Transcriptional Activation of Secondary Wall Biosynthesis by Rice and Maize NAC and MYB Transcription Factors

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The bulk of grass biomass potentially useful for cellulose-based biofuel production is the remains of secondary wall-containing sclerenchymatous fibers. Hence, it is important to uncover the molecular mechanisms underlying the regulation of secondary wall thickening in grass species. So far, little is known about the transcriptional regulatory switches responsible for the activation of the secondary wall biosynthetic program in grass species. Here, we report the roles of a group of rice and maize NAC and MYB transcription factors in the regulation of secondary wall biosynthesis. The rice and maize secondary wall-associated NACs (namely OsSWNs and ZmSWNs) were able to complement the Arabidopsis snd1 nst1 double mutant defective in secondary wall thickening. When overexpressed in Arabidopsis, OsSWNs and ZmSWNs were sufficient to activate a number of secondary wall-associated transcription factors and secondary wall biosynthetic genes, and concomitantly result in the ectopic deposition of cellulose, xylan and lignin.

Keywords: Maize • MYB46 • NAC • Rice • Secondary wall • SWN • Transcriptional regulation.

Abbreviations: CaMV, Cauliflower mosaic virus; EMSA, electrophoretic mobility shift assay; GUS, β-glucuronidase; MBP, maltose-binding protein; NST, NAC secondary wall thickening-promoting factor; SNBE, secondary wall NAC-binding elements SND, secondary wall-associated NAC domain protein; SWN, secondary wall NAC; VND, vascular-related NAC domain protein; WND, wood-associated NAC domain protein.

Introduction

The grass family is of immense importance in the supply of basic food for human survival; the plants in this family, including rice, maize and wheat, are among the world’s most extensively cultivated food crops. Tremendous efforts have been put into understanding the physiology, genetics and genomics of grass species with the goals of creating cultivars with high crop yield (Sakamoto and Matsuoka 2004, Jung et al. 2007, Penning et al. 2009). Although increasing the grain yield in these crop plants is the main focus for the breeding program, it becomes apparent that in order to withstand the heavy weight of particles it is also essential for the plants to increase the mechanical strength of stems to prevent lodging (Ching et al. 2006). The major mechanical tissues in the stems of grass species are secondary wall-containing sclerenchymatous fibers. Therefore, an increase in the number of fibers and their secondary wall thickness could lead to an enhancement in the mechanical strength of stems. Understanding the molecular mechanisms underlying secondary wall biosynthesis and fiber differentiation will potentially provide strategies and tools for breeding of cultivars with high grain yield without lodging. Recently, it has been proposed that rice and corn stover and other grass species, such as switchgrass (Panicum virgatum) and Miscanthus, could be used as a source of cellulose biomass for bioethanol production (Carroll and Somerville 2009). Hence, there is an increasing incentive to uncover the molecular mechanism controlling the biosynthesis of secondary walls, the major components of the cellulose biomass in these grass plants.

Secondary walls in grass species are mainly composed of cellulose, hemicelluloses and lignin, a composite structure similar to that in dicot species. However, there are a number of differences in terms of the types and relative abundance of hemicelluloses, lignin and phenolic compounds between grass and dicot species (McCann and Carpita 2008, Vogel 2008). For example, the major cross-linking hemicellulose in the secondary walls of grass species is glucuronoxylan, whereas that in dicot species is glucuronoxylan. In addition, lignin in the secondary walls of grass species has a higher proportion of p-hydroxyphenol units and it is cross-linked with xylan via ferulic acid residues. It is thus conceivable that while grass...
species share a suite of secondary wall biosynthetic genes with dicot species, they also evolved to have additional genes for the synthesis of wall components specific for grass secondary walls, which articulates the necessity to study cell wall biosynthesis in grass species (McCann and Carpita 2008, Vogel 2008). Although a number of genes involved in the biosynthesis of secondary wall components in grass species have recently been identified (Tanaka et al. 2003, Burton et al. 2006, Doblin et al. 2009, Zhou et al. 2009, Hirano et al. 2010, Sato et al. 2010, Xiong et al. 2010), our understanding of the transcriptional control of secondary wall biosynthesis in grass species is very limited.

Because the synthesis of secondary wall components requires a concerted action of multiple biosynthetic enzymes, it is envisaged that genes encoding secondary wall biosynthetic enzymes are coordinately activated by transcriptional switches during the development of fibers and vessels. It has recently been discovered that in dicot species, including Arabidopsis, poplar and Eucalyptus, a group of secondary wall NACs (SND1, VND6/7 and NST1/2 in Arabidopsis, PtrWNDs and EgWNDs in poplar and Eucalyptus, respectively) and MYBs (MYB46/83, PtrMYB3/20 and EgMYB2) are master transcriptional switches regulating the secondary wall biosynthetic program (Zhong and Ye 2007, Zhong et al. 2010a). These secondary wall NACs (collectively called SWNs) have been demonstrated to activate their direct targets through binding to an imperfect palindromic 19 bp consensus sequence, called the secondary wall NAC-binding element (SNBE), in the target promoters (Zhong et al. 2010c). In grass species, only a couple of secondary wall-associated transcriptional regulators, including ZmMYB31 and ZmMYB42 from maize, have been functionally characterized. It was found that overexpression of either of them in Arabidopsis causes a down-regulation of lignin biosynthetic genes and a concomitant reduction in lignin content, suggesting their involvement in the regulation of lignin biosynthesis (Sonbol et al. 2009, Fornale et al. 2010). A recent study on the gene expression profiling of maize stems has revealed a number of transcription factors that may potentially be involved in the regulation of cell wall biogenesis, but their exact functions remain to be elucidated (Bosch et al. 2011).

