BpNAC012 Positively Regulates Abiotic Stress Responses and Secondary Wall Biosynthesis[1][OPEN]

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NAC (NAM, ATAF1/2, and CUC2) transcription factors play important roles in plant biological processes and stress responses. Here, we characterized the functional roles of BpNAC012 in white birch (*Betula platyphylla*). We found that BpNAC012 serves as a transcriptional activator. Gain- and loss-of-function analyses revealed that the transcript level of *BpNAC012* was positively associated with salt and osmotic stress tolerance. BpNAC012 activated the core sequence CGT[G/A] to induce the expression of abiotic stress-responsive downstream genes, including Δ-1-pyrroline-5-carboxylate synthetase, superoxide dismutase, and peroxidase, resulting in enhanced salt and osmotic stress tolerance in *BpNAC012* overexpression transgenic birch lines. We also showed that *BpNAC012* is expressed predominantly in mature stems and that RNA interference-induced suppression of *BpNAC012* caused a drastic reduction in the secondary wall thickening of stem fibers. Overexpression of *BpNAC012* activated the expression of secondary wall-associated downstream genes by directly binding to the secondary wall NAC-binding element sites, resulting in ectopic secondary wall deposition in the stem epidermis. Moreover, salt and osmotic stresses elicited higher expression levels of lignin biosynthetic genes and elevated lignin accumulation in *BpNAC012* overexpression lines. These findings provide insight into the functions of NAC transcription factors.

Plant-specificNAC (no apical meristem [NAM], ATAF1/2, and cup-shaped cotyledon [CUC2]) proteins belong to one of the largest families of transcription factors, which are expressed in different tissues at various developmental stages. The number of NAC members in different plant species varies greatly, from 30 in the early divergent land plants to 177 genes in soybean (*Glycine max*; Zhu et al., 2012; Cenci et al., 2014). To date, 105 NAC genes have been identified in Arabidopsis (*Arabidopsis thaliana*), 113 in sorghum (*Sorghum bicolor*), 115 in maize (*Zea mays*), 138 in rice (*Oryza sativa*), and 163 in poplar (*Populus trichocarpa*; Voitsik et al., 2013; You et al., 2015). All NAC family proteins contain a conserved NAC domain with a stretch of approximately 150 conserved amino acids at the N terminus, which serves as a platform for DNA binding and also is responsible for the formation of homodimers or heterodimers with other NAC domain proteins (Ooka et al., 2003; Welner et al., 2012; Fang et al., 2014; Lindemose et al., 2014). The C-terminal regions of NAC proteins commonly contain simple amino acid repeats, including the repeats of Pro and Gln, Ser and Thr, or acidic residues (Olsen et al., 2005). The C-terminal region of NAC proteins serves as a transcriptional activator or transcriptional repressor, which is a diversified domain with variable sequence and length (Xie et al., 2000; Hao et al., 2011). As important regulatory proteins, transcription factors function by binding to cis-elements in the promoters of their target genes. The motifs bound by NAC proteins have been studied. ANAC019/055/072 specifically bind to the core DNA motif CACG (Tran et al., 2004; Zhou et al., 2013). Additionally, NAC proteins bind specifically to a DNA motif, TTNCGT[G/A], but with different affinities (Jensen et al., 2010). The target promoter regions bound by NAC proteins mostly contain the core sequence CGT[G/A] (Olsen et al., 2005; Xu et al., 2013; Lindemose et al., 2014). Secondary wall NAC master switches bind to a common cis-element, named the secondary wall NAC-binding element (SNBE), which is composed of an imperfect palindrome of 19 bp with consensus sequence (T/A)NN(C/T)(T/C/G)TNNNN NNNA(A/C)GN(A/C/T)(A/T; Zhong et al., 2010a, 2010b).

