OsSND2, a NAC family transcription factor, is involved in secondary cell wall biosynthesis through regulating MYBs expression in rice

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Abstract

Background: As one of the most important staple food crops, rice produces huge agronomic biomass residues that contain lots of secondary cell walls (SCWs) comprising cellulose, hemicelluloses and lignin. The transcriptional regulation mechanism underlying SCWs biosynthesis remains elusive.

Results: In this study, we isolated a NAC family transcription factor (TF), OsSND2 through yeast one-hybrid screening using the secondary wall NAC-binding element (SNBE) on the promoter region of OsMYB61 which is known transcription factor for regulation of SCWs biosynthesis as bait. We used an electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation analysis (ChIP) to further confirm that OsSND2 can directly bind to the promoter of OsMYB61 both in vitro and in vivo. OsSND2, a close homolog of AtSND2, is localized in the nucleus and has transcriptional activation activity. Expression pattern analysis indicated that OsSND2 was mainly expressed in internodes and panicles. Overexpression of OsSND2 resulted in rolled leaf, increased cellulose content and up-regulated expression of SCWs related genes. The knockout of OsSND2 using CRISPR/Cas9 system decreased cellulose content and down-regulated the expression of SCWs related genes. Furthermore, OsSND2 can also directly bind to the promoters of other MYB family TFs by transactivation analysis in yeast cells and rice protoplasts. Altogether, our findings suggest that OsSND2 may function as a master regulator to mediate SCWs biosynthesis.

Conclusion: OsSND2 was identified as a positive regulator of cellulose biosynthesis in rice. An increase in the expression level of this gene can improve the SCWs cellulose content. Therefore, the study of the function of OsSND2 can provide a strategy for manipulating plant biomass production.

Keywords: Secondary cell wall (SCW), Rice, Cellulose synthesis, Transcription factor (TF), NAC, MYB

Background

Plant cell wall is a unique structure that plays an important role in plant growth and development. The cell wall provides mechanical strength to the plant body and responses to environmental stimuli, such as pathogen invasion (Underwood, 2012) and stress response (Tenhaken, 2014). Plants exhibit two typical types of cell walls, namely, the primary cell walls (PCWs) that surround all cells and the secondary cell walls (SCWs), a thickened structures observed in specific cell types, such as xylem vessels and fibers (Keegstra, 2010). SCWs not only provide mechanical strength to these cells, but also greatly contribute to the bulk of renewable plant biomass (Burton and Fincher, 2014). SCWs mainly compose cellulose, hemicelluloses and lignin. Cellulose is composed of unbranched β-1, 4-glucans, and cellulose microfibrils form the main load-bearing network (Somerville, 2006). Hemicelluloses belong to a group of heterogeneous polysaccharides such as xylan, glucan, mannan, and mixed-linkage glucan (Pauly et al., 2013). Lignin is a complex phenylpropanoid polymer that provides mechanical strength to specific cell types (Boerjan et al., 2003). The understanding of the mechanism
underlying SCWs biosynthesis may provide a strategy for manipulating plant biomass production.

In the past decades, many genes involved in SCWs biosynthesis have been cloned and characterized in both dicot and monocot plants. Cellulose is synthesized in the plasma membrane by the cellulose synthase complex (CSC), which contains at least three different cellulose synthases, encoded by CESA genes (Somerville, 2006). In Arabidopsis, CESA4, CESA7, and CESA8 genes are essential for SCWs cellulose biosynthesis (Taylor et al., 2003; Taylor et al., 2000; Taylor et al., 1999). Close homologs of CESA4, CESA7 and CESA8 are required for SCWs cellulose biosynthesis in rice, and mutations of these homologs of CESA4, CESA7 and CESA8 are required for SCWs cellulose biosynthesis in rice, and mutations in any of these genes may cause a dramatic decrease in the SCWs cellulose content, resulting in the brittle culm phenotype (Song et al., 2013; Tanaka, 2003; Zhang et al., 2009). In addition to these CESA genes, some other genes are also involved in SCWs cellulose biosynthesis and assembly, such as the Arabidopsis KORRIGAN (KOR) gene, that encodes for an endo-β-1, 4-glucanase. Mutations in this gene causes reduction in the cellulose content of both PCWs and SCWs (Szyjanowicz et al., 2004). In rice, several Brittle Culm (BC) genes are involved in SCWs cellulose biosynthesis and mutations of these genes are shown to reduce the cellulose content and mechanical strength, leading to the brittle culm phenotype (Kotake et al., 2011; Wu et al., 2012; Zhang et al., 2010; Zhou et al., 2009). Xylan and mannann are the major hemicelluloses in SCWs, and they are synthesized in the Golgi apparatus and transported to the plasma membrane via Golgi vesicles (Pauly et al., 2013). In Arabidopsis, glycosyltransferase families have been implicated in hemicelluloses biosynthesis. Lignin is a complex polymer made up of p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units of lignin (Kumar et al., 2016). The monolignols are synthesized through the phenylpropanoid pathway within cells and then transported into cell walls, where they are polymerized into lignin via oxidative reactions catalyzed by oxidases, such as laccases and peroxidases (Boerjan et al., 2003). Several genes involved in SCWs biosynthesis have been reported, however the spatiotemporal expression of these genes remains unclear.