So far, no transcription factors in grasses have been demonstrated to be master transcriptional switches activating the biosynthesis of all three major secondary wall components, including cellulose, xylan and lignin. Finding such transcriptional switches may provide invaluable tools for genetically modifying grass crops tailored for biofuel production.

In this report, we have studied the functional roles of a group of rice and maize NAC domain transcription factors and MYBs transcription factors in the transcriptional control of secondary wall biosynthesis. We show that the rice and maize OsSWNs and ZmSWNs are expressed in secondary wall-forming cells and they are functional orthologs of SND1. When overexpressed in Arabidopsis, they were able to activate the secondary wall biosynthetic pathways and subsequently induce ectopic deposition of secondary wall components, including cellulose, xylan and lignin. Similarly, OsMYB46 and ZmMYB46 were found to be functional orthologs of Arabidopsis MYB46 and are capable of inducing secondary wall biosynthetic genes and subsequent ectopic deposition of secondary walls when overexpressed in Arabidopsis. We further demonstrate that SNBEs are present in the promoters of OsMYB46 and ZmMYB46 and they can be directly bound and activated by OsSWNs and ZmSWNs. Together, our results provide functional evidence demonstrating that the rice and maize secondary wall-associated SWN and MYB46 genes are key transcriptional switches regulating secondary wall biosynthesis.

**Results**

**A group of NAC domain transcription factors are expressed in secondary wall-forming cells**

To investigate whether the molecular mechanisms underlying the transcriptional regulation of secondary wall biosynthesis are conserved between dicots and monocots, we searched the rice and maize genomes for NAC domain transcription factors that exhibit high sequence similarity to the Arabidopsis secondary wall master switch, SND1. A total of seven close homologs of Arabidopsis SND1 were identified in the rice and maize genome, respectively, and they phylogenetically correspond to members of a set of Arabidopsis SWN genes that are implicated in the transcriptional regulation of secondary wall biosynthesis (Fig. 1A; Kubo et al. 2005, Mitsuda et al. 2005, Zhong et al. 2006, Mitsuda et al. 2007, Zhong et al. 2007b). Thus, these rice and maize NACs are named OsSWNs and ZmSWNs.

Three rice SWN cDNAs (OsSWN1, OsSWN3 and OsSWN7) and four maize SWNs (ZmSWN1, ZmSWN3, ZmSWN6 and ZmSWN7) were successfully cloned from stem tissues and thus they were subjected to functional analysis in this study. As a first step towards the functional characterization of these rice and maize SWNs, we examined their cell type-level expression patterns in the developing stems of rice and maize. In rice stems, the bulk of secondary wall-containing biomass comes from the cortical fibers located beneath the epidermis and the bundle sheath fibers that surround each vascular bundle (Sato et al. 2010). In situ hybridization in rice stems using gene-specific probes showed that the three OsSWN genes were expressed prominently in developing bundle sheath fibers and several layers of cells destined to become cortical fibers beneath the epidermis (Fig. 1B–D). Hybridization signals were also evident in some metaxylem cells and the phloem. In situ hybridization of ZmSWN genes in maize stems, in which the bulk of secondary wall-containing cells are bundle sheath fibers that surround the vascular bundle (Raven et al. 1999), revealed that the hybridization signals for the four ZmSWN genes were all evident in the bundle sheath fiber cells (Fig. 1E–I). The hybridization signals were less obvious in the metaxylem, probably due to a relatively low density of transcripts because of its large cell size. No hybridization signals were observed in secondary wall-forming fiber cells in the control sections hybridized with

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the sense probes for both rice and maize SWNs (Fig. 1E, J). These results demonstrate that the expression of these OsSWN and ZmSWN genes is associated with the cells undergoing secondary wall biosynthesis.

**OsSWNs and ZmSWNs are able to rescue the secondary wall defects conferred by the Arabidopsis snd1 nst1 double mutant**

Our finding that the OsSWN and ZmSWN genes are expressed in secondary wall-forming cells suggests that they may function as their Arabidopsis homologs. To study their functions, we employed the complementation approach to test whether OsSWNs and ZmSWNs are functional orthologs of Arabidopsis SND1/NST1, which are known to be master switches regulating secondary wall biosynthesis. The snd1 nst1 double mutant (Zhong et al. 2007b) exhibited the pendent inflorescence stem (Fig. 2A) and reduced stem strength (Fig. 2B) phenotypes due to a lack of lignified secondary walls in fibers (Fig. 2D). Expression of OsSWN and ZmSWN genes driven by the SND1 promoter in the snd1 nst1 mutant effectively rescued the pendent stem phenotype (Fig. 2A) and partially restored the stem strength (Fig. 2B). Examination of cross-sections of stems revealed that the expression of OsSWN and ZmSWN genes also restored the formation of lignified secondary walls in fibers in the snd1 nst1 mutant (Fig. 2E–K). The complementation analysis demonstrates that OsSWNs and ZmSWNs are functional orthologs of SND1 capable of activating the secondary wall biosynthetic program.
Overexpression of OsSWN and ZmSWN genes in Arabidopsis induces ectopic deposition of secondary walls

