Identification of Transcription Factors Involved in Rice Secondary Cell Wall Formation

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Using co-expression network analysis, we identified 123 transcription factors (TFs) as candidate secondary cell wall regulators in rice. To validate whether these TFs are associated with secondary cell wall formation, six TF genes belonging to the MYB, NAC or homeodomain-containing TF families were overexpressed or downregulated in rice. With the exception of OsMYB58/63-RNAi plants, all transgenic plants showed phenotypes possibly related to secondary cell wall alteration, such as dwarfism, narrow and dark green leaves, and also altered rice cinnamyl alcohol dehydrogenase 2 (OsCAD2) gene expression and lignin content. These results suggest that many of the 123 candidate secondary cell wall-regulating TFs are likely to function in secondary cell wall formation in rice. Further analyses were performed for the OsMYB55/61 and OsBLH6 TFs, the former being a TF in which the Arabidopsis ortholog is known to participate in lignin biosynthesis (AtMYB61) and the latter being one for which no previous involvement in cell wall formation has been reported even in Arabidopsis (BLH6). OsMYB55/61 and OsBLH6-GFP fusion proteins localized to the nucleus of onion epidermal cells. Moreover, expression of a reporter gene driven by the OsCAD2 promoter was enhanced in rice calli when OsMYB55/61 or OsBLH6 was transiently expressed, demonstrating that they function in secondary cell wall formation. These results show the validity of identifying potential secondary cell wall TFs in rice by the use of rice co-expression network analysis.

Keywords: co-expression network • lignin • rice • secondary cell wall • transcription factor.

Abbreviations: BLH, bell-like homeodomain-containing; EAR, ethylene-responsive element binding factor-associated amphiphilic repression transcription repression motif; TF, transcription factor.

Introduction

1 Owing to recent advances in molecular and bioinformatic techniques, numerous biological data are now freely available from public databases (e.g. sequence information, RNA expression profiling, protein–protein interactions); this is especially true for model species such as rice and Arabidopsis. When attempting to reveal the molecular mechanisms governing biological events, it is first necessary to identify candidate factors from these data through an efficient and accurate searching procedure.

2 In this study, we attempted to identify transcription factors (TFs) involved in secondary cell wall formation in rice. The plant cell wall is the most abundant biomaterial on earth and its major constituents, such as cellulose, xylose, and lignin, are expected to be used as sustainable energy resources (Yuan et al. 2008). Many of the current energy crops are grasses, which have a cell wall structure that is distinct from those of many other plant species (Vogel 2008). It is therefore important to understand cell wall formation in grasses to be better able to meet the challenge of modifying the cell wall for production of improved energy resources. To date, only three rice TFs have been reported to be associated with secondary cell wall formation (Zhong et al. 2011). Each of these genes is able to complement Arabidopsis mutants defective in secondary cell wall-regulating TFs. However, to our knowledge there has been no attempt to directly investigate the role of secondary cell wall TFs in rice.

3 In the accompanying paper (Hirano et al.) we present a procedure to identify candidate genes involved in rice cell wall formation. The procedure depends on creating co-expression networks of cell wall-related genes in rice and Arabidopsis (hereafter referred to as cell wall-associated genes), which are...
then plotted onto a phylogenetic tree. When both rice and Arabidopsis cell-wall-associated genes are within the same clade of the phylogenetic tree, we suppose these genes to be good candidates for functioning in cell wall formation.

To evaluate the performance of this procedure in identifying novel genes involved in rice secondary cell wall formation, this study used transgenic rice plants overproducing or downregulating cell-wall-associated TFs, including genes with a previously reported function in Arabidopsis (but not in rice) and genes with no previously reported function in rice or Arabidopsis. The transgenic rice plants showed phenotypes possibly related to cell wall alteration, suggesting that these TFs function in secondary cell wall formation. This finding demonstrates that this candidate gene identification procedure is an effective method for the identification of novel TFs involved in cell wall formation in both rice and Arabidopsis.

