High-transcriptional activation ability of bamboo SECONDARY WALL NAC transcription factors is derived from C-terminal domain

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Abstract The secondary cell wall, which is mainly composed of cellulose, hemicellulose, and lignin, constitutes woody tissues and gives physical strength and hydrophobic properties for resistance against environmental stresses. We cloned and functionally analyzed the homologous transcription factor (TF) genes of SECONDARY WALL NAC (SWN) proteins from Hachiku bamboo (Phyllostachys nigra; PnSWNs). An RT-PCR analysis showed that PnSWNs are expressed in young tissues in bamboo. Their transcriptional activation activities were higher than that of the Arabidopsis NAC SECONDARY WALL THICKENING PROMOTING FACTOR 3 (NST3) TF, which was equivalent to SWN TFs in monocot. PnSWNs preferred to activate the genes related to secondary cell wall formation but not the genes related to programmed cell death. When PnSWNs were expressed in Arabidopsis, they highly induced secondary cell wall formation, like previously-shown rice SWN1. Dissection analysis revealed that this high activity largely depends on C-terminal domain. These results demonstrate that the cloned bamboo SWNs function as regulators of secondary cell wall formation with strong activation ability derived from C-terminal domain, and could be served as new genetic tools for secondary cell wall manipulation.

Key words: bamboo, NAC transcription factor, nst1 nst3 mutant, secondary cell wall.

Introduction

There are two types of plant cell walls: the primary cell wall, which surrounds all living cells in the plant; and the secondary cell wall, which is produced beneath primary cell wall in some cells such as fiber cells and vascular vessels. The secondary cell wall provides physical strength and hydrophobicity (Albersheim et al. 2010). The components of the primary and secondary cell wall are different: the former consists of weakly crystalized cellulose, xyloglucan-based hemicellulose, and pectin; while the secondary cell wall consists of highly crystalized cellulose, xylan- or glucomanan-based hemicellulose, and lignin (Albersheim et al. 2010). Lignocellulosic biomass is mostly derived from the secondary cell wall and can be converted to biofuel and biomaterials as a sustainable natural resource. Perennial monocotyledonous plants generally grow faster and produce more biomass in a shorter time than do woody plants. Therefore, perennial monocots are considered to be next-generation biomass crops.

Understanding the regulatory mechanism of secondary cell wall formation in plants is important for the manipulation of lignocellulosic biomass to reduce its recalcitrance. Recent studies have revealed that some NAC transcription factors function as master regulators of secondary cell wall formation. VASCULAR-RELATED NAC DOMAIN (VND) 1–7 in Arabidopsis have been well characterized as the regulators of tracheary element differentiation including secondary cell wall deposition (Kubo et al. 2005; Tan et al. 2018; Zhou et al. 2014). NAC SECONDARY WALL THICKENING PROMOTING
FACTORS (NST1, NST2, and NST3, also known as SECONDARY-WALL ASSOCIATED NAC DOMAIN PROTEIN 1 (SN1D), belong to different but closely related clades to the one containing VND transcription factors, and have been identified as master regulators of secondary cell wall formation in various cells (Mitsuda and Ohme-Takagi 2008; Mitsuda et al. 2005, 2007; Zhong and Ye 2015; Zhong et al. 2006, 2007). In particular, NST1 and NST3/SND1 are essential for secondary cell wall formation in Arabidopsis fiber cells (Mitsuda et al. 2007; Zhong et al. 2007) and their genetic manipulation has been shown to improve cell wall digestibility or increase wood density in some plants (Iwase et al. 2009; Sakamoto et al. 2016; Sato et al. 2018). Although their homologues are widely conserved as regulators of secondary cell wall formation in terrestrial plants, these genes have only been functionally characterized in some model plants (Lin et al. 2017; Ohtani et al. 2011; Takata et al. 2019; Xu et al. 2014; Yoshida et al. 2013; Zhong et al. 2011).

Bamboo is a perennial monocot grown in subtropical and temperate zones of east Asia. The young shoot prior to secondary cell wall development is consumed as a food and the developed stem is used for fishing rods, furniture, and as a material for house construction because of its elastic properties. In addition to such daily uses, bamboo is a potential resource for bio-based products (Chen et al. 2017; Okahisa et al. 2011) due to high biomass production by rapid growth and xylogenesis, which initiates from cambium cells in early developmental stage (Li et al. 2016; Qu et al. 2020; Zhang et al. 2018). To understand the molecular mechanisms underlying this rapid growth and xylogenesis, RNA/ genome-sequencing was performed (Ogita et al. 2018; Peng et al. 2013; Zhang et al. 2018). Some candidate genes for secondary cell wall thickening in bamboo have been identified from their genome sequences and found to be substantially expressed even at the apical region of the bamboo shoot (Zhang et al. 2018), but they are yet to be functionally analyzed.