We next investigated the functional roles of OsSWNs and ZmSWNs in the regulation of secondary wall biosynthesis by overexpression of their cDNAs in Arabidopsis. Such an approach has been successfully applied to study the functions of maize ZmMYB31 and ZmMYB42 genes, which were found to suppress the lignin biosynthetic pathway when overexpressed in Arabidopsis (Sonbol et al. 2009, Fornalé et al. 2010). The full-length OsSWN and ZmSWN cDNAs driven by the Cauliflower mosaic virus (CaMV) 35S promoter were expressed in the wild-type Arabidopsis, and transgenic plants in their first generation were used for analyses. It was found that both OsSWN and ZmSWN overexpressors exhibited the curly leaf phenotype (Fig. 3A), which is similar to that seen in the

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**Fig. 2** Functional complementation of the Arabidopsis snd1 nst1 double mutant by OsSWNs and ZmSWNs. (A) The pendent stem phenotype exhibited by snd1 nst1 was rescued by expression of OsSWNs and ZmSWNs. (B) Measurement of the breaking strength of stems of the wild type, the snd1 nst1 double mutant and snd1 nst1 complemented with OsSWNs and ZmSWNs. Each bar represents the breaking force of the inflorescence stem of individual plants. (C) Cross-section of a wild-type stem showing lignified secondary walls in interfascicular fibers and xylem. (D) Cross-section of a snd1 nst1 stem showing lack of lignified secondary walls in interfascicular fibers. (E–K) Expression of OsSWN1 (E), OsSWN3 (F), OsSWN7 (G), ZmSWN1 (H), ZmSWN3 (I), ZmSWN6 (J) and ZmSWN7 (K) restored the lignified secondary walls in the interfascicular fibers of stems of the snd1 nst1 double mutant. The bottom parts of 8-week-old stems were sectioned and stained for lignin with phloroglucinol-HCl (C–K). if, interfascicular fiber; xy, xylem. Bar in C = 94 μm in C–I.
Fig. 3 Ectopic deposition of reticulated secondary walls in leaf mesophyll cells of OsSWN and ZmSWN overexpressors (OE). The leaves of 3-week-old OsSWN and ZmSWN overexpressors were used for examination of the secondary wall cellulose, xylan and lignin. (A) Three-week-old seedlings of the wild-type Arabidopsis and OsSWN and ZmSWN overexpressors with reduced rosette sizes and curly leaves. (B) Differential interference contrast (DIC) of a wild-type leaf showing helical secondary wall thickening in veins. (C) Lignin autofluorescence image of a wild-type leaf showing lignified veins. (D and E) Mesophyll cells of an OsSWN1 overexpressor showing the ectopic secondary wall thickening (D) and lignin autofluorescence signals (E). (F and G) Mesophyll cells of a ZmSWN1 overexpressor showing the ectopic secondary wall thickening (F) and lignin autofluorescence signals (G). (H–J) Sections of leaves of the wild type (H), OsSWN1-OE (I) and ZmSWN1-OE (J) stained for cellulose with Calcofluor White. Note the increased signal of cellulose in the epidermis and mesophyll cells of OsSWN1-OE (I) and ZmSWN1-OE (J). (K–M) Sections of leaves of the wild type (K), OsSWN1-OE (L) and ZmSWN1-OE (M) stained for xylan with the xylan-specific monoclonal antibody LM10. Note the ectopic deposition of xylan in the epidermis and mesophyll cells of OsSWN1-OE (L) and ZmSWN1-OE (M). Bars = 27 μm in B for B–G and 91 μm in H for H–M.
SND1 overexpressors (Zhong et al. 2006). At least 10 plants with the curly leaf phenotype for each construct were subjected to examination of the ectopic deposition of secondary wall components, including lignin, cellulose and xylan. In wild-type leaves, lignified helical secondary wall thickening was only seen in veins but was absent in mesophyll cells (Fig. 3B, C). In contrast, massive ectopic deposition of lignified, reticulated secondary walls was evident in the mesophyll cells of OsSWN and ZmSWN overexpressors (Fig. 3D–G). Since the images for all the overexpressors were very similar to each other, only representative images from the OsSWN1 and ZmSWN1 overexpressors were shown. In addition to lignin, ectopic deposition of secondary wall cellulose and xylan was also found in the walls of mesophyll cells and epidermis in the OsSWN and ZmSWN overexpressors compared with the wild type (Fig. 3H–M). Examination of the stems also revealed the ectopic deposition of secondary wall components, including lignin (Fig. 4A–H), cellulose (Fig. 4I–K) and xylan (Fig. 4L–N), in the epidermis and cortical cells of the inflorescence stems of OsSWN and ZmSWN overexpressors compared with the wild-type stems in which secondary wall deposition was only seen in interfascicular fibers and xylem cells. For cellulose and xylan staining, only representative images from the OsSWN1 and ZmSWN1 overexpressors were shown. Consistent with the observed ectopic deposition of secondary walls, gene expression analysis showed that the expression of...
secondary wall biosynthetic genes, represented by a cellulose synthase (CesA7; Taylor et al. 2004), a gene required for xylan biosynthesis (FRA8; Zhong et al. 2005) and a lignin biosynthetic gene (4CL1), was significantly elevated in the OsSWN and ZmSWN overexpressors (Fig. 5A). It should be noted that the activation strength of secondary wall biosynthetic genes by SWNs shown in Fig. 5 might not be directly correlated with the degree of complementation of secondary wall thickening in the snd1 nst1 mutant (Fig. 2) because the expression of SWNs in these two cases was driven by different promoters and the expression level of SWNs could also be influenced by positional effects. These results demonstrate that OsSWNs and ZmSWNs are capable of activating the biosynthetic pathways for all three major secondary wall components, i.e. cellulose, xylan and lignin.

