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Original Paper

MYB-mediated upregulation of lignin biosynthesis in *Oryza sativa* towards biomass refinery

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Abstract Lignin encrusts lignocellulose polysaccharides, and has long been considered an obstacle for the efficient use of polysaccharides during processes such as pulping and bioethanol fermentation. However, lignin is also a potential feedstock for aromatic products and is an important by-product of polysaccharide utilization. Therefore, producing biomass plant species exhibiting enhanced lignin production is an important breeding objective. Herein, we describe the development of transgenic rice plants with increased lignin content. Five *Arabidopsis thaliana* (Arabidopsis) and one *Oryza sativa* (rice) MYB transcription factor genes that were implicated to be involved in lignin biosynthesis were transformed into rice (*O. sativa* L. ssp. *japonica* cv. Nipponbare). Among them, three Arabidopsis MYBs (*AtMYB55*, *AtMYB61*, and *AtMYB63*) in transgenic rice T_1 lines resulted in culms with lignin content about 1.5-fold higher than that of control plants. Furthermore, lignin structures in *AtMYB61*-overexpressing rice plants were investigated by wet-chemistry and two-dimensional nuclear magnetic resonance spectroscopy approaches. Our data suggested that heterologous expression of *AtMYB61* in rice increased lignin content mainly by enriching syringyl units as well as *p*-coumarate and tricin moieties in the lignin polymers. We contemplate that this strategy is also applicable to lignin upregulation in large-sized grass biomass plants, such as *Sorghum*, switchgrass, *Miscanthus* and *Erianthus*.

Key words: AtMYB61, lignin, *p*-coumarate, tricin, upregulation.

Introduction

Lignocellulose biomass accounts for the highest proportion of terrestrial biomass on earth (Yoda 1982), and mainly consists of the secondary cell wall of vascular plants. It represents the most promising natural and renewable resource and, consequently, has attracted increased research interests. Future demands for lignocellulose biomass from the biomass-refinery industry will heighten the need for the methods to increase lignocellulose biomass production. Additionally, improvement in the utilization characteristics of lignocellulose biomass is also a key to boost lignocellulose utilization economy.

Lignocellulose is mainly composed of lignin and polysaccharides, i.e. cellulose and hemicelluloses. Lignin fills the spaces between cell wall polysaccharides and confers mechanical strength and imperviousness to the cell wall of vascular plants (Boerjan et al. 2003). Lignocellulose functions *in planta* as a structural material to support the plant body, and not as a storage material that is biologically recycled. Therefore, lignocellulose is inherently difficult to exploit by chemical and biochemical conversions. Lignin possesses several properties that interfere with the use of lignocellulosic polysaccharides in processes such as chemical pulping, forage digestion, and enzymatic hydrolysis. It has long been considered that plant materials with low lignin

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content or easily removed lignin may help to facilitate the polysaccharide utilization (Gressel 2008; Hisano et al. 2009; Vanholme et al. 2008). Lignin is a complex phenylpropanoid polymer that is biosynthesized via oxidative coupling of *p*-hydroxycinnamyl alcohols (monolignols) and related compounds formed in the cinnamate/monolignol pathway (Umezawa 2010). Numerous transgenic and mutant plants with downregulated expression of genes encoding enzymes of this pathway have been generated. These transgenic plants generally contain lower lignin content, leading to more efficient enzymatic saccharification, forage digestion, and pulping (Chen and Dixon 2007; Chiang 2006; Vanholme et al. 2008; Weng et al. 2008). We also reported transgenic rice (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare) lines with decreased lignin content, which exhibited significantly enhanced enzymatic saccharification efficiency (Hattori et al. 2012; Koshiba et al. 2013a, 2013b).