We previously reported that rice NST ortholog (ORYZA SATIVA SECONDARY WALL NAC 1, OsSWN1) is a key regulator of secondary cell wall formation in rice, and has a strong activation ability to induce secondary cell wall formation. Thus, it could be exploited for wood reinforcement in dicots (Sakamoto et al. 2016; Yoshida et al. 2013). The results of our previous study suggested that the activation activity of monocotyledonous SWNs in dicot plants is stronger than that of dicot NSTs. In this study, we report the molecular cloning, expression, and functional analysis of SWN orthologs from Hachiku bamboo (PHYLLOSTACHYX NIGRA). Our results show that bamboo SWN genes are strongly expressed in the young leaf and shoot, consistent with their putative function. In addition, bamboo SWNs have a stronger transcriptional activation ability to induce secondary cell wall formation in Arabidopsis than OsSWN1. We consider that the bamboo SWNs could be employed to manipulate secondary cell wall formation in plants.

**Materials and methods**

**Molecular cloning of PnSNW cDNAs**

Total RNA was isolated from leaves of Hachiku bamboo (P. nigra) maintained in a greenhouse. Leaves were frozen in liquid nitrogen and ground into a fine powder with a mortar and pestle before extraction by the SDS-phenol method. After the treatment of crude total RNA with DNase I (Takara Bio Inc., Otsu, Japan) and repurification by phenol-chloroform treatment and ethanol precipitation, cDNA was synthesized with SuperScript III reverse transcriptase (1 µg total RNA in 20 µl reaction mixture; Thermo Fisher Scientific Inc., Waltham, MA, USA). The cDNA was used as the template for PCR with degenerate primers (PnNST-deg-F8 and PnNST-deg-R4), which were designed from a conserved sequence identified by alignment of 13 NST/SWN sequences (Supplementary Table S1). The PCR cycling program was as follows: 2 min at 94°C followed by 40 cycles of amplification (30 s at 94°C, 30 s at 60°C, and 1 min at 72°C) in a 50-µl reaction mixture containing 5 ng of cDNA, 0.5 µM primers, 0.2 mM dNTPs, 1× reaction buffer, and 3.25 U Blend Taq DNA Polymerase (Toyobo Inc., Osaka, Japan). The two PCR products (around 600 and 700 bp) were separately cloned into the vector pCR2.1-TOPO-TA (Thermo Fisher Scientific Inc.) and sequenced, resulting in the isolation of fragment-1 (597 bp) and fragment-2 (666 bp).

To isolate the 5’ and 3’ missing parts of fragments-1 and -2, 5’ and 3’-rapid amplification of cDNA ends (-RACE) PCR was performed. For these analyses, poly(A) RNA was purified from the leaf total RNA using a MicroPoly(A) Purist Kit (Ambion Inc.), and cDNA was synthesized using a SMARTer RACE cDNA Amplification Kit (Takara Bio Inc.). The 5’-RACE of fragment-1 was performed with the gene-specific primer PnNST-fr1-5RACE-R1 (Supplementary Table S1) and an adaptor primer, with the following thermal cycling program; 30 s at 98°C, followed by 30 cycles of amplification (10 s at 98°C, and 90 s at 72°C). The 20-µl PCR reaction mixture containing 4 ng cDNA, 1× Universal Primer A Mix, 0.5 µM PnNST-fr1-5RACE-R1 primer, 0.2 mM dNTPs, 1× GC reaction buffer, and 0.4 U Phusion Hot Start II High Fidelity DNA Polymerase (Thermo Fisher Scientific Inc.). The smear products around 800–900 bp were purified from the agarose gel and used as the template for the second nested PCR with the gene-specific primer PnNST-fr1-5RACE-R2 (Supplementary Table S1) and a nested adaptor primer under the same conditions as used for the first PCR. The first PCR for the 3’-RACE of the fragment-1 was performed under the same conditions as used for the first 5’-RACE PCR, except that the gene-specific primer was PnNST-fr1-3RACE-F1 (Supplementary Table S1). The smear products around 800–900 bp were purified from the agarose gel and used as the template for the second nested
PCR with the gene-specific primer PnNST-fr1-3RACE-F2 (Supplementary Table S1) and a nested adaptor primer under the same conditions as used for the first PCR, except for the amplification cycle (10 s at 98°C, 30 s at 68°C, and 1 min at 72°C). The 5′-RACE of fragment-2 was performed under the same conditions as used for the first 5′-RACE PCR of fragment-1, except that the gene-specific primer was PnNST-fr2-5RACE-R1 (Supplementary Table S1). The smear products around 1 kbp were purified from the agarose gel and used as template for the second nested PCR with the gene-specific primer PnNST-fr2-5RACE-R2 (Supplementary Table S1) under the same conditions as used for the second 3′-RACE PCR of fragment-1. The 3′-RACE of fragment-2 was performed under the same conditions as used for the first 3′-RACE PCR of fragment-1, except for the use of the gene-specific primer PnNST-fr2-3RACE-F1 (Supplementary Table S1) and the amplification cycle (10 s at 98°C, and 1 min at 72°C). The 5′- and 3′-RACE products corresponding to fragments-1 and 2 were purified from the agarose gel, cloned into the vector pCR-Blunt II-TOPO (Thermo Fisher Scientific Inc.), and sequenced.