**Results**

**Analysis of rice secondary wall-associated TF genes**

In the accompanying paper (Hirano et al.) the co-expression network was created using secondary cell wall cellulose synthase genes (OsCesA4, 7, and 9), OsCAD2, rice caffeic acid 3-O-methyltransferase (OsCOMT), the homolog of the Arabidopsis xylan biosynthesis *irregular xylem* 9 gene, and the BRITTLE CULM 1 gene (Li et al. 2003, Tanaka et al. 2003, Peña et al. 2007, Hirano et al. 2012a) as guide genes at the cut-off threshold mutual rank value of 55 (see the accompanying paper for details). To evaluate the validity of the co-expression network-based screening, six rice genes belonging to the MYB, NAC, or bell-like homeodomain-containing (BLH) TF families and which appeared in the secondary cell wall network were selected (Fig. 1). These six genes comprised five genes [OsMYB58/63, OsMYB42/85, OsMYB55/61, OsMYB103, and SECONDARY WALL ASSOCIATED NAC DOMAIN PROTEIN 1 (OsSND1)] whose Arabidopsis homologs are known to function in secondary wall formation (left panel in Fig. 1A–E; Zhong et al. 2006, 2007, 2008, Mitsuda et al. 2007, Zhou et al. 2009), and another gene (OsBLH6) never before implicated in cell wall formation even in Arabidopsis (right panel in Fig. 1F). The expression profile of these genes is provided in the accompanying paper (Supplemental Figs 3 and 4 in the accompanying paper, Hirano et al.); they all show very similar expression patterns and are preferentially expressed in roots and stems, and some in lemmas and paleas.

To examine the involvement of these candidate genes in secondary cell wall formation, the genes were introduced into Arabidopsis *glabra* cv. Taichung 65 to produce transgenic plants showing both overexpression and downregulation. The rice actin promoter was used to produce overexpressors (hereafter referred to as OXs) of the candidate genes (McElroy et al. 1990). Three approaches were used for gene knockdown: RNAi, TOS17-retrotransposon insertion mutagenesis (TOS, Hirochika 2001), and overexpression of genes fused with an ethylene-responsive element binding factor-associated amphiiphilic repression (EAR) transcription repression motif (referred to as SRDX, Hiratsu et al. 2003). When there were two homologs in rice (Fig. 1A, Os02g0695200 and Os04g0594100 in the MYB58/63 clade; Fig. 1D, Os06g0131700 and Os08g0155800 in the SND1 clade; and Fig. 1E, Os01g0285300 and Os05g0140100 in the MYB55/61 clade), for simultaneous downregulation we introduced two kinds of RNAi sequences to target both genes.

Before phenotypic analysis, we confirmed changes in the expression level of the target genes in OX and knockdown plants (Supplemental Fig. 1). All lines showed increased or reduced expression of their target genes in OX and knockdown plants, respectively. However, expression of the OsMYB58/63 gene (Os04g0594100) in OsMYB58/63 RNAi plants was only reduced to 60% compared with the vector control plants. Similarly, expression of the close homolog of OsMYB58/63, Os02g0695200, which was also simultaneously targeted for RNAi suppression, was only reduced to 37% of that of the vector control plants (data not shown), indicating that suppression of both of these genes was not as strong as that of other TF genes. The expression levels of OsBLH6 in its knockdown plants were not analyzed because the SRDX system inhibits the function of the target protein without affecting the transcription level of the endogenous gene.