On the other hand, increased lignin content in plants has long been disregarded for lignocellulose utilization even though lignin has valuable attributes related to many aspects of lignocellulose production and utility. First, lignin is a potential feedstock for various bio-based aromatic chemicals and biofuels, and has generated considerable interest as a result (Marshall and Alaimo 2010; Pu et al. 2008; Zakzeski et al. 2010). For example, aromatic components can increase the heat resistance and mechanical strength of engineered plastics, and lignin represents a potential biomass-derived source of these aromatic components (Ishii et al. 2013). Second, because lignin has higher carbon content and heating values than polysaccharides, lignin-derived components in kraft-pulping effluents are being exploited as an important by-product fuel in the pulp mills. Global production of this fuel in 1999 was equivalent to 60 billion liters of crude oil (Yokoyama and Matsumura 2008). Additionally, higher lignin content is beneficial for wood and grass fuels. Third, higher plant lignin content may be correlated with higher biomass production. Relatively large graminaceous plants, such as bamboo (*Phyllostachys heterocycla*) (approximately 14 m tall) and *Erianthus* (approximately 4.5 m tall) generally have higher lignin content (approximately 26% and 23–28%, respectively) (Higuchi 1957; Itoh 1990; Yamamura et al. 2013) than smaller graminaceous plants such as rice (*O. sativa*, approximately 80 cm tall with ca. 10–15% of lignin content) (Suzuki et al. 2009). This difference may be because larger plants require greater mechanical strength than smaller plants, which can be achieved with relatively high lignin content. In addition, excessive reduction in lignin content in breeding programs by cross-fertilization often results in drastic increase in the frequency of lethality (Casler et al. 2002). Therefore, higher lignin content is important for increased lignocellulose production and utility.

The objective of this study was to produce transgenic rice plants with elevated lignin content. Various *MYB* transcription factor genes, comprising a large family of plant transcriptional factors, have been reported to directly or indirectly regulate the expression of lignin biosynthetic genes in many plant species (Nakano et al. 2015; Ye and Zhong 2015; Yoon et al. 2015). Herein, we describe the generation of transgenic rice plants overexpressing five Arabidopsis and one rice MYB genes under the control of the Cauliflower mosaic virus (CaMV) 35S promoter to represent the concept of lignin upregulation. The overexpressions of three Arabidopsis MYBs, i.e., AtMYB55, AtMYB61, and AtMYB63, led to increased lignin content in rice culms of T_1 plants. This strategy may be applicable to upregulating lignin production in large graminaceous biomass crops, such as those expected for biomass feedstock production, e.g., *Sorghum*, switchgrass, *Miscanthus*, and *Erianthus*.

Materials and methods

Instrumentation

UV absorbance was measured with a SH-1000 Lab Microplate Reader (Corona Electric Co., Ltd., Ibaraki, Japan). Gas chromatography-mass spectrometry (GC-MS) analysis was performed using Shimadzu GC-MS systems, a QP-5050A for thioacidolysis and a QP-2010 Ultra for nitrobenzene oxidation (Shimadzu Co., Ltd., Kyoto, Japan), respectively. The GC-MS conditions were as follows: Shimadzu HiCap CBP10-M25-025 column (25 m×0.22 mm for QP-5050A, 20 m×0.22 mm for QP-2010 Ultra); carrier gas, helium; injection temperature, 230°C; oven temperature, 40°C at t=0 to 2 min, then to 230°C at 40°C min^{-1}; ionization, electron-impact mode (70 eV).

Nuclear magnetic resonance spectroscopy (NMR) was conducted using a Bruker Biospin Avance III 800 system (800 MHz, Bruker Biospin, Billerica, MA, USA), and the central DMSO solvent peaks (δ_C/δ_H: 39.5/2.49 ppm) were used as an internal reference. Adiabatic heteronuclear single-quantum coherence (HSQC) experiments were carried out using a standard implementation ("hsqcgcep.3") using parameters described previously (Mansfield et al. 2012). Data processing and analysis used Bruker TopSpin 3.1 software (Bruker Biospin, Billerica, MA). HSQC plots were obtained with typical matched Gaussian apodization in F2 and squared cosine-bell apodization and one level of linear prediction (32 coefficients) in F1.

Plant transformation constructs

To overexpress *AtMYB55*, *AtMYB61*, *AtMYB63*, and *AtMYB86*, we used the previously established *Agrobacterium tumefaciens* EHA101 strains harboring pGWB2 vectors containing the cDNA inserts of RIKEN Arabidopsis full-length (RAFL) cDNA clones (RIKEN Bioresource Center, Tsukuba, Japan) (Ogawa et al. 2008). To overexpress *AtMYB103* and *OsMYB55*/*61*, we first amplified the coding sequences (CDS) by polymerase chain

Table 1. Templates and primers used for construction of plant transformation vectors.