The entire coding regions of the cDNAs corresponding to fragments-1 and -2 were obtained by PCR using leaf cDNA (synthesized as described above for 5′-RACE) as the template with primers designed from the 5′ and 3′ untranslated regions (PnNST-fr1-full-F1 and PnNST-fr1-full-R1 primers for the cDNA corresponding to fragment-1, and PnNST-fr2-full-F1 and PnNST-fr2-full-R1 primers for the cDNA corresponding to fragment-2; Supplementary Table S1). In the PCR to amplify the cDNA corresponding to fragment-1, the 50-µl reaction mixture contained 4 ng cDNA, 0.5 µM primers, 0.2 mM dNTPs, 1.5 mM MgSO4, 1× reaction buffer, and 1 U KOD-Plus Neo DNA Polymerase (Toyobo Inc.). The thermal cycling conditions were as follows; 2 min at 94°C, followed by 30 cycles of amplification (10 s at 98°C, and 1 min at 68°C). The thermal cycling conditions to amplify the cDNA corresponding to fragment-2 were as follows; 30 s at 98°C, followed by 35 cycles of amplification (10 s at 98°C, 30 s at 65°C, and 1 min at 72°C). The 50-µl reaction mixture contained 4 ng of cDNA, 0.5 µM primers, 0.2 mM dNTPs, 1× GC reaction buffer, and 1 U Phusion Hot Start II High Fidelity DNA Polymerase. The PCR products from each reaction were cloned into the vector pCR-Blunt II-TOPO and sequenced. We obtained three homologous clones corresponding to fragment-1 (PnSWN2a, PnSWN2b, and PnSWN2c) and two homologous clones corresponding to fragment-2 (PnSWN1a and PnSWN1b).

**RT-PCR analysis**

Total RNA was isolated by the SDS-phenol method from each part (young leaves, mature leaves, shoot apex, shoot internodes, young roots, mature roots, and rhizomes) of *P. nigra* plants grown in a greenhouse. Tissues other than rhizomes were frozen in liquid nitrogen and ground into a fine powder with a mortar and pestle before extraction by the SDS-phenol method. For rhizomes, freeze-dried tissue was ground to a fine powder with a grinding mill (MF10 Basic, IKA-Werke) at 3,000 rpm by setting the powder size to 0.5 mm, and then RNA was extracted from the powder. Crude total RNA samples treated with DNase I were repurified by phenol-chloroform treatment and ethanol precipitation and subsequently using the NucleoSpin RNA Plant Kit (Takara Bio Inc.).

Total RNA was isolated from suspension cells of *P. nigra* (Pn cells) cultured under distinct conditions: one that promoted proliferation (PR condition; lignification is suppressed) and two that promoted lignification (LG1 and LG2 conditions; proliferation is suppressed) (Nomura et al. 2018, 2013; Ogita et al. 2012). Total RNA was isolated from 16-day-old cells using a NucleoSpin RNA Plant Kit, followed by DNase I treatment and repurification using a NucleoSpin column.