For the phenotypic analysis, at least three independent transgenic plants were examined for each construct. OX plants showed some common morphological features, typically displaying a dwarf phenotype and producing narrow, dark green leaves (Fig. 2, Supplemental Fig. 2), although the severity of this phenotype differed depending on the gene transformed. The height of OsMYB58/63-OX and OsBLH6-OX plants was not significantly different from that of controls (Supplemental Fig. 2) but these plants did develop dark green leaves (Fig. 2A, F). OsMYB42/85-OX plants displayed a mild dwarf phenotype with narrow, dark green leaves (Fig. 2B) but were fertile and produced seeds. In contrast, OsMYB103-OX, OsSND1-OX, and OsMYB55/61-OXs showed severe dwarf phenotypes with very narrow leaves and did not grow to maturity (Fig. 2C–E). All knockdowns, with the exception of OsMYB58/63 RNAi plants, a dwarf phenotype with dark green and/or narrow leaves (Fig. 3, Supplemental Fig. 2). OsMYB58/63-RNAi plants had no observable phenotype (Fig. 3A), possibly due to mild suppression of OsMYB58/63 and its homologous gene (Supplemental Fig. 1), and were not studied further. OsMYB42/85-TOS17, OsMYB103-RNAi, and OsSND1-RNAi plants showed severe dwarf phenotypes and did not grow to maturity (Fig. 3B–D, respectively). The dwarf phenotypes of the OsMYB55/61-RNAi and OsBLH6-SRDX plants were milder than those described above and the plants were able to set seeds (Fig. 3E, F).

To confirm that these transgenic plants had altered secondary cell walls, we observed cross-sections of leaf sheaths treated with phloroglucinol or calcofluor to stain lignin and glucans, respectively (Fig. 4). Plants transformed with the vector control showed phloroglucinol staining at the vascular bundle and
Fig. 1 List of TFs analyzed in this study. (A–F) Genes encoding TFs (enclosed in orange squares) were overexpressed (shown as OX in green after the gene name) or downregulated in rice to analyze their involvement in secondary cell wall formation. RNAi, overexpression of genes fused with the SRDX motif, or TOS17 retrotransposon insertion mutagenesis was used for downregulation (shown as RNAi, SRDX, and TOS in green after the gene name, respectively). In the RNAi experiments, if two closely related genes were both found in the secondary cell wall network they were simultaneously targeted for downregulation (genes in orange and black squares).
sclerenchyma (indicated by an arrowhead and an arrow, respectively, in Fig. 4A), both of which are indicative of a well-developed secondary cell wall. Compared with control plants, all OXs showed stronger red phloroglucinol staining, especially at the sclerenchyma (indicated by an arrow), with less of a difference in the vascular bundle (arrowhead). Due to premature mortality, phloroglucinol staining was performed early (14 days after transplantation rather than 90 days after transplantation) for OsSND1-OX plants. At this stage, the development of the vascular bundle and sclerenchyma was premature and phloroglucinol staining of control plants was very faint (Fig. 4G), while OsSND1-OX showed strong staining, especially at the vascular bundle (Fig. 4H). This indicates that, among the TFs tested, OsSND1 is the most effective in increasing lignin content.

For glucan staining with calcofluor, some sections, such as those from OsMYB103-, OsBLH6- and OsSND1-OXs, tended to show stronger signals than those of control plants (right panels...
of Fig. 4A, D, F–H), although these differences were less obvious than in phloroglucinol staining. OsBLH6- and OsSND1-OXs also showed ectopic phloroglucinol and calcofluor staining in the parenchyma cells, which were stained very faintly in control plants (Fig. 5). Such ectopic staining has also been reported in Arabidopsis plants overproducing AtSND1, AtMYB46, or AtMYB83 (Zhong et al. 2006, 2007, McCarthy et al. 2009). In contrast to OXs, knockdown plants showed reduced phloroglucinol staining compared with controls (Fig. 6A), especially in the sclerenchyma (arrow in panel A), while no apparent differences in calcofluor staining were seen (right panels of Fig. 6).

For the OsMYB42/85 TOS17 insertion mutant (Fig. 6G), which is derived from Nipponbare and is not a transgenic plant (Fig. 6F), sections were compared with those taken from the same stage of wild-type Nipponbare plants. OsMYB42/85 TOS also showed reduced staining in the sclerenchyma, with no apparent difference in calcofluor staining.

Fig. 3 Phenotypes of plants with knockdown (KD) of TF genes appearing in the rice secondary cell wall network. (A–F) KD plants (right) are compared with the same stages of vector-transformed control plants (left). Except for OsMYB58/63 RNAi plants, KD plants showed a dwarf phenotype, with dark green and/or narrow leaf blades (superimposed). For downregulation, RNAi (A, C–E), TOS17 retrotransposon insertion mutagenesis (B) or overexpression of genes fused with the SRDX motif (F) was used.