In Arabidopsis, a detailed transcriptional regulation mechanism of SCWs biosynthesis has been reported. A transcriptional network comprising two large family transcription factors (TFs), NAC and MYB, are involved in SCWs biosynthesis (Zhong and Ye, 2015). In this transcriptional network, a group of NAC family TFs, including NAC SECONDARY WALL THICKENING PROMOTING FACTOR1 (NST1), NST2, NST3 (also called as SND1, SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1), VASCULAR-RELATED NAC-DOMAIN6 (VND6), and VND7, function as the top-level master switches of SCWs biosynthesis in fibers and/or vessels (Zhong and Ye, 2015). These factors directly regulate the expression of a battery of downstream TFs, including SND2, SND3, MYB20, MYB42, MYB46, MYB52, MYB54, MYB58, MYB63, MYB83, MYB85 and MYB103. Of these, MYB46 and its close homolog MYB83 act as the secondary-layer master switches to regulate SCWs biosynthesis (Hussey et al., 2013). MYB46 and MYB83 also regulate the expression of the direct targets of SND1 and its homologs, NST1, NST2, VND6 and VND7 (McCarthy et al., 2009; Zhong et al., 2007). All of these NAC and MYB TFs collectively regulate the biosynthetic genes for cellulose, xylan and lignin. The SCWs NAC family TFs activate the downstream targets through binding to a 19 base pair (bp) sequence, known as SCWs NAC-binding element (SNBE) (McCarthy et al., 2014; Zhong et al., 2010). MYB46 and MYB83 bind to a 7 bp consensus sequence, termed as SCWs MYB-responsive element (SMRE) to regulate the expression of target genes (Zhong and Ye, 2012).

Rice is one of the most important staple food crops and produces a large amount of agronomic biomass residues, which may be a potential source of bio-energy. Nevertheless, a few TFs involved in SCWs biosynthesis have been reported in rice. OsCEFI, which encodes the OsMYB103L, regulates SCWs biosynthesis by directly binding to the promoter of CESAs and BC1 genes (Ye et al., 2015). The cef1 mutant shows reduction of cellulose content, and the culm is fragile (Ye et al., 2015). OsMYB61 directly binds to the CES1 promoters and regulates their expression, and OsMYB61 can be activated by the SCWs NAC families, including NAC29 and NAC31 (Huang et al., 2015). Therefore, to unveil the master transcriptional mechanism of SCWs biosynthesis in rice may provide valuable approach for genetically modifying grass crops for biofuel production.

In this study, we isolated a NAC family TF, named OsSND2 using yeast one-hybrid screening with the SNBE site in the promoter region of OsMYB61 as bait. We demonstrated that OsSND2 directly binds to the promoter region of OsMYB61, regulates SCWs biosynthesis by directly binding to the promoter of CESAs and BC1 genes (Ye et al., 2015). The cef1 mutant shows reduction of cellulose content, and the culm is fragile (Ye et al., 2015). OsMYB61 directly binds to the CES1 promoters and regulates their expression, and OsMYB61 can be activated by the SCWs NAC families, including NAC29 and NAC31 (Huang et al., 2015). Therefore, to unveil the master transcriptional mechanism of SCWs biosynthesis in rice may provide valuable approach for genetically modifying grass crops for biofuel production.

**Methods**

**Plant materials and growth conditions**

The all rice (*Oryza sativa*) plants were used in this study, including the *japonica* cultivar wild-type plants, wuyunjing7 (WYJ7) and the overexpression and knock-down of OsSND2 transgenic plants were grown in the experimental fields at the Institute of Technical Biology and Agriculture Engineering, Hefei Institute of Physical
Science, Chinese Academy of Sciences (Hefei, China) and Sanya (Hainan province, China) during the natural growing season.

**Yeast one-hybrid screening**

Five OsMYB61 bait fragments of pMYB61–1 (−1946, −1258), pMYB61–2 (−1607, −1258), pMYB61–3 (−1258, −897), pMYB61–4 (−870, −356) and pMYB61–5 (−356, −1) were cloned into the pHIS2 vector between EcoRI and SacI sites and integrated into the genome of yeast strain Y187 (MATα, ura3–52, his3–200, ade2–101, trp1–901, leu2–3, 112, gal4Δ, met+, gal80Δ, URA3:: GAL1_LUAS·GAL1_TATA·lacZ, MEL1). For the self-activation test, promoter bait strains were grown on the SD/-Trp, -His (a synthetic Trp and His dropout medium) media in the presence of 0 mM, 10 mM, 30 mM and 50 mM 3-amino triazole (3-AT). We performed the yeast one-hybrid screening using the BD Matchmaker One-hybrid Library Construction and Screening Kit (K1617–1, Clontech) according to the user manual (PT3529–1, Clontech). The cDNA library of the internodes tissue was constructed with the pGADT7-Rec2 vector (Clontech). The promoter bait strains were then mated with the "pGADT7-Rec2-cDNA" library and screened on the SD/-Leu -Trp -His selection media containing 30 mM 3-AT. Positive colonies were selected for yeast plasmid isolation or PCR with primers AD-F and AD-R. The PCR was performed according to the following program, 95 °C 5 min, 95 °C 30 s, 56 °C 30 s, 72 °C 2 min, 36 cycles, 72 °C 10 min, 12 °C pause.

**Bioinformatics analysis of OsSND2**

A search for OsSND2 homologs in rice and Arabidopsis was performed using the NCBI BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The alignment was performed using DNAMAN software. An unrooted phylogenetic tree of OsSND2 homologs in rice and Arabidopsis was constructed using MEGA5 software with 1000 bootstrap replications (Tamura et al., 2011). The co-expression analysis of OsSND2 with candidates in cell wall synthesis was performed using the expressing database at http://www.ricearray.org.