The cDNA was synthesized from the purified total RNA with SuperScript III reverse transcriptase (1 µg total RNA in a 20-µl reaction mixture). The PCRs for PnSWN2a/2b, PnSWN2c, and PnSWN1a/1b were performed with the primer sets specific to each of them; i.e., PnNST2/4-rt-F2 and PnNST2/4-rt-R2 primers, PnNST9-rt-F4 and PnNST9-rt-R2 primers, and PnNST14/22-rt-F1 and PnNST14/22-rt-R1 primers, respectively (Supplementary Table S1). The specificity and equality of amplification among the primer sets were verified by PCR using the plasmid DNA harboring each PnSWN cDNA as the template. The PCR cycling conditions were as follows; 2 min at 94°C, followed by 40 cycles of amplification (30 s at 96°C, and 1 min at 68°C) in a 20-µl reaction mixture containing 50 ng of cDNA, 0.5 µM primers, 0.2 mM dNTPs, 1× reaction buffer, and 1.25 U Blend Taq DNA Polymerase (or 0.5 U for amplification of the *P. nigra* Actin gene based on the information of *Phyllostachys edulis* Actin gene [GenBank accession no. F601918] as the endogenous standard). The amplified products were detected with ethidium bromide staining after agarose gel electrophoresis.

**Protein and phylogenetic analysis**

Sequence alignment and phylogenetic analyses were performed using the ClustalW program at the web site (https://www.genome.jp/tools-bin/clusterw) with the dataset listed in Supplementary Table S2. The phylogenetic tree was drawn using MEGA software (ver. 10.0.5, https://www.megasoftware.net/; Kumar et al. 2018) and information listed in Supplementary Table S3.

**Vector construction**

The sequences of VAMP722, OsSWN1, PnSWN1a, PnSWN2a, and PnSWN2c subcloned by PCR amplification were inserted into the pDONR207 vector by the BP reaction and then transferred into destination vectors for transient gene expression and for expression in the *Arabidopsis*nst1nst3 double mutant. Each gene was transferred into the pDEST35SHSP vector for transient effector-reporter assay and pDEST430T1.2 vector for the transactivation assays by the LR reaction. pDEST430T1.2 was constructed by inserting the Gateway cassette (amplified with primers listed in Supplementary Table S1) into the Smal site of the 430T1.2 vector.
Cloning and functional analyses of SWN orthologs in bamboo

To express genes in the nst1 nst3 double mutant under the control of the NST3 promoter, NST3pro::VAMP722::HSPter, NST3pro::OsSWN1::HSPter, NST3pro::PnSWN1a::HSPter, and NST3pro::PnSWN2c::HSPter were each inserted into the pDEST_NST3pro_HSPter_GWB4 vector by the LR reaction. Each construct was derived from the entry clone of each gene, which was prepared from the PCR product using the primers listed in Supplementary Table S1. The pDEST_NST3pro_HSPter_GWB4 vector was constructed by inserting a ca. 3-kb NST3 promoter fragment, which was amplified with the primer sets listed in Supplementary Table S1, into pDEST_HSPter_GWB4. Finally, pHSPter_GWB4 was made by inserting the Arabidopsis HSP 18.2 terminator (Nagaya et al. 2010) into pGW4B4 (Nakagawa et al. 2015). For the construction of reporter plasmid, 1-kb 5’ upstream region amplified by specific primers listed in Supplementary Table S1 was subcloned into pDONR-P4P1R vector (Thermo Fisher Scientific Inc.) by BP reaction, and then transferred into R4L1pDEST_LUC_HSP (Oshima et al. 2013) vector by LR reaction. For domain swapping, N-terminal end from NST3 and C-terminal ends from PnSWNs were amplified with specific primers listed in Supplementary Table S1. Each PCR amplicon was mixed and connected each other by the overlap extension PCR using primer sets of “attB1_AT1g32770”–“attB2_PnNST12_C” and “attB1_AT1g32770”–“attB2_PnNST9_C”.

Transactivation assay

Mesophyll protoplasts were isolated from true leaves of 4-week-old Arabidopsis thaliana ecotype Columbia-0 plants grown on soil under a 16-h light (60–80 mol m⁻² s⁻¹)/8-h dark photoperiod using the tape-sandwich method as described previously (Sakamoto et al. 2016). Transient gene transfection was carried out using the PEG/Ca²⁺ method in a 96-well plate. The reporter construct consisted of firefly luciferase driven by the promoter containing 5×Gal4 DNA binding sites, and was co-introduced with the effector construct containing NST3 or the rice or bamboo orthologs driven by the 35S promoter and the reference plasmid containing codon-optimized Renilla luciferase (hRLUC, Promega Inc.) driven by the 35S promoter and terminated by the HSP terminator (Nagaya et al. 2010) (phRLHSP). The transfected protoplasts were rinsed with W5 buffer [150 mM NaCl, 125 mM CaCl₂, 5 mM KCl, and 2 mM MES (pH 5.7)] and then incubated at 22°C for 18 h in the dark. Dual-luciferase activity was determined using the Pikkagene-Dual kit (TOYO B-Net Inc.). “Relative luciferase activity” was calculated by dividing the reporter activity by the reference activity.