Target gene name	Template	Forward primer	Reverse primer
AtMYB103	Arabidopsis cDNAs	5′-CAC CAT GGG TCA TCA CTC ATG CT-3′	5′-AAA CGA AGA AGG GAA AGA AGA AGA TAA GGC-3′
OsMYB55/61	J013087D08	5′-CAC CAT GGG GAG ACA TTC CTG-3′	5′-GAT ATT CTC AAA AGA CAA GGA CAT CCT TTG-3′
YFP	pH35YG	5′-CACCATGGTGAGCAAGGGC-3′	5′-CTTGTACAGCTCGTCCATGCC-3′

reaction (PCR) using KOD Plus DNA polymerase (TOYOBO, Osaka, Japan) or *pfu* polymerase (Promega KK, Tokyo, Japan), gene specific primer sets, and Arabidopsis cDNAs (Noda et al. 2013) or a cDNA clone (Rice Genome Resource Center, National Institute of Agrobiological Sciences, Tsukuba, Japan) as the templates (Table 1). We also amplified a *yellow fluorescent protein* (*YFP*) gene to generate transgenic control plants using pH35YG vector as the template (Table 1). The PCR products were then subcloned into the entry vector pENTR/D-TOPO (Life Technologies Corporation, Tokyo, Japan). The accuracy of the constructs was confirmed by DNA sequencing. The rice transformation vector was produced by an LR Clonase (Life Technologies Corporation) reaction between the entry vector and the pGWB2 destination vector (Nakagawa et al. 2007).

Preparation of transgenic rice (T_0 generation)

The prepared vectors were introduced into *A. tumefaciens* strain EHA101 using a freeze-thaw method (Holsters et al. 1978). Primary rice transformants (T_0) were generated from *O. sativa* L. ssp. *japonica* cv. Nipponbare calli as previously described (Hattori et al. 2012) using the *Agrobacterium* strains.

Cultivation of rice plants

Transgenic rice plants (T_0 generation, approximately 3 months after inoculation with *Agrobacterium*) were transplanted into 1/10,000a Wagner pots containing a 1 : 1 mixture of vermiculite and "Hanasaki Monogatari" garden soil (Akimoto-Tensanbutsu, Iga, Japan). The transplanted rice plants were grown to maturity at 27°C under a 12-h light/12h-dark photoperiod in a Koitotoron growth chamber (Koito Industries, Yokohama, Japan). The pot bases were constantly immersed in water (a few centimeters deep). The first-generation progeny of the transformants (T_1) and wild-type (WT) plants were cultivated as previously described (Koshiba et al. 2013a).

Preparation of dried cell wall samples

Two months after transplanting, the youngest leaves (approximately 30 cm long, including the blade and sheath) were harvested from T_0 rice plants and oven-dried overnight at 60°C. About 4 months after transplanted into containers filled with nutrient solution, the aerial parts of WT and T_1 rice plants were harvested and dried at room temperature for 1 month. The panicles were removed from the aerial parts, and the remaining plant material was separated into leaves, leaf sheaths, and culms. The separated plant samples were individually cut into pieces with scissors and then pulverized with a TissueLyser (Qiagen, Hilden, Germany), extracted sequentially with methanol, hexane, and distilled water, and then freeze-dried as described previously (Koshiba et al. 2013a, 2013b).

Lignin analysis

Lignin quantitation by the thioglycolic acid method, microscale nitrobenzene oxidation analysis, and thioacidolysis were conducted according to the methods as previously described (Koshiba et al. 2013a, 2013b; Suzuki et al. 2009; Yamamura et al. 2010, 2012). Two-dimensional NMR (2D-NMR) analysis was conducted as previously reported (Mansfield et al. 2012). Briefly, extractive-free and dried cell wall samples were ball-milled using a planetary micro mill Pulverisette 7 (FRITSCH GmbH, Idar-Oberstein, Germany) with ZrO_2 vessels containing ZrO_2 ball bearings (600 rpm, 10 cycles of 10 min with 5 min interval). The obtained ball-milled cell wall samples (ca. 60 mg) were then dispersed in DMSO-d_6/pyridine-d_5 (4 : 1, vol/vol) and subjected for NMR measurement.