To investigate whether our candidate rice cell wall TFs control the expression of OsCAD2, a rice lignin biosynthesis gene whose mutants show reduced lignin accumulation (Zhang K et al. 2006), we analyzed OsCAD2 expression in the roots of each transgenic plant by qRT-PCR. When TFs fused with the glucocorticoid receptor (GR) were overexpressed in rice, treatment with dexamethasone (DEX) enhanced the expression level of OsCAD2 with differing effectiveness (Fig. 7A, relative expression of DEX untreated plants = 1). To confirm that GR-fused TFs could function in rice, we grew OsMYB58/63–GR plants with or without DEX until maturity and stained the internode section with phloroglucinol or calcofluor (Supplemental Fig. 4). Similar to what was observed for OsMYB58/63-overexpressing plants, DEX-treated plants showed stronger phloroglucinol staining of the sclerenchyma compared with plants not treated with DEX, while no apparent difference could be observed for calcofluor staining. In contrast to DEX-treated, GR-fused TFs,
knockdown plants showed reduced OsCAD2 expression compared with control plants carrying an empty vector insert (Fig. 7B), indicating that these TFs regulate transcription of OsCAD2. The lignin contents of each transgenic plant was also measured directly. As expected, OX or knockdown plants accumulated higher or lower amounts of monolignol than control plants, respectively (Supplemental Fig. 3), confirming that these TFs affect lignin biosynthesis.

**Further analyses of novel rice secondary cell wall-associated TFs**

For the TFs OsMYB55/61 and OsBLH6, we performed further analyses of TF activity. First, by transient expression in the bombarded onion epidermal cells we analyzed the cellular localization of OsMYB55/61 and OsBLH6 chimeric proteins fused with Green Fluorescent Protein (GFP) (Supplemental Fig. 5). In contrast to GFP protein, whose fluorescence signal was broadly observed in the cytoplasm and nucleus (Supplemental Fig. 5A), signals of GFP-fused OsMYB55/61 or OsBLH6 were restricted to the nucleus, indicating that they localize to the nucleus (Supplemental Fig. 5B, C). We next examined whether these TFs induce the expression of OsCAD2 in rice callus using an engineered reporter construct containing the promoter of OsCAD2 driving Renilla reniformis luciferase (hRluc) reporter gene expression (Supplemental Fig. 6A). Rice callus was co-bombarded with the reporter construct and an effector (OsMYB55/61 or OsBLH6) under the control of the maize ubiquitin promoter (Supplemental Fig. 6A).
expression of hRluc controlled by the OsCAD2 promoter was enhanced 7.0- and 1.7-fold by OsMYB55/61 and OsBLH6, respectively, compared with the vector control (Supplemental Fig. 6B), although enhancement by OsBLH6 was less than that by OsMYB55/61. This result demonstrates that OsMYB55/61 and also OsBLH6 may possibly function as secondary cell wall-associated TFs by enhancing the transcription of cell wall biosynthesis genes.

**Discussion**

Among the nine MYB family clades containing both rice and Arabidopsis secondary cell wall-associated genes, six clades contained known Arabidopsis MYBs (MYB58/63, MYB42/85, MYB55/61, MYB103, MYB20/43, and MYB52/54; see the accompanying paper, Hirano et al.). We hypothesized that rice MYBs in these clades are strong candidates for secondary cell wall regulating factors and selected MYBs Os04g0594100 (OsMYB58/63), Os09g0532900 (OsMYB42/85), Os01g0285300 (OsMYB55/61), and Os08g0151300 (OsMYB103) for analysis of biological function through transgenic experiments in rice (enclosed in orange squares in Fig. 1A–C, E). With the exception of OsMYB58/63-RNAi plants, all MYB-OX and knockdown plants showed abnormal phenotypes, which may be related to the secondary cell wall, including dwarfism and narrow, dark green leaves. Furthermore, altered OsCAD2 gene expression and lignin content were also observed. There were three other MYB clades consisting of rice and Arabidopsis secondary wall-associated genes with no prior reported functions. These rice genes found in the three remaining MYB clades may also be involved in secondary wall formation.