Transformation into nst1 nst3 double mutant

The nst1-1 nst3-1 double mutant (Mitsuda et al. 2007) of A. thaliana was grown in soil at 22°C under a 16-h (60–80 mol m⁻² s⁻¹)/8-h light/dark photoperiod. Two-month-old double mutant plants were used for transformation with the floral-dip method (Clough and Bent 1998). Transgenic T1 seeds were sown on Murashige and Skoog solid medium supplemented with kanamycin and grown for 2 weeks. The kanamycin-resistant plants were transplanted into soil and grown for 5 weeks before harvesting all the inflorescence stems for analysis.

Microscopic observation of stem cross-section

The stem slice sections were prepared as described by Sakamoto et al. (2016). The 3-cm lowermost part of the inflorescence stem from a 7-week-old Arabidopsis plants grown in soil was embedded in 5% agar and then sliced into 50-μm-thin sections with a vibrating microtome (Microm HM-650 V, Thermo Fisher Scientific Inc.). Sliced sections were immediately observed under bright-field and UV-illumination, or fixed and stored in FAA solution (50% ethanol, 5% formaldehyde, and 5% acetic acid) until use. Lignin was stained with Mäule stain as described by Sakamoto et al. (2016) using the fixed-slice sections mentioned above. All images were captured with the Axioscop2 Plus System (Carl Zeiss Inc.).

Monosaccharide composition analysis

Monosaccharide composition analysis was performed as described previously (Sakamoto and Mitsuda 2015; Sakamoto et al. 2015). The lowermost 10-cm portion of the inflorescence stem from an 8-week-old Arabidopsis plant was used for these analyses. To ensure sufficient tissue for various analyses, the lower stem portions harvested from four to five plants were combined as one sample. The methanol-fixed inflorescence stem was treated with methanol at 80°C for 10 min twice, acetone at 70°C for 5 min twice, methanol: chloroform (1:1, v/v) at 70°C for 5 min twice, and then rinsed with 100% ethanol. After drying at 65°C overnight, the stem segment was pulverized with 3 zirconia beads (3 mm, Nikkato Inc.) and a stainless bead (6 mm, Biomedical Science Inc.) in 2 ml-screw cap tube. The powdered stem material was destarched with α-amylase solution [500 U ml⁻¹ α-amylase (Megazyme Inc.) and 0.33 U ml⁻¹ amylo-glucosidase (Megazyme Inc.) in 0.1 M sodium malate buffer (pH 6.0)] for 18 h at 37°C, and then rinsed with ultrapure water. After desiccation in a sample heater, the destarched powder was weighed in 2–3 mg portions into tightly capped 2 ml-microtubes (Eppendorf Inc.) for subsequent analyses.

To determine monosaccharide composition, the destarched sample was hydrolyzed by the two-step sulfuric acid degradation method as described previously (Sakamoto et al. 2015). The hydrolysate was neutralized with calcium carbonate powder, and then 5 μl supernatant was mixed with 20 μl p-aminobenzoic ethyl ester (ABEE) solution containing 330 mg ml⁻¹ p-aminobenzoic ethyl ester, 70 mg ml⁻¹ sodium cyanoborohydride, 8% (v/v) acetic acid, and 70% (v/v) methanol, and then derivatized at 80°C for 30 min. After removing excess ABBE by the liquid-liquid extraction with...
chloroform, the aqueous layer was loaded into a UPLC system. The components of the mixture were separated with boric acid and acetonitrile as the eluent, and detected with a fluorescence detector (ACQUITY UPLC FLR Detector, Waters Inc.) at 305 nm (excitation) and 360 nm (emission). Products were quantified by comparison with a standard curve prepared from detected area indexes of authentic sugar solutions.

Lignin quantification
Lignin content was determined by the method described by Sato et al. (2018). Cell wall residue (2–3 mg) was hydrolyzed by a two-step sulfuric acid hydrolysis at 121°C for 60 min. The resultant residue was rinsed with ultrapure water and dried at 65°C overnight. The complete dried residue was weighed as acid-insoluble lignin. Acid-soluble lignin was calculated from the UV absorption value at 205 nm using the lignin extinction coefficient (110 l/g/cm).