Quantitative reverse transcription polymerase chain reaction analysis

Total RNAs were extracted from flag leaves at the heading stage of rice T_1 and WT rice plants. Quantitative reverse-transcription PCR (qRT-PCR) was conducted as previously described (Koshiba et al. 2013a, 2013b). Each gene was amplified using specific primers: 5′-TTG GTA TGT GGC CAT GTA ACC A-3′ and 5′-GGC CAT ATG CAT GTT GCT GA-3′ for *AtMYB55*, 5′-AAC ATG GTT GGT TCT GTC CTT CA-3′ and 5′-AAT CGA GGG CTT TAC GCA TAC T-3′ for *AtMYB61*, 5′-ACA AAC CCG ATC TGC TGG AG-3′ and 5′-TCC AAA TGT CAG GAT CTG AAT CAA-3′ for *AtMYB63*, respectively. A ubiquitin gene (*OsUBQ5*; accession no. AK061988) was amplified using specific primers 5′-ACC ACT TCG ACC GCC ACT ACT-3′ and 5′-ACG CCT AAG CCT GCT GGT T-3′ and used as an internal control. The ΔΔCt method was adopted for calculation of gene expression (User Bulletin #2, Applied Biosystems).

Phylogenetic analysis

Full-length Arabidopsis and rice R2R3-MYB protein sequences (Hirano et al. 2013a; Zhao and Bartley 2014) were downloaded from The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org), the Rice Annotation Project (RAP-DB, http://rapdb.dna.affrc.go.jp/), and the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) databases. Multiple alignments were generated from the conserved R2R3 domain (Stracke et al. 2001) with ClustalX (Thompson et al. 1997) under default parameters. Phylogenetic trees (Figure 1) were generated from the alignments using the neighbor-joining method program in ClustalX with the following parameters:

"1,000 bootstrap replicates" and "exclude position with gaps". Then, we specified Arabidopsis AtMYB4 as an outgroup in the phylogenetic tree using NJplot (Perrière and Gouy 1996) and visualized the tree using TreeView (Page 1996).

Results

Lignin upregulation by MYB genes

A number of NAC and MYB transcription factors were proved or suggested to be involved in lignification (Nakano et al. 2015; Umezawa 2010; Ye and Zhong 2015; Yoon et al. 2015). For example, AtNACs such as AtNST1, AtNST2, AtVND6, AtVND7, and AtSND1, and AtMYBs such as AtMYB46 and AtMYB83 were found to be located in upstream steps of transcriptional network for secondary cell wall formation (Nakano et al. 2015; Ye and Zhong 2015; Yoon et al. 2015). Heterologous expression of these upstream regulatory genes in rice affects biosynthesis of all cell wall components, resulting in severe modification of the cell wall formation and eventually growth of transformants. On the other hand, selective upregulation of lignin biosynthesis is probably beneficial to avoid or minimize negative effects of metabolic engineering on the growth. Overexpression of AtMYB61 (Newman et al. 2004), AtMYB85 (Zhong et al. 2008), AtMYB58 (Zhou et al. 2009), and AtMYB63 (Zhou et al. 2009) induced ectopic lignification, while AtMYB103 was reported to activate syringyl (S)-lignin biosynthesis (Öhman et al. 2013). In the present study, as the first preliminary experiment to represent the concept of lignin upregulation for biomass refinery, we selected three of these activator-type MYB genes, *AtMYB61*, *AtMYB63*, and *AtMYB103*. In addition, because Arabidopsis transcription factors may function differently when heterologously expressed in rice, we also included one close rice homolog of *AtMYB61*, *OsMYB55/61* (BAF04687) (Hirano et al. 2013a) (Figure 1), and two Arabidopsis homologs of *AtMYB61*, namely *AtMYB55* and *AtMYB86* (Figure 1).

The selected genes were then overexpressed in rice plants under the control of the CaMV 35S promoter. To screen for the *MYB* genes that are potent for lignin upregulation in rice, we measured the lignin content of ca. 30-cm-long leaves sampled from regenerated T_0 plants in the vegetative phase with a plant height of ca. 40 cm. The average lignin content in the transgenic lines is provided in Table 2. The overexpression of *AtMYB55*, *AtMYB61*, and *AtMYB63* significantly ($p<0.05$, Table 2) increased lignin content compared with that of the control (Table 2). Therefore, the T_1 generation of these transgenic lines was further subjected to more detailed analysis using the plants. The expression of *AtMYB55*, *AtMYB61*, and *AtMYB63* in the flag leaves of transgenic T_1 rice plants was confirmed by qRT-PCR (Figure 2). The lignin content of leaves, leaf sheaths, culms and panicles collected from mature plants was analyzed, and the data for culms are presented in Figure 2. The gene expression and the lignin content seemed inconsistent. In particular,