**Subcellular localization of OsSND2**

To observe the subcellular localization of OsSND2, a green fluorescent protein (GFP) fused to the C-terminus of OsSND2 and inserted into the pCAMBIA1300 between the KpnI and BamHI sites to create the 3SS::OsSND2-GFP vector, which was transformed into rice protoplasts by polyethylene glycol (PEG) mediated transformation method. The subcellular distribution of the OsSND2-GFP protein was observed using confocal laser scanning microscope (Leica TCS SP5).

**Binary vectors construction and rice transformation**

For the overexpression construct of OsSND2, the full-length coding sequence of OsSND2 was amplified using gene-specific primers, OE-F, 5′-CCAAGCTTATGACGTGGTGCAACAGCTT-3′ and OE-R, 5′-GGGG ATCCCTAAGGCCACCAAGCTGT-3′, which contain HindIII and BamHI restriction sites. The PCR fragment was cloned into the intermediate vector N-Tagged SK (−), which encodes Myc-tag protein. Then, the sequencing-confirmed vector was digested using KpnI/BamHI and inserted into the pCAMBIA2300 between the KpnI and BamHI sites to create the p35S::Myc-OsSND2 vector.

We used CRISPR/Cas9 system for creating snd2 mutants. The CRISPR/Cas9 binary vectors were constructed as previously described (Ma et al., 2015). The Cas9 plant expression vector (pYLCRISPR/Cas9Pubi-H) and sgRNA expression vector (pYlgRNA) were provided by Prof. Yao-Guang Liu (South China Agricultural University). We selected the Target1 (CAGCGACGTCCGCACCGCCG) and Target2 (GGAGGGGCACATCTTGACG) in the first exon of OsSND2 (Fig. 5a) as candidate target sequences according to the design principles of the target sequences in the CRISPR/Cas9 system. Then, they were ligated into two sgRNA expression cassettes of a Cas9 binary vector, driven by OsU6 and OsU3 promoters, respectively.

These constructs were introduced into a japonica cultivar, wuyunjing7 (WYJ7) by the Agrobacterium-mediated transformation procedure as described previously (Raineri et al., 1990).

**RNA extraction and quantitative real-time PCR (qRT-PCR)**

Total RNA was extracted from various rice tissues using TRizol reagent (Invitrogen), as described previously (Wadsworth et al., 1988). The first strand of cDNA was synthesized using a reverse transcriptional kit (TransGen). qRT-PCR was performed using relevant primers and qRT-PCR kit (TransGen) on a quantitative 7500 PCR system (ABI). All assays were repeated at least three times, the Actin1 gene was used as an internal control.

**Electrophoretic mobility shift assay (EMSA)**

The coding sequence of OsSND2 was amplified and cloned into the pGEX-4 T-1 vector (GE Healthcare). GST and GST-OsSND2 fusion proteins were purified as described previously (Wang et al., 2015). DNA fragments for EMSA were obtained by PCR amplification and labeled using a biotin labeling kit (Invitrogen). DNA gel shift assays were performed using the LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific).

**Chromatin immunoprecipitation (ChIP) analysis**

The above-ground portion of p35S::Myc-OsSND2 transgenic rice plants was harvested between 2 and 3 g after
growth on soil for 3 to 4 weeks and immediately cross-linked with 1% formaldehyde under vacuum for 15 min at 15–25 °C. The cross-linking was stopped by adding glycerol to the final concentration of 0.125 M for 5 min under vacuum. The cross-linked samples were rinsed twice with double distilled water. The further ChiP assay based on an antibody to Myc (9E10, Santa Cruz Biotechnology) was performed as described previously (Wang et al., 2015). Chromatin samples without Myc antibody immunoprecipitation were used as the control. Enrichment of DNA fragments was determined using qRT-PCR analysis performed on three biological replicates. The Actin1 gene exon used as negative controls.

**Transactivation analysis in yeast cells and Rice protoplasts**

Transactivation analysis in yeast was performed as described previously (Wang et al., 2012). The full length coding sequence of OsSND2 was amplified and cloned into pGBKT7 vector, and then transformed into the yeast strain AH109 (MATa, trp1−/−, leu2−/−, 112, ura3−/−, his3−/−, 200, gal4Δ, gal80Δ, LYS2::GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ΔDE2, URA3::MEL1UAS-MEL1TATA-lacZ, MEL1). The empty pGBKT7 (BD) and fusing the GAL4 vectors were used as negative and positive controls, respectively. The transactivation activity was evaluated according to the growth on SD/−Trp and SD/−Trp − His − Ade.

Transactivation analysis was also performed in rice protoplasts as described previously (Wang et al., 2015). For the effector vector, the full length coding sequence of TFs were amplified and fused with GAL4 binding domain (GAL4BD). The empty GAL4BD and fused with VP16 were used as negative and positive controls, respectively. For the reporter vectors, the pUC19 containing the firefly luciferase (LUC) reporter gene driven by the minimal TATA box of the 35S promoter plus five GAL4 binding elements was used for self-activation test. The 2 kb fragments of upstream sequence from start codon of the candidate genes were amplified and fused with LUC protein to generate reporter plasmids for targets transactivation analysis. A pTRL plasmid containing Renilla LUC gene driven by the CaMV (Cauliflower mosaic virus) 35S promoter, was used as an internal control. The pTRL, effector and reporter were simultaneously transformed into the rice protoplast system, then kept in dark for 16 h. The LUC activity was measured as described previously (Ohta et al., 2000).