For the NAC family, among the five clades containing rice and Arabidopsis cell wall-associated genes (see accompanying paper, Hirano et al.), OsSND1 (Os06g0131700) was previously
shown to function in secondary wall formation in rice and, together with two other homologous rice NACs (Os08g0103900 and Os06g0104200), was shown to functionally complement the Arabidopsis snd1/nst1 double mutant, to bind the promoter region of AtMYB46, and to cause ectopic deposition of cellulose, xylan, and lignin when overexpressed in Arabidopsis (Zhong et al. 2011). Based on these observations, Zhong et al. (2011) suggested the possibility that rice SND1 and VASCULAR-RELATED NAC-DOMAIN (VND) homologs, both belonging to the NAC family, function as master regulators in rice. In Arabidopsis, a hierarchy of TFs exists for secondary cell wall formation (McCarthy et al. 2009, Demura and Ye, 2010) and SND1 and VND6/7 are regarded as master regulators required for activating a cascade of downstream TFs, including MYBs, NACs (SND2 and -3) and homeodomain (HD)-containing protein (Knotted1-like homeodomain protein, KNAT7) (Mitsuda et al. 2007, Zhong et al. 2008, Ohashi-Itoh et al. 2010). Our observation that OsSND1/Os06g0131700 had significant effects on secondary cell wall formation in rice, presenting the most severe phenotype among the TFs tested, suggests that OsSND1 has a strong effect on secondary cell wall formation.

OsBLH6 (Os03g0165300) was categorized into a clade containing rice and Arabidopsis secondary wall-associated genes with no previously reported function. OsBLH6-OX and SRDX plants shared similar phenotypes with other OX and knockdown plants, suggesting this HD-containing TF is involved in cell wall formation in rice. This is significant as there have been no previous reports describing the involvement of this type of TF in cell wall formation in either rice or Arabidopsis. As there

**Fig. 6** Phloroglucinol (left) and calcofluor staining (right) of transverse sections of leaf blades in knockdown plants. All knockdown plants showed weaker phloroglucinol staining than control plants, especially in the sclerenchyma (white arrow in A). (A–E) Plants were compared with vector control plants at the same stage (A). An OsMYB42/85 TOS17 insertion mutant was compared with wild-type (Nipponbare) plants at the same stages (F, G). Bar = 50 μm.
had been only three TFs experimentally proved to regulate rice cell wall formation before this study (Zhong et al. 2011), the results on the six genes presented here clearly demonstrate the validity of identifying secondary cell wall TFs through the analysis of TFs appearing in the rice secondary cell wall network; however, more in-depth studies are necessary to fully understand the molecular mechanism of rice secondary cell wall formation. Furthermore, this approach may prove useful for the identification of novel types of secondary wall-regulating TFs in Arabidopsis, as well as novel glycosyltransferases (GTs) and glycosyl hydrolases (GHs) involved in cell wall formation, for which we have conducted network analysis (see accompanying paper, Hirano et al.).