We next investigated the ability of OsSWN and ZmSWN to activate the expression of SND1-regulated downstream transcription factors, including SND3, MYB103, MYB46, MYB83, KNAT7, MYB85, MYB58 and MYB63 (Zhong et al. 2008, Zhou et al. 2009). Quantitative PCR analysis revealed that the expression levels of these SND1 downstream transcription factors were drastically increased in the overexpressors compared with the wild type (Fig. 5B). It was noticed that the induction level of each transcription factor by different rice and maize SWNs varied dramatically. For example, the expression of MYB46 was only induced about 2-fold by OsSWN1, whereas it was induced in a range of 20- to 300-fold by other SWNs. This differential induction of the target gene expression may reflect the different degrees of transcriptional activation strength exhibited by different rice and maize SWNs. A similar scenario was observed for poplar PtrWNDs, different members of which showed variable transcriptional activation strength toward downstream targets (Zhong and Ye 2010, Zhong et al. 2010b). These results described above demonstrate that OsSWNs and ZmSWNs activate the same transcriptional program as Arabidopsis SND1 and suggest that the molecular mechanisms underlying the transcriptional activation of secondary wall biosynthesis are evolutionarily conserved between dicots and monocots.

**Secondary wall-associated OsMYB46 and ZmMYB46 are functional orthologs of Arabidopsis MYB46 and are capable of activating the secondary wall biosynthetic program**

The finding that OsSWNs and ZmSWNs were able to activate the SND1-regulated downstream targets when overexpressed in Arabidopsis prompted us to investigate the functional roles of additional rice and maize transcription factors potentially involved in the regulation of secondary wall biosynthesis. MYB46 together with MYB83 are direct targets of SND1 and act as the second-level master switches regulating secondary wall biosynthesis in Arabidopsis (Zhong et al. 2007a, McCarthy et al. 2009). Since OsSWNs and ZmSWNs induced the expression of MYB46 and MYB83 when overexpressed in Arabidopsis, the expression of secondary wall biosynthetic genes and secondary wall-associated transcription factors is increased in OsSWN and ZmSWN overexpressers (OE). Real-time quantitative PCR was used to examine the expression of genes of interest in the seedlings of 3-week-old wild type and OsSWN-OE and ZmSWN-OE. The expression level of genes of interest in the wild type was set to 1. Error bars denote the SE of three biological replicates. (A) Induction of secondary wall biosynthetic genes for cellulose (CesA7), xylan (FRA8) and lignin (4CL1) in OsSWN-OE ZmSWN-OE. (B) Induction of secondary wall-associated transcription factors in OsSWN-OE and ZmSWN-OE.
we reasoned that there may exist homologs of Arabidopsis MYB46/MYB83 in the rice and maize genome, which might be downstream targets regulated by OsSWNs and ZmSWNs.

Sequence analysis of the rice and maize genomes revealed one close homolog of Arabidopsis MYB46/MYB83 in each genome, and they were named OsMYB46 and ZmMYB46, respectively. OsMYB46 and ZmMYB46 are phylogenetically closely related to Arabidopsis MYB46 and other MYB46 homologs from poplar, Eucalyptus and pine (Fig. 6A), all of which have been known to be transcription regulators of secondary wall biosynthetic pathways (Patzlaff et al. 2003, Goicoechea et al. 2005, McCarthy et al. 2010, Zhong et al. 2010a). In situ hybridization demonstrated that the expression of both OsMYB46 and ZmMYB46 was closely associated with secondary wall-forming cells, i.e. cortical fibers and bundle sheath fibers in rice stems (Fig. 6B) and bundle sheath fibers in maize stems (Fig. 6D). The control rice and maize stem sections hybridized with the sense probes of OsMYB46 and ZmMYB46, respectively, did not exhibit positive signals (Fig. 6C, E).

To investigate the possible involvement of OsMYB46 and ZmMYB46 in the regulation of secondary wall biosynthesis, we first tested their ability to rescue the secondary wall defects conferred by the Arabidopsis myb46/myb83 double mutant. Simultaneous mutations of MYB46 and MYB83 genes in Arabidopsis result in severe phenotypes, including a loss of helical secondary wall thickening in vessels and a concomitant growth arrest (Fig. 7; McCarthy et al. 2009). Expression of OsMYB46 and ZmMYB46 driven by the Arabidopsis MYB46 promoter effectively complemented the myb46/myb83 double mutant phenotypes (Fig. 7), indicating that they are functional orthologs of Arabidopsis MYB46 involved in the regulation of secondary wall biosynthesis.

Fig. 6 Phylogenetic and expression analyses of OsMYB46 and ZmMYB46. (A) Phylogenetic relationship of rice and maize MYB46 with their orthologs from Arabidopsis (MYB46/83), poplar (PtrMYB2/3/20/21), pine (PtMYB4) and Eucalyptus (EgMYB2). The phylogenetic tree was shown using the TREEVIEW program (Page 1996). The 0.1 scale denotes 10% change. (B–E) Cross-sections of developing rice (B and C) and maize (D and E) stems were hybridized with digoxigenin-labeled antisense OsMYB46 (B) and ZmMYB46 (D) or sense OsMYB46 (C) and ZmMYB46 (E) RNA probes, and the hybridization signals were detected with alkaline phosphatase-conjugated antibodies and are shown as purple color. cf, cortical fiber; bs, bundle sheath fiber; mx, metaxylem. Bars = 12 μm (B–E).