**Yeast one-hybrid assay**

The OsSND2, OsMYB61L and OsMYB86L encoding sequence was amplified and inserted into the unique EcoRI and Xhol sites of the pB42AD vector (Takara) to construct effector. For the reporter vectors, the 2 kb DNA fragments corresponding to the promoter of candidate genes were amplified and cloned into the pLacZI2u vector to drive lacZ reporter gene expression. The effectors and reporters were simultaneously transformed into the yeast strain EGY48. The transformants were grown on synthetic drop-out plates without tryptophan and uracil containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for colony coloration. The empty pB42AD and pLacZI were used as negative control.

**Cell wall composition analysis**

The second internodes of wild type and transgenic plants at mature stage were ground into powder under liquid nitrogen and prepared alcohol-insoluble residues (AIRs). De-starched AIRs and trifluoroacetic acid (TFA) treatment were hydrolyzed in Updegraff reagent (acetic acid: nitric acid: water: 8:1:2 v/v). The cooled pellets were washed and hydrolyzed with 72% sulfuric acid. The cellulose content was measured by the anthrone assay (Updegraff, 1969). The monosaccharide composition was determined by gas chromatography-mass spectrometry as described previously (Xiong et al., 2010). The lignin content was measured by the acetyl bromide method as described previously (Huang et al., 2015).

**Microscopy**

For the scanning electron microscope (SEM) observation, the second internodes segments were sliced with Gillette razor blades and then fixed in 4% paraformaldehyde. After dehydration through a gradient of ethanol and critical point drying, the samples were sprayed with gold particles and observed with a scanning electron microscope (SEM) (S-3000 N; Hitachi, Tokyo, Japan).

**Accession numbers**

Sequence data used in this manuscript can be found in the rice genome annotation database (http://rice.plantbiology.msu.edu) and in the Arabidopsis information resource (TAIR, http://www.arabidopsis.org) under the following accession numbers: OsMYB61 (Os01g18240), OsSND2 (Os05g48850), OsCESA4 (Os01g54620), OsCESA7 (Os10g32980), OsCESA9 (Os09g25490), OsCESA11 (Os06g39970), OsMYB86L (Os08g36460), OsMYB61L (Os05g04820), AtSND2 (At4g28500).

**Results**

Identification of the interaction between OsSND2 and OsMYB61 promoter

To understand the hierarchical regulatory mechanism controlling SCWs biosynthesis in rice, we conducted the yeast one-hybrid screening using five different length sequences of OsMYB61 promoter (Additional file 1: Figure S1a) fused to HIS3 reporter as baits (Additional file 1:
Figure S1b) to search for novel transcription factors involved in the regulation of OsMYB61 expression. The cDNA library from the second internodes harvested during the heading stage of rice fused to yeast GAL4 activation domain (AD) was used as a prey. To test the bait construct self-activation, promoter bait strains were grown on the SD/−Trp -His media in the presence of 0, 10, 30 and 50 mM of 3-AT, a competitive inhibitor of HIS3 protein. As a result, only the yeast strain with OsMYB61-P5 bait construct was completely suppressed in the presence of 30 mM 3-AT (Additional file 1: Figure S1c). Yeast strains harbouring the other four constructs were not suppressed even with 50 mM of 3-AT (Additional file 1: Figure S1c). Hence, we chose the construct OsMYB61-P5 to perform screening experiment with 30 mM 3-AT. Through the screening of $3.2 \times 10^5$ cDNA clones, one positive clone was obtained (clone 13). We isolated the yeast plasmid and subjected it to sequencing and BLAST search against NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The BLAST search found a the rice full-length cDNA, NM_001062858. Further sequence analysis and annotation of this clone using RGAP database (http://rice.plantbiology.msu.edu/) showed that this gene is on the locus LOC_Os05g48850, which has three exons and two introns. LOC_Os05g48850 encodes for a NAC family transcription factor with a length of 314 amino acids and a molecular mass of approximately 35 kD. Phylogenetic analysis showed that LOC_Os05g48850 is closely related to NAC family transcription factors in Arabidopsis At4g28500 (AtSND2) (Fig. 1a). Protein sequence alignment revealed that they are highly conserved in the predicted NAC DNA-binding domains (Fig. 1b). Therefore, we designated LOC_Os05g48850 as OsSND2 (Oryza sativa SND2).

One of the significant features of transcription factors is nuclear localization. To determine the subcellular localization of OsSND2, the construct of OsSND2 with C-terminus green fluorescent protein (GFP) tag was cloned into a 35S::OsSND2-GFP vector. Using confocal

![Fig. 1 OsSND2 is a NAC family transcription factor and has a very high homology to AtSND2. a] Phylogenetic analysis of the secondary wall NACs in Rice and Arabidopsis. The red rectangle indicate the OsSND2. An unrooted phylogenetic tree was generated with the full-length amino acid sequences. b] Protein sequences alignment of OsSND2 and AtSND2. Black shadings indicate identical amino acids. The red underline indicate NAC domain
laser scanning microscopy, we confirmed that the OsSND2-GFP fusion protein was located predominantly in the nucleus (Fig. 2a).

To investigate whether OsSND2 has a potential transcriptional activity, we used the yeast assay system to investigate OsSND2. The growth of transformants carrying pGBK7-OsSND2 on selective medium (SD/-Trp) and (SD/-Trp -His -Ade) indicated the OsSND2 protein has transcriptional activity, the pGBK7-OsMYB103L and empty pGBK7 were used as positive and negative control, respectively (Fig. 2b). We also used a dual-luciferase reporter (DLR) assay system in the rice protoplast to test the transcriptional activation of OsSND2. In comparison with the GAL4-BD negative control, OsSND2 can activate the LUC gene, similarly to the activation by VP16 as positive control (Fig. 2c). These results indicate that OsSND2 protein exhibits transcriptional activity (Fig. 2b and c).