In the present study, transgenic rice with altered TF expression levels (OX or knockdown) displayed similar phenotypes of dwarfism and dark green leaves. Dwarf phenotypes of TF-OX plants have been extensively reported in Arabidopsis (Mitsuda et al. 2005, Zhong et al. 2006, McCarthy et al. 2009, Zhou J et al. 2009), indicating that such a phenotype is shared between TF-OX plants of the two species. However, when downregulated, many of the Arabidopsis TFs show reduced mechanical strength without accompanying dwarfism (Mitsuda et al. 2007, Zhong et al. 2006, 2007, Zhou J et al. 2009), which was not what we observed in our rice TF knockdown plants. Among the TF knockdown rice plants, we also did not observe any phenotypes related to reduced mechanical strength (such as brittle culm phenotype) or plants that were unable to support their body weight. These observations indicate that rice and Arabidopsis show different phenotypes upon downregulation of TFs involved in secondary cell wall formation. Relative to reduced mechanical strength of the rice cell wall, Gui et al. (2011) reported that downregulation of Os4CL3, a rice lignin biosynthesis gene, leads to significant dwarfism compared with normal plants, but reduced mechanical strength was not reported. A rice CAD2 mutant, gh2, possessing slightly reduced lignin content, had no reported mechanical strength phenotype (Zhang K et al. 2006). On the other hand, rice brittle culm mutants, all of which are defective in secondary cell wall formation, show reduced mechanical strength with mixed instances of accompanying dwarfism (Tanaka et al. 2003, Li et al. 2003, Zhang B et al. 2009, Zhou Y et al. 2009, Hirano et al. 2010a, Xiong et al. 2010, Zhang M et al., 2010, Kotake et al. 2011, Wu et al. 2012). The reasons behind these different outcomes should be investigated in future work.

### Materials and Methods

#### Plasmid construction

The sequences of primers used in this study are listed in Supplemental Table 1. All PCR fragments were sequenced to confirm that no mutations had been introduced.
For transformation to overexpress TFs, proAct-FLAG/pCAMBIA was used as the binary vector (Hirano et al. 2010b). TF genes were PCR-amplified and cloned into the XbaI–SpeI site of proAct-FLAG/pCAMBIA, which deletes the FLAG tag. For transformation of TFs fused with the GR, proAct-GR/pBI101 was used as the binary vector. TF genes were PCR-amplified and cloned into the XbaI–SpeI site of proAct-GR/pBI101.

To construct SRDX transgenic plants, a region of the cDNA of each TF gene was amplified in both sense and antisense directions using PCR. For simultaneous knockdown of two genes, each region of the cDNA was fused by fusion PCR in both sense and antisense directions. The resulting PCR fragments were ligated into 933 bp of a partial GUS sequence/pBluescript vector, in a position sandwiching the GUS sequence. Sense–GUS–antisense sequences were ligated between the Act1 promoter and nos terminator in the binary vector pBI101–Ham2.

To construct the SRDX transcriptional repressor fusion, TF genes were cloned into the XbaI–SmaI site of proAct–omega-SRDX/pCAMBIA to produce proAct–omega-TF gene-SRDX/pCAMBIA.

To construct GFP-fused OsMYB55/61 and OsBLH6 for the onion epidermal cell bombardment experiment, OsMYB55/61 and OsBLH6 were cloned into the SmaI site of 35S-GFP/pUC19 to produce 35S-OsMYB55/61-GFP and 35S-OsBLH6-GFP, respectively. The following method was used to construct plasmids for rice callus bombardment experiment. The Renilla reniformis luciferase (hRluc) gene was inserted into the Smal–SacI site of pUC19 to produce hRluc/pUC19. The sequence of OsCAD2 1013 bp upstream of its start codon was PCR-amplified with forward and reverse primers that possess an HindIII and SmaI site at their 5’ ends, respectively, and the PCR product was inserted into the HindIII–SmaI site of hRluc/pUC19 to produce pOsCAD2-hRluc/pUC19. Effector constructs were generated as follows. OsMYB55/61 and OsBLH6 were cloned into the Smal site of maize ubiquitin promoter (pUbi-Ω)/pUC19 (Hirano et al. 2012b) to produce pUbi-Ω-OsMYB55/61/pUC19 and pUbi-Ω-OsBLH6/pUC19, respectively.

To construct BD-OsMYB55/61 and BD-OsBLH6 for Y1H experiments, both genes were cloned into the SmaI site of pGBK7T (Clontech, Mountain View, CA).

Plant transformation

Constructs were introduced into Agrobacterium tumefaciens strain EHA105 and used to infect callus of rice cv. Taichung 65 according to Ozawa (2009). Transformed cells and plants were selected by hygromycin resistance, and regenerants were grown to maturity in pots in a greenhouse. Transgenic plants of the T0 generation were used for the analysis.