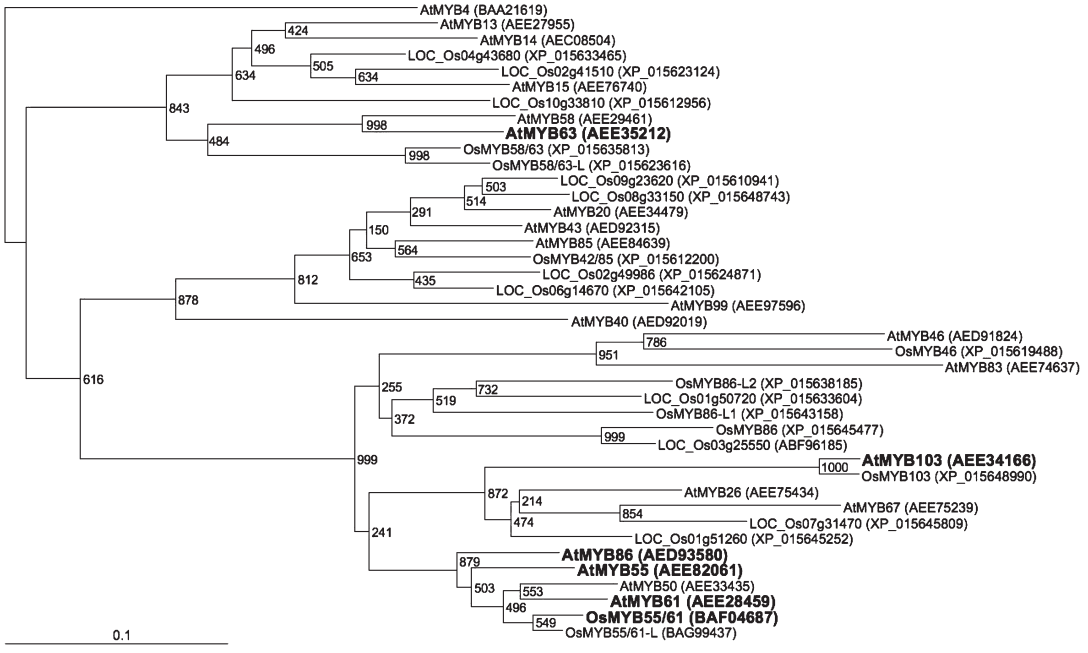

Figure 1. Phylogenetic relationships among Arabidopsis and rice MYB proteins. The numbers at nodes represent bootstrap values obtained after 1000 replicates. AtMYB4 is specified as an outgroup. MYB proteins targeted in this study are noted in bold type. The prefixes At and Os indicate Arabidopsis and rice proteins, respectively. Note that not all MYB proteins in the Arabidopsis and rice genomes are shown. The GenBank accession numbers are given in parentheses after gene names. Rice gene names are based on Hirano et al. (2013b), Zhao and Bartley (2014), and Noda et al. (2015).

the *AtMYB55* transgene expression was as low as less than 0.004 relative to the *OsUBQ5* expression. This awaits further studies to elucidate the mechanisms, but might be due to the difference of the organs. We could not obtain both data with the same organ specimens. Because lignin measurement must be done with mature tissues, in which lignification has already ceased, we used alive flag leaves for the gene expression analysis and tried to make sure that the target genes were surely expressed in the plants. The genes were driven by CaMV 35S promoter and therefore the genes should be expressed in the culms as well as the flag leaves. *AtMYB55* and *AtMYB61* were found to be particularly effective for lignin upregulation. Transgenic rice plants overexpressing these genes contained approximately 1.5-fold lignin than the WT controls (*AtMYB55-2-3*, 50% increase; *AtMYB61-3-3*, 53% increase; and *AtMYB61-3-4*, 50% increase; compared with the WT) (Figure 2). Upregulated lignin production was also observed in an *AtMYB63*-overexpressing line (*AtMYB63-3-4*, 25%), albeit at lower levels than the *AtMYB55*- and *AtMYB61*-overexpressing lines. There were no noticeable differences in the growth of transgenic lines with increased lignin content and WT lines under the cultivation conditions used in this study.