**OsSND2 can directly bind to the promoter of OsMYB61**

To confirm the interaction between OsSND2 and OsMYB61 promoter, we used the yeast one-hybrid system with LacZ reporter gene (Fig. 3a). The yeast one-hybrid assay revealed the predominant activation of LacZ reporter gene expression by OsSND2 under the control of OsMYB61 promoter. On the contrary, pB42AD without OsSND2 failed to activate LacZ expression (Fig. 3b).

We further performed the dual-luciferase reporter (DLR) assay system in rice protoplasts to explore the effect of OsSND2 on the transcriptional regulation of OsMYB61 expression using a reporter construct carrying the firefly luciferase (LUC) driven by the 2 kb fragment of OsMYB61 promoter. DLR assay revealed an 11-fold increase in the transcriptional activation in the protoplasts co-expressing an effector carrying OsSND2 (Fig. 3c) and a reporter containing OsMYB61 promoter to drive luciferase as compared with the negative control (Fig. 3d). This result suggests that OsSND2 functions as a transcriptional activator to directly regulate OsMYB61 expression.

Secondary wall-related NAC proteins regulate target genes expression through binding to the SNBE element, (T/A)NN(C/T)(T/C/G)TNNNNNNNA(A/C)GN(A/C/T)(A/T) (Zhong et al., 2010). To determine whether the interaction between OsSND2 and OsMYB61 promoter occurs through binding to SNBE site, we performed sequence searching within the promoter of OsMYB61 and found it contains two SNBE sites (SNBE1 and SNBE2) (Additional file 2: Figure S2a). We further conducted chromatin immunoprecipitation (ChIP) assay in wild-type and p35S::Myc-OsSND2 overexpression...
transgenic rice plants. The results showed that the two fragments (P3 and P9) containing the SNBE sites were significantly enriched in Myc-OsSND2 overexpression plants (Fig. 3e). We used an electrophoretic mobility shift assay (EMSA) to examine whether OsSND2 bind to P9 and P3 fragments containing the SNBE1 and SNBE2, respectively. P9 and P3 were bound by the recombinant OsSND2 protein fused to glutathione S-transferase (GST-OsSND2), which resulted in a mobility shift (Fig. 3f and Additional file 2: Figure S2b). GST alone, as a negative control, failed to induce the mobility shift (Fig. 3f and Additional file 2: Figure S2b), thereby confirming the binding specificity.

Taken together, the above results demonstrate the function of OsSND2 as a transcription activator through its direct binding to SNBE sites in the promoter of OsMYB61 in vitro and in vivo.

OsSND2 was mainly expressed in internodes and panicles

To investigate whether the expression of OsSND2 is associated with SCWs biosynthesis, the expression pattern of OsSND2 was examined by quantitative real-time PCR (qRT-PCR) using RNAs isolated from various organs of WYJ7 plants. OsSND2 expression was detected in all organs, with relatively higher levels observed in internodes and panicles. The expression level of OsSND2 was relatively low in leaves, sheaths, and roots during the heading and seedling stages (Fig. 4). We examined the expression pattern of OsMYB61 and found it to be consistent with OsSND2 expression (Additional file 3: Figure S3).

Mutation of OsSND2 decreased cellulose content and down-regulated SCWs gene expression

To investigate the biological function of OsSND2, we generated OsSND2 mutants using CRISPR/Cas9 system. We designed two sequence-specific single guide RNA (sgRNA) target sites, Target1 and Target2, which were 76-bp apart in the first exon of OsSND2 (Fig. 5a). Two transgene-free homozygous knockout lines with different genotypes, snd2-c1 and snd2-c2 were obtained (Fig. 5b). Protein sequence alignments of the two homozygous mutants and the wild type protein revealed that snd2-c1 and snd2-c2 showed coding frame shifts and premature translational stops (Fig. 5c).

No obvious morphological changes, except for a little early flowering in ossnd2 mutant (Fig. 5d). However, a significant decrease in the cellulose content was detected in snd2-c1 and snd2-c2 mutants (Fig. 5e). No significant alteration in the contents of xylose and lignin were
detected (Additional file 4: Table S1) We determined the expression levels of OsMYB61 and secondary wall CESA genes in two mutants and found that the expression levels of OsMYB61 and CESA genes were down-regulated (Fig. 5f). We further analyzed the wall thickness of sclerenchyma cells in the internodes of wild-type and snd2 mutant plants by SEM and found that snd2 mutant plants showed obviously thinner walls than the wild-type plants in sclerenchyma cells (Fig. 5g).

**Overexpression of OsSND2 increased cellulose content and up-regulated SCWs gene expression**

To further elucidate the biological function of OsSND2, we generated OsSND2 overexpression (OX) transgenic plants. Seventeen OX transgenic lines were obtained. We used qRT-PCR analysis to examine the expression level of OsSND2 in these transgenic plants. Transgenic lines with significant alterations in the expression level of OsSND2 were selected for further study (Fig. 6a).

The overexpression of OsSND2 resulted in the phenotypic characteristics such as semi-dwarf plant height and significant leaf rolling. The degree of leaf rolling increased with an increase in the expression level of

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**Fig. 4** Expression pattern of OsSND2. qRT-PCR analysis of OsSND2 expression in various rice organs and different developmental stage, the heading stage and seedling stage indicate the tenth day after flowering and the two weeks old seedlings, respectively. The Actin1 gene was used as an internal control. Error bars, SD of three biological replicates.