Plant NAC proteins play important roles in diverse processes, such as control of boundary cell formation (Aida et al., 1997), lateral root development (Xie et al., 2000), leaf senescence (Guo and Gan, 2006), flowering (Kim et al., 2007; Ying et al., 2014), boundary and shoot

[1]This work was supported by the National Natural Science Foundation of China (31470663), the 111 Project (B16010), and the National High Technology Research and Development Project (863 Program) of China (2013AA102704).

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C.P.Y. and P.H. conceived the original screening and research plans; P.H. performed most of the experiments and analyzed the data; K.M.Z. provided technical assistance to P.H.; P.H. conceived the project and wrote the article with contributions of all the authors; C.P.Y. supervised and complemented the writing.

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www.plantphysiol.org/cgi/doi/10.1104/pp.18.01167

meristem formation (Vroemen et al., 2003), secondary cell wall synthesis (Mitsuda et al., 2005, 2007; Zhong et al., 2006), and xylem vessel differentiation (Kubo et al., 2005; Yamaguchi et al., 2011). Overexpression of the NAC domain genes *PtVNS* (for VND-, NST/SND-, and SMB-related proteins)/*PtrWND* (for wood-associated NAC domain transcription factor) from poplar induced ectopic secondary wall thickening in poplar leaves, suggesting that wood formation in poplar is controlled by cooperative functions of the NAC proteins (Ohtani et al., 2011). PtrWND2B and PtrWND6B activate the promoter activities of a number of wood-associated transcription factors and wood biosynthetic genes in poplar, suggesting that PtrWNDs and their downstream transcription factors form a transcriptional network to regulate wood formation in poplar (Zhong et al., 2010a). Moreover, NAC proteins also play important roles in plant responses to abiotic stresses. A rice stress-responsive NAC gene (*SNAC1*) is highly induced by osmotic stress and can improve drought and salt tolerance by enhancing root development and reducing transpiration rates (Liu et al., 2014). SNAC1 also activates the expression of genes involved in abiotic stresses and abscisic acid (ABA) signaling, including Suc phosphate synthase, 1-phosphatidylinositol-3-phosphate-5-kinase, regulatory components of ABA receptor, and type 2C protein phosphatases (Saad et al., 2013). A rice NAC protein (OsNAP) enhances salinity and drought tolerance by inducing the expression of *OsPP2C06*/*OsABI2*, *OsPP2C09*, *OsPP2C68*, and *OsSalT* as well as transcription factors involved in stress (OsDREB1A, OsMYB2, OsAP37, and OsAP59; Chen et al., 2014). Overexpression of *TaNAC2*, a NAC member from wheat (*Triticum aestivum*), enhances tolerances to drought, salt, and freezing stresses in transgenic Arabidopsis lines by inducing the expression of the abiotic stress-responsive genes *DREB2A*, *RD22*, *ABI2*, and *ABI5* (Mao et al., 2012).

Although many NACs have been cloned and identified in various plant species, only a few of them have been characterized functionally (You et al., 2015). White birch (*Betula platyphylla*) is one of the main broad-leaf tree species with a characteristic of fast growth. This widely grown tree is tolerant to cold and drought and has important applications in biofuels and pulp industries (Li et al., 2002; Borrega et al., 2013). In this study, we characterized the function of BpNAC012, a NAC transcription factor from the white birch. We found distinct roles of BpNAC012 in regulating abiotic stress responses and secondary wall biosynthesis. Chromatin immunoprecipitation (ChIP) assays revealed the binding specificity of BpNAC012 to the core sequence CGT[G/A] and the SNBE site in the promoters of abiotic stress-responsive downstream genes and secondary wall-associated downstream genes, respectively. This novel mechanism may be relevant to other transcription factors that generate specific patterns of gene expression in plant development and stress responses.