Lignin analysis of AtMYB61-overexpressing lines

We conducted a series of structural analyses using the extractive-free cell wall samples prepared from *AtMYB61*-overexpressing lines to obtain structural information regarding the lignins. Nitrobenzene oxidation analysis revealed the syringaldehyde/vanillin (Sa/Va) and *p*-hydroxybenzaldehyde/vanillin (Ha/Va) ratios were slightly higher for the *AtMYB61*-overexpressing lines than for the controls (Table 3). This observation suggested that upregulated lignin biosynthesis through the overexpression of *AtMYB61* modified the cell wall aromatic composition in addition to increasing the lignin content. However, it is important to note that the degradation of *p*-coumarates and ferulates in lignin and/or hemicelluloses of grass cell walls also release the same *p*-hydroxybenzaldehyde derivatives as those released from monolignol-derived typical lignin moieties (Chen 1992). In addition, it is conceivable that syringaldehyde is also formed from the S aromatic ring on tricin units by nitrobenzene oxidation. Hence, we also conducted a thioacidolysis analysis to determine lignin aromatic composition associated with *β*-*O*-4 substructures in the lignin polymers. As summarized in Table 4, the syringyl/guaiacyl (S/G) ratios

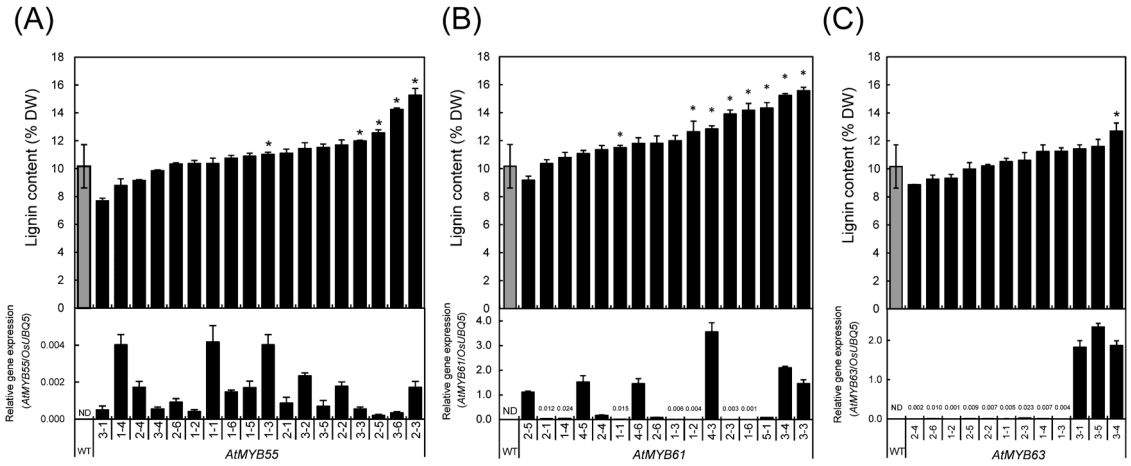

Figure 2. Relative gene expression rate (lower panels) and lignin content (upper panels) in the culm of wild-type (WT) and *AtMYB*-overexpressing rice plants. (A), *AtMYB55*-, (B), *AtMYB61*-, and (C), *AtMYB63*-overexpressed rice plants. Each value in the lower panels is the mean of three replicates±SD, and expressed relative to the expression of *OsUBQ5*. ND, Not Detected; *, Significant difference (Student's *t*-test; $p<0.05$); DW, dry weight.

Table 2. Thioglycolic acid lignin content in ca. 30-cm-long leaves of T_0 transgenic rice lines.

Overexpressed genes	Numbers of transgenic lines	Lignin content (%)		*p*-Value
		Average	SD	
YFP (control)	10	5.99	1.00	—
AtMYB55	16	7.31	1.37	0.005**
AtMYB61	14	7.15	1.81	0.030*
AtMYB63	15	8.35	2.16	0.019*
AtMYB86	9	5.54	1.06	0.177
AtMYB103	24	6.30	1.13	0.221
OsMYB55/61	14	7.09	2.40	0.072

The *p*-Value was calculated using Student's *t*-test to control. SD, standard deviation; *, $p<0.05$; **, $p<0.01$ (Student's *t*-test).

were higher for the three *AtMYB61*-overexpressing lines than for the controls, implying the abundance of S lignin increased in the cell walls of *AtMYB61*-overexpressing rice plants.