Fig. 7 Complementation of the myb46 myb83 double mutant by expression of OsMYB46 and ZmMYB46. The growth arrest defect (arrow in the upper panel; inset showing high magnification of the plant) and the vessel wall-thickening defect (arrowhead in the lower panel) conferred by myb46 myb83 were rescued by expression of OsMYB46 and ZmMYB46. The secondary wall thickening in leaf veins is shown below the corresponding plants.

overexpressed in Arabidopsis. Transgenic Arabidopsis plants overexpressing OsMYB46 and ZmMYB46 exhibited a strong curly leaf phenotype (Fig. 8A, B). Histological examination of these curly leaves showed an ectopic deposition of lignin and xylan and an increased accumulation of cellulose in the walls of epidermis (Fig. 8C–N). As a result, the epidermal walls in the overexpressors were significantly thickened compared with the wild type (Fig. 8C, E, G). It was also noticed that the morphology of the leaf epidermal cells was altered by OsMYB46 and ZmMYB46 overexpression. Instead of the jigsaw shapes seen in the wild-type epidermal cells (Fig. 8C), the epidermal cells in the overexpressors have a relatively straight contour.
Fig. 8  Overexpression of OsMYB46 and ZmMYB46 results in ectopic deposition of secondary walls in the epidermal cells of Arabidopsis leaves. The leaves of 3-week-old OsMYB46 and ZmMYB46 overexpressors (OE) were used for examination of the secondary wall cellulose, xylan and lignin. (A) Reverse transcription–PCR analysis showing OsMYB46 (left panel) and ZmMYB46 (right panel) transcripts in the seedlings of four representative transgenic Arabidopsis lines. The transcript of EF1α is shown as an internal control. (B) Three-week-old seedlings showing reduced rosette sizes and curly leaves in OsMYB46-OE (middle) and ZmMYB46-OE (right) compared with the wild type (left). (C) Differential interference contrast (DIC) of epidermal cells of a wild-type leaf. (D) Lignin autofluorescence image of epidermal cells of a wild-type leaf. (E and F) Leaf epidermis of OsMYB46-OE showing ectopic deposition of secondary walls (E) and lignin autofluorescence (F). (G and H) Leaf epidermis of ZmMYB46-OE showing ectopic deposition of secondary walls (G) and lignin autofluorescence (H). (I–K) Calcofluor White staining of leaf sections showing an increased cellulose signal in the epidermis (arrows) of OsMYB46-OE (J) and ZmMYB46-OE (K) compared with the wild type (I). (L–N) Immunostaining of xylan in leaf sections showing ectopic deposition of xylan in the epidermis (arrows) of OsMYB46-OE (M) and ZmMYB46-OE (N) compared with the wild type (L). Bars = 27 μm in C for C–H and 107 μm in I for I–N.
This observation indicates that the ectopic deposition of secondary walls may have occurred early during cell expansion, thereby affecting the normal epidermal cell development and subsequently the leaf morphology. Ectopic deposition of secondary wall components was also detected in the stems of OsMYB46 and ZmMYB46 overexpressors. The walls of both epidermis and cortical cells, which were devoid of secondary wall components in the wild type, were heavily stained for lignin, cellulose and xylan in the overexpressors (Fig. 9).

Gene expression analysis demonstrated that the overexpression of OsMYB46 and ZmMYB46 led to a significant induction of secondary wall biosynthetic genes, including CesA7, FRA8 and 4CL1 (Fig. 10A), as well as several SND1-regulated downstream targets, including KNAT7, MYB85, MYB58 and MYB63 (Fig. 10B). Taken together, these results demonstrate that OsMYB46 and ZmMYB46 are capable of activating the biosynthetic pathways of cellulose, xylan and lignin, leading to ectopic deposition of secondary walls.

OsSWNs and ZmSWNs directly bind to and activate the SNBE sequences in the promoters of OsMYB46 and ZmMYB46

The fact that OsSWNs and ZmSWNs could activate the same downstream transcription factors as SND1 when overexpressed in Arabidopsis indicates that OsSWNs and ZmSWNs may bind to the same cis-DNA elements as SND1 and thereby activate their direct target gene expression. SND1 was previously demonstrated to bind to a 19 bp semi-palindromic DNA sequence named SNBE, and this binding has been shown to be essential for the activation of SND1 direct target genes (Zhong et al. 2010c). To find out whether OsSWNs and ZmSWNs were able to bind to the SNBE sequences, we first tested their ability to activate the Arabidopsis MYB46 SNBE-driven β-glucuronidase (GUS) reporter gene. Co-transfection of OsSWN and ZmSWN overexpression constructs together with the Arabidopsis MYB46 SNBE–GUS construct into Arabidopsis protoplasts resulted in an activation of the
expression of the GUS reporter gene (Fig. 11). While mutations of the non-critical nucleotides (M1) did not reduce the expression of the GUS reporter gene, mutations of the nucleotides (M2 and M3) that are critical for SND1 binding (Zhong et al. 2010c) resulted in a near abolishment of the GUS gene activation.