RESULTS

Cloning and Sequence Analysis of *BpNAC012*

Previously, we generated transcriptomes of birch, and a NAC homolog that was highly expressed in birch stems was identified and investigated in this study (Wang et al., 2014). The coding sequence (CDS) of this NAC gene is 1,281 bp, encoding a deduced protein of 426 amino acids. The phylogenetic tree was constructed for this birch NAC gene with 24 Arabidopsis NAC genes that are representative of different NAC subfamilies by the neighbor-joining method using MEGA5. The result revealed that this birch NAC gene is closely related to the Arabidopsis *SND1* gene (AT1G32770, also called *NST3*/*ANAC012*; Supplemental Fig. S1). Therefore, we designated the birch NAC gene as *BpNAC012*, which was deposited in GenBank with accession number KT344119.

Expression Patterns of *BpNAC012*

The transcript level of *BpNAC012* was determined using reverse transcription quantitative PCR (RT-qPCR) in different tissues. The transcript level of *BpNAC012* is highest in stems, followed by the leaves and apical buds; *BpNAC012* expression is lowest in the roots, which was used as a calibrator to normalize the expression level in other tissues (Fig. 1A). These results indicated that *BpNAC012* has a tissue-specific expression profile and is expressed predominantly in stems. Additionally, *BpNAC012* is expressed preferentially in mature stems or leaves rather than in young stems or leaves, indicating that BpNAC012 also is involved in plant development.

The expression of *BpNAC012* in birch leaves in response to salt, osmotic, and drought stresses or ABA treatment was studied further. The results showed that the expression of *BpNAC012* is induced by both NaCl and mannitol treatments and reached peak levels at 24 h of stress (Fig. 1B). We further studied the expression of *BpNAC012* in the leaves of birch plants grown in soil without water for 14 d. The results showed that the expression of *BpNAC012* increased from 3 to 14 d without watering (Fig. 1B). Under ABA treatment, the expression of *BpNAC012* was induced and peaked at 9 h (Fig. 1B). These results indicated that *BpNAC012* expression responds to salt, osmotic, and drought stresses, suggesting an involvement in abiotic stress responses.

Determination of the Transcriptional Activation Domain of BpNAC012

To determine the transcriptional activation domain of BpNAC012, the full-length or truncated CDS of *BpNAC012* was fused with the *GAL4* DNA-binding domain and transformed into Y2H Gold Yeast Strain (Clontech) for transcriptional activity detection. The