**Fig. 5** Generation and analysis of snd2 mutants. **a** Schematic diagram of OsSND2 gene structure and two CRISPR/Cas9 target sites. UTRs, exons, and introns are indicated by blank rectangles, black rectangles, and black lines, respectively. **b** DNA sequence alignments for the two homozygous snd2 mutants identified in the T1 generation, together with a wild-type (WT) control. The numbers on the right side are the sizes of the indels, with “−” and “+” showing deletion and insertion of nucleotides involved, respectively. **c** Deduced OsSND2 amino acid sequence alignments for the two homozygous mutants and WT. **d** Three-month-old plant of wild type (WT) and snd2-c1 mutant. Bar = 10 cm. **e** Measurement of cellulose content in WT and snd2 mutants. **f** Relative expression of OsMYB61 and SCWs-related CESA genes in WT and snd2 mutants. The Actin1 gene was used as internal control. Error bars, SD of three biological replicates. **g** Observation of sclerenchyma cell walls in the internodes from the three-month-old wild-type and snd2 mutant plants via transmission electron microscope. Bar = 2 μm.
OsSND2 (Fig. 6b). We measured the cellulose content in OsSND2-OX plants and found that OsSND2-OX plants have significantly increased cellulose content (Fig. 6c), but showed no significant alterations in the contents of xylose and lignin (Additional file 4: Table S1). We further examined the expression levels of OsMYB61 and secondary wall CESA genes using qRT-PCR. Consistent with the increased cellulose content, OsMYB61 and CESA genes expression level were higher in OsSND2-OX plants (Fig. 6d). The results of anatomical analysis revealed the obvious thickness in the sclerenchyma cell wall of OsSND2-OX plants as compared to wild-type plants (Fig. 6e). Collectively, these results suggest that OsSND2 may be involved in the regulation of the biosynthetic pathways involved in SCW cellulose synthesis and affect the thickness of sclerenchyma cell wall.

OsSND2 directly regulates the expression of other R2R3-MYB family TFs

Secondary wall-related NAC proteins can activate lots of R2R3-type MYB family TFs expression to start the entire transcription regulation network controlling SCWs biosynthesis in Arabidopsis (Zhong et al., 2008). To investigate whether OsSND2 regulates other SCWs-related R2R3-MYB family TFs expression in rice, we performed co-expression analysis of OsSND2 with SCWs-related CESAs and R2R3-type MYBs. We identified several MYB candidates that may be involved in SCWs biosynthesis (Table 1). We further examined the expression levels of these R2R3-type MYBs in snd2-c1 mutants and OsSND2 overexpression transgenic plants. qRT-PCR assay showed that OsMYB86L (LOC_Os08g36460), OsMYB61L (LOC_Os05g04820), and OsMYB58/63 (LOC_Os04g50770) were down-regulated in snd2-c1 mutants and up-regulated in the transgenic OsSND2-OX plants (Fig. 7a). The expression of OsMYB103L (LOC_Os08g05520) had no obvious difference in these transgenic lines (Fig. 7a).

To further investigate if OsSND2 can directly regulate the expression of these MYBs, transcriptional activation assays were performed in yeast and rice protoplasts. OsMYB86L and OsMYB61L, but not OsMYB58/63 were directly regulated by OsSND2 (Fig. 7b and c). In addition,
we investigated the distribution of SNBE sites and found two and three SNBE sites in the promoter region (1.5-kb upstream sequence of the start codon) of OsMYB86L and OsMYB61L, respectively. Hence, OsSND2 directly regulates the expression of OsMYB86L and OsMYB61L, probably through its binding to SNBE sites in the promoter regions of OsMYB86L and OsMYB61L.

OsMYB86L and OsMYB61L directly activate the transcription of CESAs
OsCESA4, OsCESA7 and OsCESA9 are essential for SCWs cellulose biosynthesis in rice. To investigate whether OsMYB86L and OsMYB61L are involved in SCWs biosynthesis, we performed yeast one-hybrid using OsMYB86L and OsMYB61L fusion proteins and OsCESA4 promoter region (~600 to ~1 bp upstream of the start codon), which contains three SCWs MYB-responsive elements (SMRE) (Fig. 7d). These results provided evidence that OsMYB61L and OsMYB86L bind to the promoter region of OsCESA4 (Fig. 7e).

We performed the transcriptional activation assay in rice protoplasts to investigate whether OsMYB86L and OsMYB61L can activate the transcription of OsCESA4, OsCESA7 and OsCESA9. The assay results show that the luciferase activity was significantly higher for protoplasts co-expressing the reporter carrying three CESAs gene promoters driving luciferase and the effector containing OsMYB86L or OsMYB61L than the negative control (Fig. 7f). Thus, OsMYB86L and OsMYB61L directly activate the transcription of OsCESA4, OsCESA7, and OsCESA9.

Discussion
Secondary cell walls play a critical role in plant growth and development, and they also contain high amounts of lignocellulose, a key feedstock for the production of bio-energy and bio-based products. In rice, OsMYB61 is a key regulator that binds to the promoter of CESAs genes and regulates their expression (Huang et al., 2015). Yeast one-hybrid screening is a powerful tool for the identification and isolation more transcription factors using promoter segments or regulatory elements of targets as baits. In this study, we isolated a NAC transcription factor from the yeast one-hybrid screening using OsMYB61 promoter region containing a SNBE site as bait (Additional file 1: Figure S1). We named it as the OsSND2 based on its closed relationship with AtSND2. We confirmed the direct binding of OsSND2 on OsMYB61 promoter in vitro and in vivo (Fig. 3 and Additional file 2: Figure S2). We also further investigated OsSND2 protein function and downstream genes.