We next investigated whether the promoters of OsMYB46 and ZmMYB46 contain the SNBE sites and whether they can be activated by OsSWNs and ZmSWNs. Sequence analysis of the 1.5 kb promoters of OsMYB46 and ZmMYB46 revealed three and one SNBE sites, respectively, that were well matched with the SNBE consensus sequences (Fig. 12A). Two copies of these OsMYB46 and ZmMYB46 SNBE sequences linked with the GUS reporter gene were co-transfected with OsSWN and ZmSWN overexpression constructs into Arabidopsis protoplasts to test whether they could be activated by OsSWNs and ZmSWNs (Fig. 12B). It was found that while the OsMYB46-SNBE1 sequence was activated by all the OsSWNs and ZmSWNs tested, the OsMYB46-SNBE2/3 sites were activated by some of the OsSWNs and ZmSWNs, and the ZmMYB46-SNBE site was only activated by ZmSWN6 (Fig. 12C). This observation indicates a wide variation of the activation strength of OsSWNs and ZmSWNs toward different SNBE sequences. The direct binding of OsSWNs and ZmSWNs to SNBEs was proven by electrophoretic mobility shift assay (EMSA) showing a specific retardation in the mobility of the OsMYB46-SNBE1 sequence by the binding of OsSWNs and ZmSWNs (Fig. 12D). Together, these results indicate that OsSWNs and ZmSWNs directly bind to and activate the SNBE sites in the promoters of their target genes, such as OsMYB46 and ZmMYB46.
Discussion

Cellulosic biomass from grass species, such as rice, maize, switchgrass and Miscanthus, has recently been proposed to be an important source for biofuel production (Carpita and McCann 2008). Since the bulk of grass biomass is derived from secondary walls, it is crucial to dissect the molecular mechanisms underlying secondary wall biosynthesis in grass species in order to better utilize the grass biomass for biofuel production. Although a significant advance in our understanding of transcriptional regulation of secondary wall biosynthesis in dicot species, such as Arabidopsis and poplar, has been achieved in the past several years (Zhong et al. 2010a), little is known about how the secondary wall biosynthetic program is switched on in monocots. Currently, no transcriptional switches responsible for the activation of the entire secondary wall biosynthetic program have been characterized in grass species. Our studies on the rice and maize secondary wall transcriptional switches represent a significant advance toward dissection of the transcriptional control of secondary wall biosynthesis in grass species.

We have demonstrated that a group of rice and maize NAC domain transcription factors, namely OsSWNs and ZmSWNs, are transcriptional switches that are able to activate the secondary wall biosynthetic program. There are seven OsSWNs and four ZmSWNs shown to be expressed in secondary wall-forming cells and are functional orthologs of Arabidopsis SND1/NST1. When overexpressed in Arabidopsis, these rice and maize SWNs are capable of inducing the expression of SND1-regulated downstream transcription factors and secondary wall biosynthetic genes, and concomitantly resulting in the ectopic deposition of secondary walls. These findings suggest that these SWNs are master switches involved in transcriptional activation of the secondary wall biosynthetic program in secondary wall-forming cells in rice and maize. Rice and maize SWNs join a list of secondary wall NACs (collectively called SWNs) previously characterized in Arabidopsis, poplar and Eucalyptus (Zhong et al. 2010a). The findings from this study together with those from previous studies support the hypothesis that the SWN-mediated transcriptional regulation of secondary wall biosynthesis is a conserved mechanism throughout vascular plants (Zhong et al. 2010a).

Fig. 12 Presence of SNBE sequences in the promoters of OsMYB46 and ZmMYB46 and their direct activation by OsSWNs and ZmSWNs. (A) The promoters of OsMYB46 and ZmMYB46 harbor SNBE sequences. The consensus nucleotides in the SNBE sequences are shaded. The number shown at the left of each sequence is the position of the first nucleotide relative to the start codon. (B) Diagrams of the GUS reporter and the effector constructs. The GUS reporter constructs consist of the GUS reporter gene driven by two copies of the SNBE sequences from rice and maize MYB46 promoters. (C) Transactivation analysis of the OsMYB46 and ZmMYB46 SNBE sequences by OsSWNs and ZmSWNs. Activation of the SNBE sequences by SWNs was analyzed in Arabidopsis protoplasts co-transfected with the GUS reporter and the effector constructs. (D) EMSA showing the direct binding of OsSWNs and ZmSWNs to the OsMYB46-SNBE1 sequence. Purified recombinant SWNs fused with maltose-binding protein (MBP) was incubated with biotin-labeled OsMYB46-SNBE1 oligonucleotide and subjected to EMSA. Note the band shift resulting from binding of SWNs to the labeled SNBE sequence.
We have also demonstrated that rice and maize MYB46 genes are functional orthologs of Arabidopsis MYB46/MYB83, which are direct targets of Arabidopsis SWNs. OsMYB46 and ZmMYB46 are secondary wall associated and able to activate the secondary wall biosynthetic program when overexpressed in Arabidopsis, indicating that similar to Arabidopsis MYB46, they are second-level key transcriptional switches of the secondary wall biosynthetic program in rice and maize. The findings that MYB46 and its close homologs from both dicots and monocots are functionally conserved lend further support to the hypothesis that the transcriptional program mediated by SWNs and their downstream targets is an ancient mechanism that vascular plants evolved to activate secondary wall biosynthesis.