Results and discussion
Isolation of SWN orthologs from bamboo
By using RT-PCR primers designed from a conserved sequence among 13 NST/SWN orthologs from Arabidopsis and monocots, we successfully cloned a partial cDNA fragment with high homology to O. sativa SWNs (OsSWNs) using RNA isolated from Hachiku bamboo young leaves. The full-length cDNA was obtained by RACE. We finally obtained five full-length clones with high similarity to OsSWNs. These sequences encompass open reading frames of 1,128 bp (PnSWN1a [GenBank accession no. LC497864]; PnSWN1b [GenBank accession no. LC497865]), 1,170 bp (PnSWN2a [GenBank accession no. LC497861]), 1,173 bp (PnSWN2b [GenBank accession no. LC497862]), and 1,125 bp (PnSWN2c [GenBank accession no. LC497863]). The UTR sequences of PnSWN1a and PnSWN1b are very similar as well as PnSWN2a and PnSWN2b, suggesting that these may be derived from same loci but no concrete evidence (Supplementary Figure S1). We found that all the PnSWNs have typical conserved motifs; a NAC domain, four other domains (D1–D4 in Figure 1A), and two domains specific to monocot SWNs (Figure 1A and Supplementary Figure S2). According to the coding sequence data of Moso bamboo (Phyllostachys heterocycla) (Peng et al. 2013), the Moso bamboo genome has two OsSWN1 homologous genes (PH01000352G0610 and PH010000352G0610) and two OsSWN2 homologous genes (PH010000352G1230 and PH01001896G0600) (Zhao et al. 2014). Our cloned genes correspond to three of them (PnSWN1a and 1b match to PH01000439G0460 with 100% and 99% identity in amino acid level, respectively. PnSWN2a and 2b match to PH010000352G1230 with 99% identity in amino acid level. PnSWN2c match to PH01001896G0600 with 98% identity in amino acid level.) (Supplementary Table S4). One OsSWN1 homologue (PH01000352G0610), which has 92% identity in amino acid level with the other one (PH01000439G0460), was not able to be cloned maybe due to low expression in the tissues employed in this study or not encoded in the genome of Hachiku bamboo. The phylogenetic analysis showed all cloned PnSWNs belong to the OsSWN subclade of the NST/SWN clade (Figure 1B), indicating that the cloned PnSWNs are orthologs of NST/SWNs in Hachiku bamboo.

Preferential expression of PnSWNs in young bamboo tissues
To determine the tissue-specific expression patterns of PnSWNs, we carried out semi-quantitative RT-
PCR using RNA isolated from several tissues and plants at different growth stages. We employed specific primer sets to distinguish PnSWN1a/PnSWN1b, PnSWN2a/PnSWN2b, and PnSWN2c under high stringent condition and detected the transcripts of PnSWN1a/PnSWN1b and PnSWN2c in some tissues of bamboo, while not those of PnSWN2a/PnSWN2b probably due to low expression levels in the examined tissues (Figure 2). Transcripts of PnSWN2c were detected in leaves, shoots, and rhizome, while transcripts of PnWSN1a/1b were detected in all of the tested tissues except for proliferating suspension cells. Zhang et al. (2018) reported that the expression level of SWN orthologs in Moso bamboo is high in middle part of the bamboo shoot but is adequate both in the apex and the bottom parts, consistent with our findings. These results suggest that bamboo SWNs are widely expressed in bamboo tissues and may not have a functional divergence in fibers and vessels.

**PnSWNs have strong and distinct transcriptional activation ability**

Next, we analyzed the transcriptional activation abilities of PnSWN1a and PnSWN2c, which were expressed in bamboo tissues (Figure 2), by the protein fused to the Gal4 DNA-binding domain in a transient reporter-effector system using Arabidopsis mesophyll protoplast. Considering the similarity of the PnSWNs to OsSWN1, which has a stronger transcriptional activity than that of Arabidopsis NST3/SND1 (Sakamoto et al. 2016), we expected that the PnSWNs would also have stronger transcriptional activation activity than that of Arabidopsis NST3. We selected PnSWN1a and PnSWN2c as representatives based on homology and expression pattern. In our analyses, PnSWN1a and PnSWN2c had stronger transcriptional activation ability than those of OsSWN1 and NST3/SND1 (Figure 3A, B).