Lastly, 2D-NMR was used for a more in-depth analysis of lignin structures. All HSQC spectra for the cell wall samples of *AtMYB61*-overexpressing and control lines (Figure 3) displayed typical lignin aromatic signals for G and S units, as well as those from *p*-hydroxyphenyl (H) unit, albeit at low levels. Aromatic signals from *p*-coumarate, ferulate and tricin residues in lignin and/or hemicelluloses were clearly observed. A quantitative analysis of the HSQC contour signals revealed that the S/G signal ratio increased in all the three *AtMYB61*-overexpressing lines. The result was well consistent with the thioacidolysis data (Table 4), and further confirmed that *AtMYB61* overexpression increased S lignin content. More significantly, our NMR analysis revealed that the signals from *p*-coumarate and tricin units were greater in the spectra of *AtMYB61*-overexpressing lines than in the WT spectrum. Increased *p*-coumarate and tricin units in the *AtMYB61*-overexpressing cell wall samples were in line with the increased *p*-hydroxybenzaldehyde and syringaldehyde yields upon nitrobenzene oxidation (Table 3). Overall, our data suggested that *AtMYB61* overexpression increased lignin content in rice cell walls

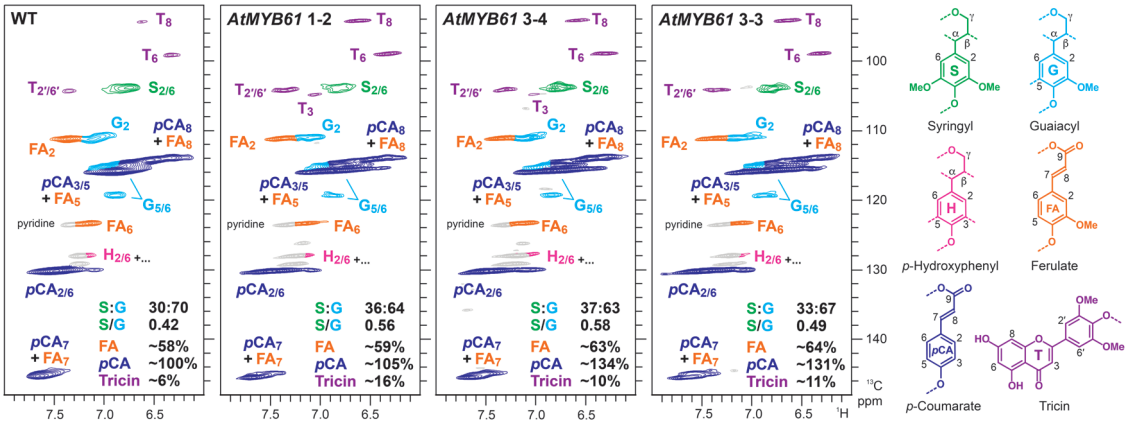

Figure 3. Aromatic subregions of short range ^{1}H–^{13}C correlation (HSQC) NMR spectra of cell wall samples isolated from wild-type (WT), and *AtMYB61*-overexpressing rice plants. Volume integrals are given for the aromatic units that are color-coded to match their assignments in the spectrum. The percentages reported are integrals relative to total of G and S aromatic units.

Table 3. Nitrobenzene oxidation analysis on *AtMYB61*-overexpressing rice transgenic lines.

Line	Vanillin (Va)	Syringaldehyde (Sa)	*p*-Hydroxybenzaldehyde (Ha)	Vanillic acid (VA)	Syringic acid (SA)	*p*-Hydroxybenzoic acid (HA)	Sa/Va	Ha/Va
			μmol g^{-1} cell wall residue (CWR)					
WT	208.69±4.03	50.29±0.74	52.62±2.37	13.67±0.04	9.57±0.34	15.96±0.10	0.24±0.00	0.25±0.01
AtMYB61 1–2	196.39±6.36*	50.39±0.21	56.00±3.32	12.96±0.16**	9.05±0.27	15.72±0.07*	0.26±0.01*	0.29±0.03
AtMYB61 3–4	164.86±7.96**	48.52±2.72	55.83±4.60	12.89±0.05**	9.07±0.55	15.66±0.04**	0.29±0.00**	0.34±0.01**
AtMYB61 3–3	212.51±14.19	61.04±0.63**	65.23±2.23**	13.67±0.16	10.51±0.32*	16.02±0.11	0.29±0.02	0.31±0.03*

Data represent the average±SD (n=3). Asterisks indicate significant differences (Student's t-test; p<0.05 and 0.01 for * and **, respectively). Sa/Va and Ha/Va: Molar ratios of Sa to Va and Ha to Va, respectively.

Table 4. Changes in lignin *β*-*O*-4 substructure composition in the *AtMYB61*-overexpressed plants.