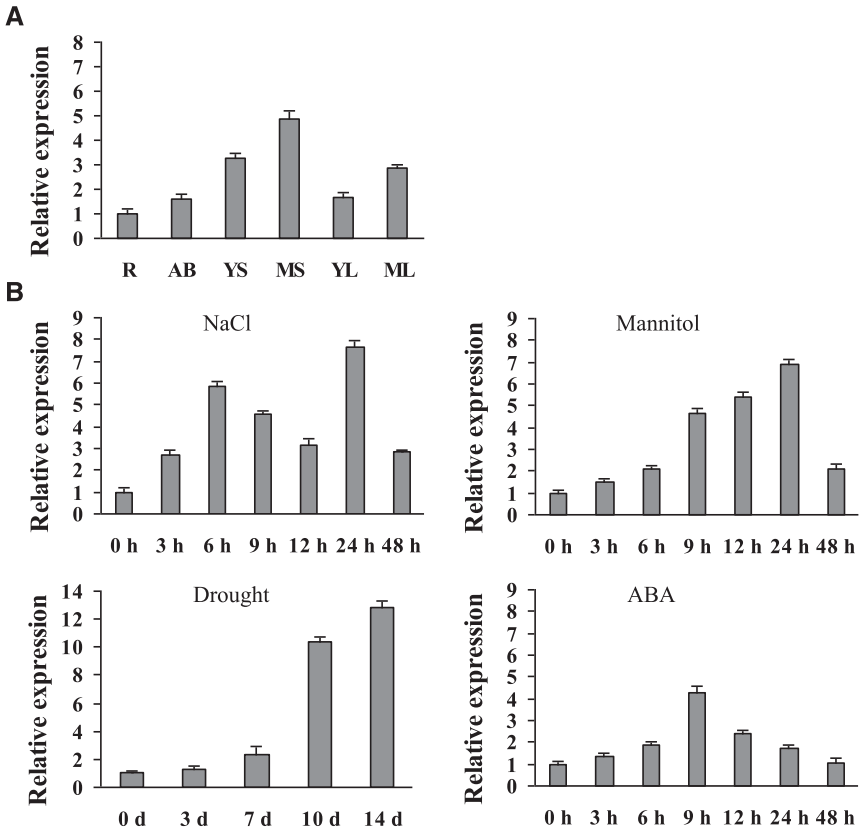

Figure 1. Expression pattern analysis of *BpNAC012*. A, Expression of *BpNAC012* in different birch tissues: roots (R), apical buds (AB), young stems (YS), mature stems (MS), young leaves (YL), and mature leaves (ML). The relative expression in other tissues was normalized by that in roots, which was set as 1. B, Expression patterns of *BpNAC012* in birch leaves responding to 200 mM NaCl, 300 mM mannitol, drought stress, or 100 μM ABA treatment. Birch plants without stress were used as the mock controls to normalize the expression in the stress treatments. Error bars indicate the SD of three biological replicates.

full-length CDS demonstrated transactivation ability, indicating that BpNAC012 is a transcriptional activator (Supplemental Fig. S2). Additionally, the minimal truncated region that showed transcriptional activity consists of amino acids 145 to 215; further deletion of this region completely abolished the transactivation activity (Supplemental Fig. S2), suggesting that the transcriptional activation domain of BpNAC012 is in amino acids 145 to 215.

Generation of Transgenic Birch with Overexpression or Suppression of *BpNAC012*

To investigate the function of BpNAC012 using gain- and loss-of-function methods, transgenic birch plants that overexpressed *BpNAC012* or had *BpNAC012* suppressed by RNA interference (RNAi) were generated using *Agrobacterium tumefaciens*-mediated transformation. Sixteen lines overexpressing *BpNAC012* (OE) and 17 lines with RNAi-suppressed *BpNAC012* (RS) were generated. The expression levels of *BpNAC012* in the transgenic lines were studied using RT-qPCR (Supplemental Fig. S3). Two *BpNAC012* overexpression lines, OE3 and OE8, were randomly selected for gain-of-function study; two RNAi-suppressed *BpNAC012* transgenic lines, RS1 and RS6, where the expression of *BpNAC012* was decreased greatly, were selected for loss-of-function analysis (Supplemental Fig. S3).

Salt and Osmotic Stress Tolerance Test

The NaCl- and mannitol-inducible expression of *BpNAC012* prompted us to analyze its potential role in salt and osmotic resistance (Fig. 1B). The plantlets of BpNAC012-OE, the wild type, and BpNAC012-RS of similar sizes were cultured on one-half-strength Murashige and Skoog (1/2MS) medium containing 80 mM NaCl or 100 mM mannitol for 21 d, and plantlets grown on 1/2MS medium were used as a control. Under normal conditions, there was no difference in the growth and phenotype among these lines. Under NaCl and mannitol stress conditions, the transgenic OE plants grew better than the wild-type and RS plantlets and remained green; however, the RS lines wilted and died (Fig. 2A). Additionally, the fresh weight and root length of the OE lines were the highest, followed by the wild-type plants; the RS lines had the lowest fresh weight and root length (Fig. 2, B and C). These results indicated that overexpression of *BpNAC012* confers substantially improved tolerance to salt and osmotic stresses in transgenic plants.

