008; Hirano et al. 2012). However, it remains unclear whether such irregularities result directly from those modifications to lignin deposition or are instead a consequence of the accumulation of certain intermediates or byproducts (Bonawitz and Chapple 2013).

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