Line	Gt	St	Ht	S/G	H/G
		% for total peak area of trithioethylpropane compounds			
WT	58.92±0.29	31.38±0.39	9.70±0.48	0.53±0.01	0.16±0.01
AtMYB61 1–2	57.09±0.68	32.56±0.66	10.35±0.81	0.57±0.01 **	0.18±0.02
AtMYB61 3–4	52.10±1.12	37.16±2.32	10.74±1.69	0.71±0.06 **	0.21±0.03 *
AtMYB61 3–3	54.11±0.30	35.92±1.85	9.97±1.61	0.66±0.04 **	0.18±0.03

Data represent the average±SD (n=3). Asterisks indicate significant differences (Student's t-test; p<0.05 and 0.01 for * and **, respectively. Gt, guaiacyltrithioethylpropane (m/z 269, base-ion); St, syringyltrithioethylpropane (m/z 299, base-ion); Ht, *p*-hydroxyphenyltrithioethylpropane (m/z 239, base-ion) formed following thioacidolysis of lignin. S/G (syringyl/guaiacyl) and H/G (*p*-hydroxyphenyl/guaiacyl), peak area ratios of St to Gt and Ht to Gt, respectively.

mainly by enriching the abundance of S lignin units as well as *p*-coumarate and tricin moieties.

Discussion

The present study demonstrated that upregulated lignin biosynthesis in rice plants was stimulated by heterologous expression of the genes encoding *Arabidopsis* transcription factors AtMYB55, AtMYB61, and AtMYB63. Our results indicated that these *Arabidopsis* MYBs were also functional in rice plants, although, to the best of our knowledge, there are no reports describing a conclusive functional analysis of AtMYB55 in *A. thaliana* plants. On the other hand, AtMYB61 has been known to play a pleiotropic role (Dubos et al. 2010), affecting lignification (Newman et al. 2004), mucilage production (Penfield et al. 2001) and stomatal aperture (Liang et al. 2005). It was also suggested to potentially control resource acquisition and allocation (Romano et al. 2012). We observed that the overexpression of *AtMYB61* in rice plants resulted in increased production of lignin, which is similar to the findings of a previous study in which CaMV 35S-regulated overexpression of *AtMYB61* in Arabidopsis plants caused ectopic lignification (Newman et al. 2004). Interestingly, however, *AtMYB61* overexpression in rice increased the abundance of S lignin units and also *p*-coumarate and tricin moieties that are the lignin partial structures characteristic of grasses (Lan et al. 2015; Ralph 2010) (Table 3, 4 and Figure 3). This result may help characterize the transcriptional control for the biosynthesis of grass lignins.

The lignin content in T_1 transgenic rice plants was up to 53% higher than that of WT plants (Figure 2), implying it may be possible to produce graminaceous biomass plants with greater lignin content and heating values. The higher heating value (HHV) is highly correlated with lignin content and is calculated for wood using the following equation:

$$HHV = 17.61 + 0.0854X_L,$$

where HHV = higher heating value of extractive-free wood ($MJ\,kg^{-1}$) and X_L = lignin content (%) (White 1987). A 53% increase in lignin abundance in lignocellulosic biomass samples originally containing 10% and 20% lignin content gives rise to 15.3% and 30.6% lignin content, resulting in 2.5% and 4.7% increase in HHVs, respectively.

In addition to transcriptional activators, several transcriptional suppressors (Nakano et al. 2015; Xu et al. 2014; Ye and Zhong 2015; Yoon et al. 2015) are known to regulate lignin biosynthesis. Downregulation of these suppressors is likely to induce upregulation of lignin biosynthesis. Knockout of the suppressor genes may be achieved by non-transgenic technologies such as mutagenesis using chemicals or ion beam irradiation.

These strategies describe herein for lignin upregulation may be applicable to large-sized graminaceous plants such as *Sorghum*, *Erianthus*, napier grass, switchgrass, and *Miscanthus*. The increased lignin content in these plants may be beneficial for fuel use and aromatic feedstock production, and ultimately lead to greater biomass productivity, which will help decrease the land area required for biomass production.

In conclusion, we have generated transgenic rice lines with lignin content approximately 1.5-fold higher than that of control plants by upregulation of *MYB* transcription factor genes. This approach may be applicable for lignin upregulation in large graminaceous plants, which are biomass resources potentially useful for the production of fuels and aromatic industrial feedstock production.

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