Pro Biosynthesis Is Regulated by BpNAC012

The Pro content was measured among the BpNAC012-OE, wild-type, and BpNAC012-RS plants. Under normal conditions, each studied line had similar Pro content. Under salt and osmotic stress treatments,

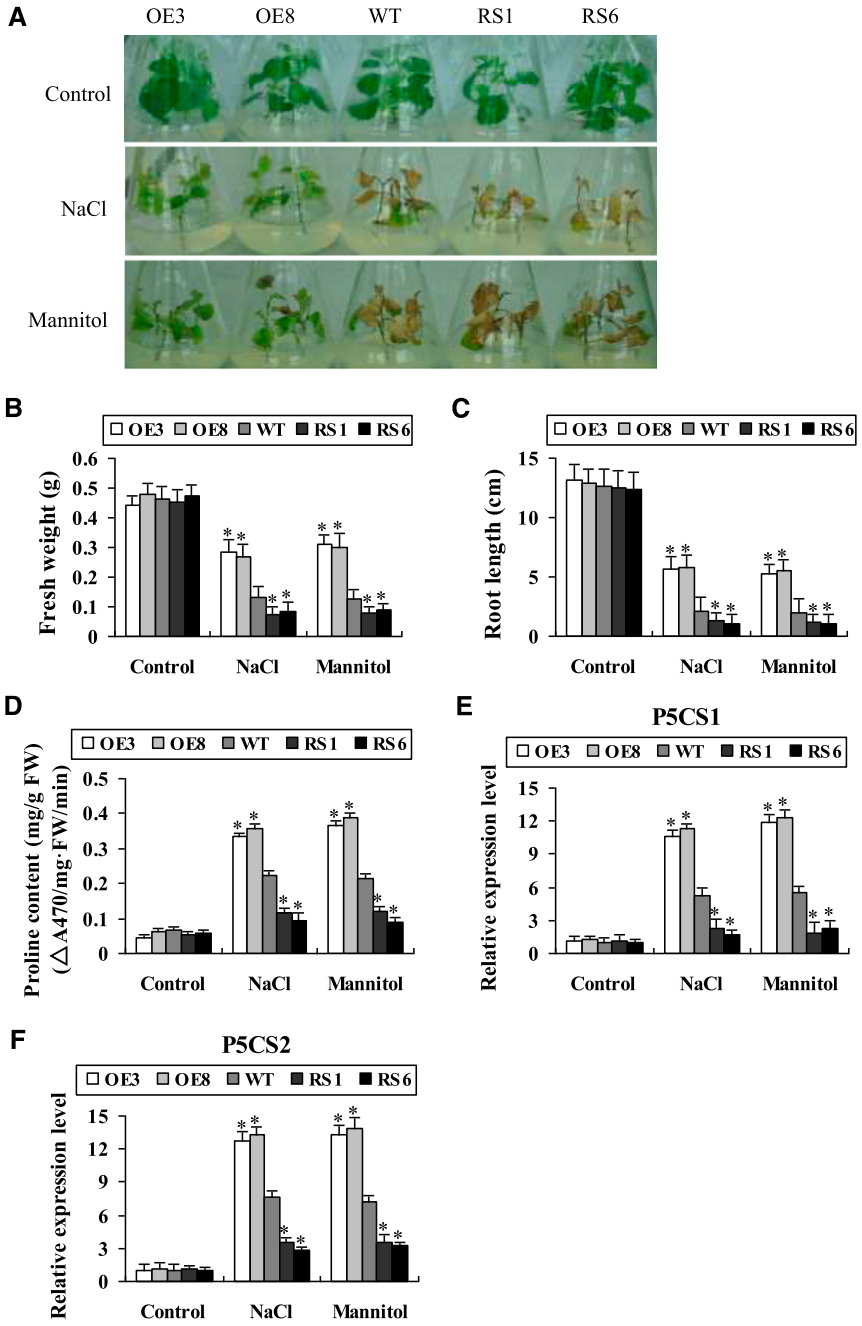

Figure 2. Plant growth and Pro biosynthesis under NaCl and mannitol stresses. A, Plant growth assay. The BpNAC012-overexpression (OE3 and OE8), RNAi (RS1 and RS6), and wild-type (WT) plantlets were cultured on 1/2MS medium containing 80 mM NaCl or 100 mM mannitol for 21 d. Plantlets grown on normal 1/2MS medium were used as controls. B and C, Fresh weight (B) and root length (C) of the seedlings after growth in normal, NaCl, or mannitol medium for 21 d. D, Measurement of Pro contents. FW, Fresh weight. E and F, Expression levels of *BpP5CS1* (E) and *BpP5CS2* (F) genes. Birch plants were subjected to 200 mM NaCl or 300 mM mannitol for 24 h. The expression of the target genes in wild-type plants under normal conditions was used to normalize their expression under stress treatments. Error bars represent the SD of three biological replicates. Asterisks indicate statistically significant differences between the wild type and transgenic lines at the 0.05 level by one-way ANOVA.

all lines displayed increased Pro contents relative to those under normal conditions. Additionally, the OE lines accumulated significantly higher Pro contents and the RS lines exhibited significantly decreased Pro contents, compared with wild-type plants (Fig. 2D).

As the Pro contents were altered in the different birch lines, we further examined the expression of two birch Pro biosynthesis-related genes, *BpP5CS1* and *BpP5CS2* (for Δ-1-pyrroline-5-carboxylate synthetase). Under normal conditions, there was no difference in the expression levels of *BpP5CS1* and *BpP5CS2* among the studied lines. Under salt and osmotic stress conditions, the expression levels of *BpP5CS1* and *BpP5CS2* increased significantly in the OE lines but were reduced significantly in the RS lines, compared with the wild-type plants (Fig. 2, E and F). These results indicated that BpNAC012 can regulate the biosynthesis of Pro in response to salt and osmotic stresses.