OsSND2 directly activate OsMYB61 expression
We have demonstrated that OsSND2 can directly bind to the promoter of OsMYB61 (Fig. 3b and d). In Arabidopsis, secondary wall NAC family proteins (SWNs) activate their direct target genes through binding to the SNBE sites, and the binding affinities vary with different SWNs and SNBE sequences (McCarthy et al., 2014; Zhong et al., 2010). We have found two SNBE sites in OsMYB61 promoter region. EMSA and ChIP assay showed that OsSND2 binds to the two SNBE sites (Fig. 3e, f and Additional file 2: Figure S2). In previous study, NAC29 and NAC31 were shown to bind only to the SNBE site farther from the start codon (Huang et al., 2015).

The NAC family transcription factors are highly conserved at the N-terminal NAC binding domain and have a highly variable C-terminal domain, which may function as a transcriptional activator or repressor (Olsen et al., 2005). A large number of SWNs function as a transcriptional activator to regulate downstream genes expression in Arabidopsis, such as SND1 and its close homologs (Zhong et al., 2008). Transactivation analysis indicated that OsSND2 exhibits transcriptional activity (Fig. 2b and c) and functions as a transcriptional activator to initiate the transcription of OsMYB61 (Fig. 3d). The expression of OsMYB61 was up-regulated in OsSND2-OX transgenic lines (Fig. 6d) and down-regulated in snd2 mutants (Fig. 5f). We also detected similar expression patterns for OsSND2 and OsMYB61 (Fig. 4 and Additional file 3: Figure S3). Previous study shows that the transcription of OsMYB61 is mainly mediated by NAC29 and NAC31 (Huang et al., 2015). Therefore, we reported OsSND2 as a new transcriptional activator to directly regulate OsMYB61 expression.

OsSND2 regulate secondary wall cellulose biosynthesis
In rice, the genome was predicted to contain 151 NAC genes (Nuruzzaman et al., 2010). The NAC family transcription factors play important roles in plant growth and development (Olsen et al., 2005), especially in response to different abiotic stresses (Fujita et al., 2004;
Hegedus et al., 2003; Tran et al., 2004) and SCWs formations (Zhong and Ye, 2015), NAC proteins may have contributed to the evolution of both water-conducting and supporting cells during the adaptation of plants to land (Xu et al., 2014). In Arabidopsis, SWNs can originate entire regulation network controlling SCWs biosynthesis, SND1 and its homologs act directly upstream of MYB46 and MYB83, which have been reported to bind the promoter of SCWs CESA genes to regulate SCWs cellulose biosynthesis (Wang and Dixon, 2012). OsCESA4, OsCESA7 and OsCESA9 are responsible for secondary wall cellulose biosynthesis in rice, mutation or down-regulation expression of these CESA genes results in brittle culm phenotype and reduction of the cellulose
content (Kotake et al., 2011; Tanaka, 2003; Zhang et al., 2009). OsMYB61 has been reported to directly bind to the promoters of CESA genes to regulate their expression and cellulose biosynthesis (Huang et al., 2015). NAC29-OX, NAC31-OX and OsMYB61-OX transgenic plants have thick internodes, upward curved leaves, and significantly increased cellulose content (Huang et al., 2015). Our findings revealed the up-regulated and down-regulated expression of OsMYB61 in OsSND2-OX lines (Fig. 6d) and snd2 mutants (Fig. 5f), respectively. Consistent with the expression level of OsMYB61, up-regulation of CESA genes expression (Fig. 6d) and increased cellulose content were observed in OsSND2-OX lines (Fig. 6c). On the other hand, down-regulated CESA genes expression (Fig. 5f) and decreased cellulose content were found in snd2 mutants (Fig. 5e). Unlike OsMYB103L, whose mutation lead to the reduction in the cellulose content and a brittle culm phenotype (Ye et al., 2015), snd2 mutants showed normal culm and no obvious change in plant morphology except for lower cellulose content and thinner sclerenchyma cell wall (Fig. 5b and g). These observations suggest that the function of OsSND2 may be redundant to its close homolog in rice. The contents of xylose and lignin were almost unchanged in snd2 mutants and SND2-OX plants (Additional file 4: Table S1). Thus, OsSND2 functions as a regulator to control SCWs cellulose biosynthesis.

Hierarchical transcriptional network regulating the SCWs biosynthetic program is present in rice

In Arabidopsis, the detailed transcriptional network regulating the SCWs biosynthesis has been revealed (Zhong and Ye, 2015). In this transcriptional network, the secondary wall NAC families (SWNs) function as the top-layer master switches to regulate a battery of downstream transcription factors, including SND2, SND3, MYB20, MYB42, MYB46, MYB83 and MYB103 to start the entire SCWs biosynthetic program (Zhong et al., 2008). MYB46 and MYB83 function as the regulators of the secondary-layer and regulate the expression of other MYBs and biosynthetic genes for cellulose, xylan and lignin (Zhong and Ye, 2012). A few studies have investigated the hierarchical transcriptional network regulating SCWs formation in rice. We demonstrated that OsSND2 functions as a regulator to control the expression of MYBs (Fig. 7a), which can further activate the SCWs CESA genes expression. Furthermore, we proved that AtSND2 (At4g28500) can bind to the promoter of AtMYB61 (At1g09540) in yeast one-hybrid assay (Additional file 5: Figure S4). This result suggests the conservation of the regulatory mechanism in dicot and monocot plants. In Arabidopsis, AtSND2 acts downstream of AtSND1 and its close homologs (Zhong et al., 2008). As OsSWN1 and OsSWN2/OsNAC29 are close homologs of AtSND1 in rice (Fig. 1a), they may function as regulators to activate OsSND2 expression and initiate the entire SCWs biosynthetic program. OsMYB58/63, OsMYB61L and OsMYB86L act downstream of OsSND2 (Fig. 7). We have demonstrated that OsMYB61L and OsMYB86L directly activate the transcription of SCWs-related CESA genes (Fig. 7f). OsMYB58/63 was shown to directly up-regulate the expression of OsCESA7 (Noda et al., 2015). Therefore, OsSND2 may act as the secondary-layer master switch involved in the controlling of SCWs biosynthesis, thus, a hierarchical transcriptional network similar to that of Arabidopsis also exists in rice (Additional file 6: Figure S5). These results are consistent with the previous study for survey of involved in rice SCWs formation through a co-expression network (Hirano et al., 2013).