SWNs from several different species, such as poplar, Eucalyptus, rice and maize, have been shown to be capable of complementing the secondary wall defects conferred in the Arabidopsis snd1 nst1 double mutant and activate the secondary wall biosynthetic program when overexpressed in Arabidopsis, indicating that these SWNs all recognize the same downstream targets as Arabidopsis SND1. SND1 has recently been revealed to bind to an imperfect palindromic 19 bp consensus sequence designated as SNBE, (T/A)(A/T)(A/T)(C/G)(C/G)A(T), in the promoters of its direct targets and thereby activate their expression (Zhong et al. 2010c, McCarthy et al. 2011). We have shown that OsSWNs and ZmSWNs also bind to the SNBE sequences, which explains why they can complement the snd1 nst1 mutant and activate the expression of SND1 target genes when overexpressed in Arabidopsis. Similarly, poplar SWNs (PrtWNDs) were found to activate their direct targets by binding to the SNBE sequences (R. Zhong and Z.-H.Ye, unpublished data). These findings suggest that the DNA binding specificity of SWNs from diverse plant species was well conserved during evolution, which is a necessity for vascular plants to make secondary walls essential for their survival, and thus little room was left for revamping the transcriptional activation mechanism by SWNs.

In summary, we have provided functional evidence that the rice and maize NAC domain transcription factors, OsSWNs and ZmSWNs, and the MYB transcription factors, OsMYB46 and ZmMYB46, are master transcriptional switches involved in the regulation of secondary wall biosynthesis. OsSWNs and ZmSWNs are able to bind to and activate the SNBE sites in the promoters of OsMYB46 and ZmMYB46, suggesting that OsMYB46 and ZmMYB46 are direct targets of OsSWNs and ZmSWNs, respectively. The identification of OsSWNs/ZmSWNs and OsMYB46/ZmMYB46 as master switches of secondary wall biosynthesis provides an unprecedented tool for further analysis of the transcriptional program controlling the production of secondary walls, the major biomass in grass species. Considering that grass species, such as switchgrass and Miscanthus, which are an important source of cellulosic biomass for biofuel production, all contain close homologs of SWNs and MYB46, further characterization of the SWN- and MYB46-mediated transcriptional regulation of secondary wall biosynthesis in rice and maize may generate new means for rational design of grass biomass composition suited for more efficient production of biofuels.

Materials and Methods

Phylogenetic analysis

The amino acid sequences of rice and maize SWNs were analyzed for their phylogenetic relationship with Arabidopsis SND1, NSTs and VNDs using the ClustalW program (Thompson et al. 1994), and the phylogenetic tree was shown using the TREEVIEW program (Page 1996).

In situ hybridization

The developing stems of rice (Oryza sativa) and maize (Zea mays) growing in a greenhouse were fixed in 2.5% formaldehyde and 0.5% glutaraldehyde, embedded in paraffin, and sectioned (12 μm thick) for in situ mRNA localization according to McAbee et al. (2005) and Zhou et al. (2007). The 300 bp 3’ sequences of SWN or MYB46 cDNAs were used for synthesis of digoxigenin-labeled antisense and sense RNA probes with the DIG RNA labeling mix (Roche). Stem sections were hybridized with the antisense and sense probes, and the hybridization signals were detected by incubating with alkaline phosphatase-conjugated antibodies against digoxigenin and subsequent color development with alkaline phosphatase substrates.

Complementation of Arabidopsis mutants

The full-length cDNAs of rice and maize SWN genes driven by the 3 kb SND1 promoter were cloned into the Xbal site of the pGPTV binary vector and introduced into the Arabidopsis snd1 nst1 double mutant (Zhong et al. 2007b) by Agrobacterium-mediated transformation (Bechtold and Bouchez 1994). The primer sequences and engineered cloning sites for each cDNA are as follows: OsSWN1 (5′-atgagccttcggatacaagggg-3′ and 5′-ttctacgtccctgctgagac-3′; Nhel), OsSWN3 (5′-atgtactcttgatgaaggatc-3′ and 5′-catctctcctctctgagac-3′; SpeI), OsSWN7 (5′-atgtactctctctctgagac-3′ and 5′-cttctctctctctcagac-3′; Nhel), ZmSWN1 (5′-atgagccttcggatacaagggg-3′ and 5′-ttctacgtccctgctgagac-3′; Nhel), ZmSWN3 (5′-atgagccttcggatacaagggg-3′ and 5′-ttctacgtccctgctgagac-3′; Nhel), ZmSWN6 (5′-atgagccttcggatacaagggg-3′ and 5′-ttctacgtccctgctgagac-3′; Nhel), and ZmSWN7 (5′-atgagccttcggatacaagggg-3′ and 5′-ttctacgtccctgctgagac-3′; Nhel), and 5′-cttctctctctctcagac-3′; Nhel), and ZmMYB46 (5′-atgagccttcggatacaagggg-3′ and 5′-ttctacgtccctgctgagac-3′; Nhel). Similarly, the full-length cDNAs of OsMYB46 (5′-atgagccttcggatacaagggg-3′ and 5′-ttctacgtccctgctgagac-3′; Nhel) and ZmMYB46 (5′-atgagccttcggatacaagggg-3′ and 5′-ttctacgtccctgctgagac-3′; Nhel) driven by the 3 kb MYB46 promoter were cloned into the Xbal site of the pGPTV vector and introduced into the myb46 (−/−; homozygous) myb83 (+/−; heterozygous) double mutant (McCarthy et al. 2009). More than 72 transgenic plants were generated for each construct, and 10 representative plants with homozygous mutations for both MYB46 and MYB83 were used for further
analyses. Double homozygous mutants were selected by PCR amplification of T-DNA insertions as described previously (McCarthy et al. 2009). Basal parts of the main inflorescence of 8-week-old plants were measured for the breaking force using a digital force/length tester (Zhong et al. 1997). The breaking force was calculated as the force needed to break apart a stem segment. The basal parts of stems were cut into 50 μm thick sections and then stained for lignin with phloroglucinol-HCl.