Reactive Oxygen Species Scavenging Assay

Hydrogen peroxide (H_2O_2) and superoxide (O^{2-}), two prominent reactive oxygen species (ROS), were determined by 3,3′-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) in situ staining, respectively, which

were visualized as deep brown and dark blue products, respectively. Similar results were observed in two OE lines or in two RS lines, and representative images are shown in Figure 3, A and B. H_2O_2 and O^{2-} were largely reduced in leaves of the two OE lines compared with the wild-type plants under salt or osmotic stress conditions (Fig. 3, A and B). Meanwhile, H_2O_2 and O^{2-} in RS1 and RS6 lines were substantially higher than in the wild-type plants (Fig. 3, A and B). Measurement of H_2O_2 also confirmed that both RS lines had the highest H_2O_2 content, followed by the wild type, and the OE lines had the lowest H_2O_2 levels (Fig. 3C), which was consistent with the DAB staining.

We further studied whether the altered ROS level reflects a changed ROS-scavenging capability in the plants. The activities of superoxide dismutase (SOD) and peroxidase (POD), the two main antioxidant enzymes involved in ROS scavenging, were determined. Under normal conditions, there was no difference in SOD and POD activities among the BpNAC012-OE, wild-type, and BpNAC012-RS lines (Fig. 3, D and E). Under NaCl and mannitol treatments, both SOD and POD activities were enhanced significantly in the OE lines but were reduced in the RS lines compared with the wild-type plants (Fig. 3, D and E).

Next, we examined the expression of *SOD* and *POD* genes to determine whether the altered SOD and POD activities were caused by the altered expression of *SODs* and *PODs*. Six SOD and eight POD genes that are homologous to the corresponding Arabidopsis genes with definite SOD and POD activity were included. Under NaCl and mannitol treatment conditions, the expression levels of *SODs* and *PODs* were increased significantly in the OE lines and reduced significantly in the RS lines compared with the wild-type plants (Fig. 3F), indicating that BpNAC012 could induce the expression of *SODs* and *PODs*.