OsSND2 has a potential value in rice straw management

As one of the most important staple food crops, rice produces huge amount of agronomic biomass residues. The handling of biomass is a challenge for breeders, as rice straw decomposes take a long time. Farmers prefer straw burning, which is economic and convenient, but may causes environmental problems. The major reason underlying the difficult treatment procedure is the high cellulose content of the cell wall of straws (Tian et al., 1992). The brittle culm (bc) rice mutants are the ideal breeds for straw treatments owing to lower cellulose content and finer breakage at harvest (Cabiles et al., 2008; Johnson et al., 2006). However, not all bc mutants can be used for breeding because of their concomitant phenotypes, such as dwarfism, low fertility and withering of leaf apex (Zhang et al., 2009; Zhang et al., 2010; Zhou et al., 2009). Mutations in OsMYB103L, a TF regulating SCWs-related genes expression, lead to the decreased cellulose content and brittle culm phenotype without morphological abnormalities (Ye et al., 2015). We have demonstrated that OsSND2 can regulate MYBs and SCWs CESA genes expression (Figs. 5f, 6d and 7a), and that snd2 mutants have lower cellulose contents (Fig. 5e) and exhibit no change in morphology (Fig. 5d). The snd2 mutant plants exhibit a little early flowering may be caused by the effects of the flowering genes expression (Fig. 5d), but show normal morphology. Hence, snd2 mutants have the potential value for rice straw management.

Conclusion

In this study, OsSND2 was identified as a positive regulator of cellulose biosynthesis in rice. Increasing the expression level of this gene can improve the SCWs cellulose content, but the content of xylose and lignin were not affected. Therefore, study the function of OsSND2 can provide a strategy for manipulating plant biomass production.
Additional file 1: Figure S1. Yeast one-hybrid screening using different fragments of OsMYB61 promoter as baits. a. Diagram of OsMYB61 with five different fragments using for bait constructs. b. Diagram of bait construct in yeast one-hybrid screening. c. Self-activation test of five different bait constructs. The transformants harbouring the different bait construct were streaked onto SD-Trp. His media in the presence of 0 mM, 10 mM, 30 mM and 50 mM 3-amino-triazole (3-AT) to determine growth. (TIF 2102 kb)

Additional file 2: Figure S2. OsSND2 binds to the SNBE sites in the OsMYB61 promoter. a. Diagram of OsMYB61 promoter containing two SNBE sites. Dark brown boxes indicate the SNBE elements. The DNA sequences containing the SNBE sites (the red bases) were subjected to the EMSA assay. b. EMSA assay showing that the recombinant OsSND2 protein directly bound to the biotin-labeled sequence containing SNBE2 site. (TIF 1061 kb)

Additional file 3: Figure S3. Expression pattern of OsMYB61. qRT-PCR analysis of OsMYB61 expression in various rice organs and different developmental stage, the heading stage and seedling stage indicate the tenth day after flowering and the two weeks old seedlings, respectively. The Actin1 gene was used as an internal control. Error bars, SD of three biological replicates. (TIF 1176 kb)

Additional file 4: Table S1. Composition analysis of sugar and lignin content of wall residues of the intermodomes from wild type and transgenic rice plants. (DOC 30 kb)

Additional file 5: Figure S4. AtSND2 directly binds to the promoter of Actin1. Yeast one-hybrid assay showing the activity of LacZ reporters driven by Actin1 promoter (2 kb length sequence from the start codon) and activated by AtSND2 fusion with activation domain (AD). The empty pBAD24 and pLacZ were used as negative control. (TIF 331 kb)

Additional file 6: Figure S5. The transcriptional regulatory model of SCW formation in rice. Arrows indicate transcriptional activation, whereas flat-ended arrows indicate transcriptional repression. Solid arrows indicate direct transcriptional activation. Dashed arrows indicate indirect transcriptional activation. (TIF 136 kb)

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Availability of data and materials
All data supporting the conclusions of this article are provided within the article and its additional files (Additional file 1: Figure S1, Additional file 2: Figure S2, Additional file 3: Figure S3, Additional file 5: Figure S4, and Additional file 6: Figure S5).

Authors’ contributions
YW, BL and XF together designed the experiments. YY and BL performed most of the experiments and wrote the manuscript. BL analyzed motifs in promoter regions and performed the ChIP analysis. YY and KW performed the yeast one hybrid screening. KW and QL performed the EMSA and yeast one hybrid analysis. JC performed the subcellular localization of OsSND2 and transcriptional activation. All authors have discussed the results and contributed to the drafting of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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