Observation of secondary cell wall

For histochemical staining, transformed plants were cut into pieces, fixed in 5% (w/v) agar dissolved in water, and sectioned with a microtome at a thickness of 80–100 μm.

For phloroglucinol staining, sections were incubated for 5 min in phloroglucinol solution [2% in ethanol:water (95:5)], the phloroglucinol was removed and the sections were treated with 18% HCl for 5 min before being observed with an Olympus BX51 microscope (http://www.olympus.co.jp/jp/lisg/bio-micro/product/bx51/). For calcofluor staining, sections were treated with 0.01% calcofluor for 8 min and examined immediately under UV excitation.

DEX treatment

For plants expressing a TF fused with GR, roots of size and shape similar to those of plants generated from the same callus were selected and treated with or without DEX at a concentration of 0.2 M for 1 hour. For rice into which OsMYB58/63-GR had been introduced, the plant was grown to maturity under constitutive treatment with 0.2 M DEX (DEX+) or without DEX treatment (DEX–), and a transverse section of the internode at the heading stage was stained with either phloroglucinol or calcoflour.

RNA isolation and quantitative RT-PCR analysis

Total RNA was prepared from leaf sheaths [for quantitative real-time RT-PCR (qRT-PCR) analysis of genes introduced for overexpression or downregulation] or roots (for OsCAD2 gene expression analysis) of transgenic plants, as described by Chomczynski and Sacchi (1987). First-strand cDNA was synthesized from total RNA using an Omniscript Reverse Transcription Kit (Qiagen, Tokyo, Japan). qRT-PCR was performed with the LightCycler System (Roche, Basel, Switzerland) and the SYBR Green PCR Kit (Qiagen). The results were confirmed using three independent biological replicates. The ubiquitin gene from rice was used as an internal standard to normalize cDNA concentration variations.

Lignin measurement

The amount of lignin in the leaf sheath of transgenic plants was determined as thioglycolic acid lignin by the method of Suzuki et al. (2009).

Transient expression of GFP protein-fused OsMYB55/61 and OsBLH6 in onion epidermal cells

Particle bombardment was carried out using the PDS-1000/He Biolistic Particle Delivery System (Bio-Rad Laboratories, Tokyo, Japan). Plasmid DNA was precipitated onto gold particles (1.6 μm; Bio-Rad Laboratories) by the CaCl2/spermidine method (Klein et al. 1987) and delivered into onion epidermal cells or calli of rice cv. Taichung 65. The parameters for bombardment were 1350 psi and a microcarrier flight distance of 6 cm. For transient expression in onion epidermal cells, after bombardment onion peels were incubated for 16 h in the dark at 30°C. The peels were screened for GFP fluorescence using a Olympus BX51 microscope. The GFP was excited with a 488 nm laser line and GFP fluorescence was detected with a band-pass 505–550 nm filter. To visualize the nucleus, the bombarded
peels were stained with DAPI (4',6-diamidino-2-phenylindole; 0.4 mg/ml). For transient expression using rice calli, after bombardment samples were immersed in a 0.02% Tween 20 solution and incubated for 16 h in the dark at 30°C. Crude protein extracts of calli were prepared under ice by grinding the calli with a pestle and mortar in the presence of extraction buffer (100 mM potassium phosphate, pH 7.8, 2 mM DTT, 2 mM EDTA, and 5% (w/v) glycerol). Renilla luciferase activity was assayed using the Renilla Luciferase Assay System (Promega, Tokyo, Japan) and firefly luciferase activity was assayed using Picagene (Toyo Ink) and measured using a Lumat LB9507 (Berthold Technologies, Bad Wildbad, Germany). Firefly luciferase expressed under a pUb1 promoter was used as an internal standard for determining bombardment efficiency, while the Renilla luciferase gene was driven by the OsCAD2 promoter. Target plasmid and control plasmid were mixed at equivalent molar ratios. The relative specificity of Renilla luciferase was calculated by dividing the activity of Renilla luciferase by the firefly luciferase activity.

Supplementary data
Supplementary data are available at PCP online.

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Disclosures
The authors declare that they have no conflict of interest concerning this research.

References