The NAC transcription factors generally have two structural characteristics, a highly conserved N-terminal NAC domain for DNA binding and divergent C-terminal intrinsically disordered region (IDR) (Ernst et al. 2004). Considering the high similarity of N-terminal domains among NACs, the differences in the C-terminus IDR are probably responsible for their high transcriptional activation ability. Thus, we created domain-swapped chimeric TFs consisting of N-terminal half of NST3 and C-terminal half of PnSWN1a or PnSWN2c (Figure 3C). As expected, these chimeric TFs showed higher transcriptional activation ability than NST3, indicating that C-terminal region is responsible for the high transcriptional activation ability of PnSWNs (Figure 3D).

We further analyzed the transcription activation ability of them as well as VNDs for the promoters of genes related to tracheary element differentiation (XCP1 and XCP2) and secondary cell wall formation (MYB46, 4CL, LAC12, CESAs8, and IRX9) in a transient effector-reporter analysis, in which ca. 1 kb promoter regions of Arabidopsis were employed (Figure 4). As previously demonstrated for VNDs (Yamaguchi et al. 2011), VND6 and VND7 highly activated XCP-promoters-driven reporter while the activation by NST/SWNs was limited (Figure 4B). In contrast, NST/SWNs showed higher activation ability for promoters of genes related to secondary cell wall formation than VNDs (Figure 4B). Clustering analysis of the promoter activation ability suggested that VNDs and NST/SWNs including PnSWNs belong to different distinct class (Figure 4C).

Kang et al. (2018) suggested that the C-terminal domain determines the residence time near DNA via electrical interactions, based on the results of an analysis of changes in transcriptional activation ability after a D24-H135 histidine-switch in the NAC domain.
also reported that the negatively charged C-terminal domain controls the structure of the N-terminal for DNA binding. We calculated the pI value, which reflects the electric charge of the protein complex, for the C-terminal domains of NST/SWNs orthologs tested in this study. The calculated pI values were 4.53 for NST3, 5.69 for OsSWN1, 5.56 for PnSWN1a, and 5.34 for PnSWN2c. These pI values were less correlated with transcriptional activity. These findings suggest that the strong activation ability of OsSWN and PnSWNs is not due to the level of negative charge at the C-terminal domain. Further structural analyses are required to clarify the mechanism of the strong transcriptional activation ability of monocot SWNs.

**Enhanced cell wall formation in Arabidopsis inflorescence stem by PnSWNs expression**

Previously, we showed that OsSWN1 is able to induce ectopic and strong secondary cell wall formation in a dicot plant (Sakamoto et al. 2016). To determine whether PnSWNs have similar characteristics to induce secondary cell wall, we first investigated the phenotypic restoration of transgenic *nst1 nst3* double mutant lines expressing PnSWNs under the control of the NST3 promoter (Figure 5). Although the *nst1 nst3* double mutant and negative-control transgenic lines (expressing VAMP722) showed a pendent stem phenotype, the PnSWNs transgenic lines (*nst1 nst3 NST3pro::PnSWNs*) showed a clearly restored phenotype, like the OsSWN1 transgenic lines (Figure 5).

Next, we examined lignin distribution in the stem cross section by monitoring UV autofluorescence of lignin and Mäule staining (Figure 6). The stem section of the wild-type plant clearly showed UV autofluorescence and lignin staining in interfascicular fiber cells of the inflorescence stem while that of *nst1 nst3* double mutant did not. The color of Mäule staining in interfascicular fiber cells was red in the wild type, indicating enrichment of syringyl units in the lignin in interfascicular fiber cells.
In contrast, the PnSWN1a and PnSWN2c transgenic lines showed ectopic secondary cell wall formation in the pith of the inflorescence stem and increased secondary cell wall formation in interfascicular fiber cells, as previously shown and discussed for OsSWN1 transgenic line (Sakamoto et al. 2016).

We quantitatively determined the AIR/FW ratio (ratio of amount of cell wall to fresh weight of plant tissue) (Figure 7). The AIR/FWs of PnSWN1a and PnSWN2c and the OsSWN1 transgenic lines were significantly higher than that those of the wild type and the nst1 nst3 double mutant. Together, these results indicate that PnSWNs under the control of the NST3 promoter have a strong ability to induce secondary cell wall formation in the inflorescence stem in the nst1 nst3 double mutant. The transcriptional activation ability shown in Figure 3 did not necessarily correspond to the AIR/FW value in PnSWNs transgenic lines. This could be explained by the limited sink capacity; that is, it may have already reached the maximum in the OsSWN1 transgenic line. Further analyses in woody plants with a larger sink capacity in the stem are required to compare the ability to induce secondary cell wall formation between OsSWN1 and PnSWNs.
Increased abundance of secondary cell wall-related components in PnSWN lines