**Overexpression**

The full-length cDNAs of rice and maize SWN genes and MYB46 (the same primer sequences and engineered cloning sites as those used for complementation constructs) were inserted in the XbaI site downstream of the CaMV 35S promoter in a modified pBI212 to create the overexpression constructs. The constructs were introduced into wild-type Arabidopsis plant (ecotype Columbia) by Agrobacterium-mediated transformation. At least 64 transgenic plants were generated for each construct; 10 representative plants exhibiting severe phenotypes were used for phenotypic characterization and the representative results were presented. Leaf and stem tissues were fixed and sectioned for cellulose, xylan and lignin staining. Lignin was examined by staining sections with phloroglucinol-HCl or visualized using a UV fluorescence microscope (Zhong et al. 2006). Secondary wall cellulose staining was done by incubating 1 μm thick sections with 0.01% Calcofluor White (Hughes and McCully 1975). Xylan was detected by using the monoclonal LM10 antibody against xylan, and fluorescein isothiocyanate-conjugated goat anti-rat secondary antibodies according to McCartney et al. 2005.

**Gene expression analysis**

Total RNA from Arabidopsis tissues was isolated using a Qiagen RNA isolation kit. Quantitative PCR analysis was done using the first-strand cDNA as templates with the QuantiTect SYBR Green PCR Kit (Clontech). The PCR primers for the genes examined were described previously (Zhong et al. 2006, Zhou et al. 2009). The relative mRNA levels were determined by normalizing the PCR threshold cycle number of each gene with that of the EF1α reference gene. The expression level of each gene in the wild type was set to 1 and the data were the average of three biological replicates.

**Transcriptional activation analysis**

For analysis of the transactivation of SNBEs, two or three copies of SNBEs linked with the CaMV 35S minimal promoter (from −1 to −46) were ligated upstream of the GUS reporter gene in pBI221 (Clontech) to create the reporter constructs. The effector constructs were created by placing the full-length cDNA of SWN genes (the same primer sequences and engineered cloning sites as those used for complementation constructs) between the CaMV 35S promoter and the nopaline synthase terminator in a modified pBI221. Arabidopsis leaf protoplasts were co-transfected according to Sheen (2001) with the reporter and effector constructs together with a reference construct containing the firefly luciferase gene driven by the CaMV 35S promoter for determination of the transfection efficiency. After 20h incubation, soluble extracts isolated from the transfected protoplasts were used for analysis of the GUS and the luciferase activities. The GUS activity was normalized against the luciferase activity in each transfection and the data were the averages of three biological replicates.

**Electrophoretic mobility shift assay**

Rice and maize SWNs (the same primer sequences and engineered cloning sites as those used for complementation constructs, except with no stop codon) were cloned into the XbaI site of a pMAL vector (New England BioLabs) and expressed in *Escherichia coli*. The recombinant SWNs fused with the maltose-binding protein (MBP) were purified using amylose resin according to the manufacturer’s instructions (New England BioLabs), and then used for EMSA with the SNBE sequence. The SNBE fragments were biotin labeled at the 3’ end using the Biotin 3′ End DNA Labeling Kit (Pierce). The biotin-labeled DNA fragments were incubated with 100 ng of SWN–MBP in the binding buffer [10 mM Tris, pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 2.5% glycerol, 5 mM MgCl2, 0.05% NP-40, 100 ng μl−1 poly(dI–dC)]. The SWN-bound DNA probes were separated from the unbound probes by PAGE. The DNA was electroblotted onto a nitrocellulose membrane and detected by the chemiluminescent method.

**Statistical analysis**

The experimental data of quantitative PCR and GUS activity measurement were subjected to statistical analysis using the Student’s t-test program (http://www.graphpad.com/quickcalcstest1.cfm), and the quantitative difference between the two groups of data for comparison was found to be statistically significant (P < 0.001).

**Accession numbers**

The Genbank accession numbers for the rice and maize genes investigated in this study are OsSWN1 (JN634070; RAP-DB ID # Os06g04090/Os06g0131700), OsSWN2 (JN634071; Os08g02300/Os08g0115800), OsSWN3 (JN634072; Os08g01330/Os08g0103900), OsSWN4 (JN634073; Os10g38834/Os10g0532000), OsSWN5 (JN634074; Os03g03540/Os03g0127200), OsSWN6 (JN634075; Os04g53430/Os04g0536500), OsSWN7 (JN634076; Os06g01480/Os06g0104200), ZmSWN1 (JN634077), ZmSWN2 (JN634078), ZmSWN3 (JN634079), ZmSWN4 (JN634080), ZmSWN5 (JN634081), ZmSWN6 (JN634082), ZmSWN7 (JN634083), ZmMYB46 (JN634084; Os12g33070/Os12g0515300) and ZmMYB46 (JN634085). The Arabidopsis Genome Initiative locus identifiers for genes used in this study are MYB83 (At3g08500), MYB46 (At5g12870), SND3 (At1g28470), MYB58 (At1g16490), MYB63 (At1g79180), MYB85 (At4g22680), MYB103 (At1g3910), KNAT7.
References