To assess whether cell wall composition is also affected by the increase in secondary cell wall formation in PnSWNs lines, the monosaccharide composition and lignin content in the inflorescence stem of each line were chemically determined (Figure 8). In our previous study, we determined that the contents of glucose (Glc), xylose (Xyl), 4-O-methyl-D-glucuronic acid (mGlcA), and glucuronic acid (GlcA), the main components of the secondary cell wall, were lower in the nst1 nst3 double mutant than in wild type, while the contents of galacturonic acid (GalA), galactose (Gal), rhamnose (Rha), arabinose (Ara), and fucose (Fuc), which are main monosaccharide of primary cell wall, were higher (Sakamoto and Mitsuda 2015). Those results were consistent with the cell wall compositions in the wild type and nst1 nst3 double mutant determined in this study.

Figure 5. Phenotypic restoration of nst1-1 nst3-1 pendent phenotype by monocot SWNs. Pendent phenotype of nst1-1 nst3-1 mutant was recovered by introducing OsSWN1, PnSWN1a, and PnSWN2c, but not VAMP722 (negative control).

Figure 6. Monocot SWNs induce ectopic lignification in pith of Arabidopsis inflorescence stem. Cross sections of Arabidopsis inflorescence stem of wild-type, nst1-1 nst3-1 double mutant, VAMP722, OsSWN1, PnSWN1a, and PnSWN2c lines (n=4). Different letters indicate significant differences among lines (p<0.05; Tukey–Kramer test).

Figure 7. Increased amount of cell wall in monocot SWN lines. AIR/FW of bottom 10-cm long Arabidopsis inflorescence stem of wild-type, nst1-1 nst3-1 double mutant, VAMP722, OsSWN1, PnSWN1a, and PnSWN2c lines (n=4). Different letters indicate significant differences among lines (p<0.05; Tukey–Kramer test).

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study (Figure 8). The increase in the amounts of primary cell wall constituents is due to the relative changes in the proportions of monosaccharides. The contents of Xyl, mGlcA, and GlcA were clearly higher in the OsSWN1, PnSWN1a and PnSWN2c lines than in the nst1 nst3 and VAMP722 (negative control) lines. However, the contents of GalA, Gal, Rha, Ara, and Fuc were lower in the OsSWN1, PnSWN1a, and PnSWN2c lines than in the nst1 nst3 and VAMP722 lines. These results indicate that secondary-wall-related monosaccharides are enriched in the cell wall residues of the PnSWN lines.

The lignin contents of the lines were also determined. Acid-insoluble lignin contents were comparable among PnSWNs, wild-type, and OsSWN1 lines, but the lignin contents in the nst1 nst3 and VAMP722 lines were about one-third that in wild type (Figure 9). The acid-soluble lignin contents in OsSWN1 and PnSWNs lines were much lower than that in wild type, while those in...
nst1 nst3 and VAMP722 transgenic lines were higher than that in wild type. The acid soluble-lignin content tends to correlate with the ratio of syringyl to guaiacyl units (S/G ratio) in lignin (Al-Haddad et al. 2013; Kishimoto et al. 2010), suggesting that the S/G ratio will be lower in the OsSWN1 and PnSWNs lines than in wild type. Indeed, the S/G ratio was found to be lower in OsSWN1-overexpressing poplar, which produced abundant secondary cell wall materials, than in wild type (Nuoendagula et al. 2018). Taken together, these results show that the reconstituted cell wall in the PnSWNs transgenic lines is overproduced secondary cell wall, like in OsSWN1 lines.

Conclusion
In this study, we cloned and functionally analyzed bamboo orthologous genes of NST/SWN transcription factors. Our results suggest that PnSWNs are regulators of secondary cell wall formation in Hachiku bamboo. We also demonstrated that cloned bamboo SWNs have stronger activation ability than OsSWN1, and induce ectopic secondary cell wall formation as effectively as OsSWN1. Considering that their strong activities, these genes are potential resources to reinforce wood density and biomass production in dicot plants. The genetic modification or genome editing of PnSWNs in bamboo could be a practical strategy for the engineering of lignocellulose biomass. Bamboo is an economically important non-timber forest plant, and the flexible regulation of secondary cell wall formation has great potential to expand its industrial uses. Further detailed analyses of the regulators of secondary cell wall formation in bamboo will provide us with more critical information for this.

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