Overexpression of *BpNAC012* Decreases Cell Death

Cell death was determined by Evans Blue staining in leaves. The result showed that cell death was decreased

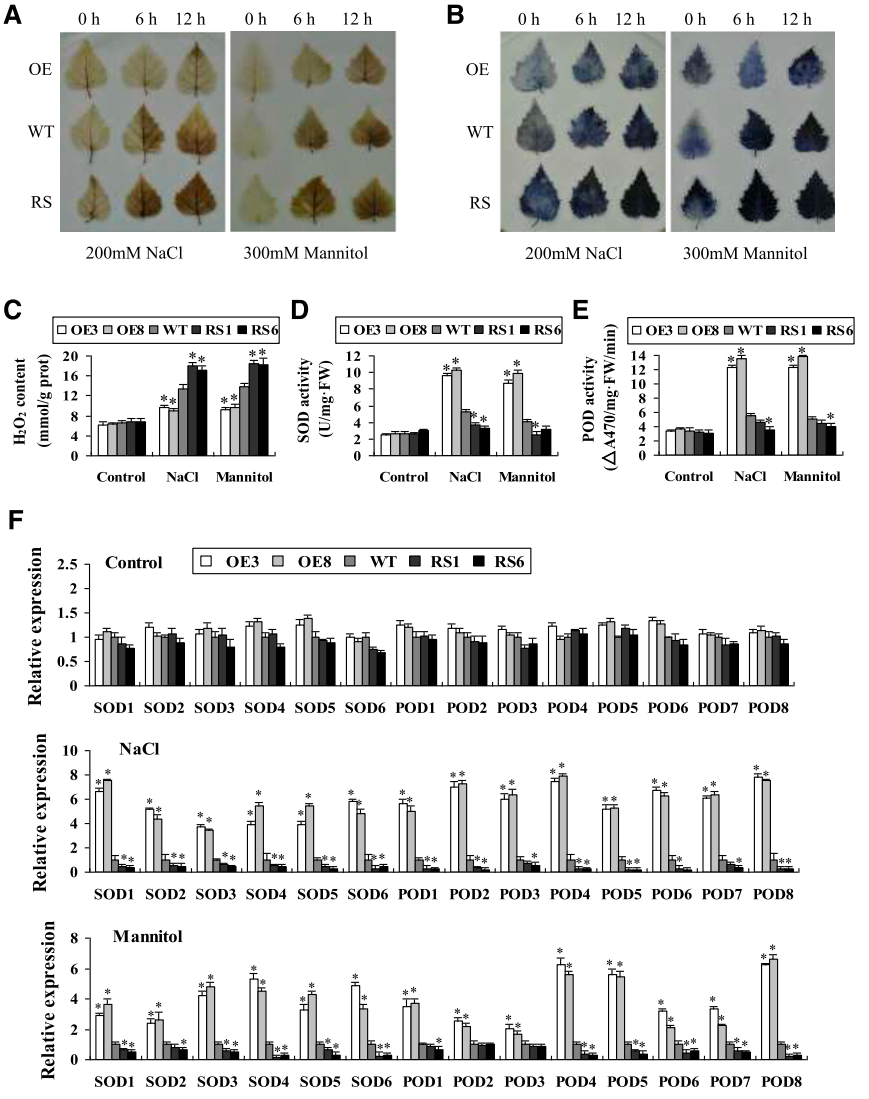

Figure 3. Analysis of ROS scavenging mediated by BpNAC012. A and B, Detection of ROS by DAB and NBT staining. Leaves from birch plants treated with NaCl or mannitol were infiltrated with DAB (A) or NBT (B) for detection of H_2O_2 or O^{2-}, respectively. C, H_2O_2 level assay. D, Measurement of SOD activity. E, Measurement of POD activity. F, Analysis of the expression of *SOD* and *POD* genes in wild-type (WT), OE, and RS plants after stress treatments. The expression level of each gene in the wild type was set to 1. Error bars represent the SD of three biological replicates. Asterisks indicate statistically significant differences between the wild type and transgenic lines at the 0.05 level by one-way ANOVA. FW, Fresh weight.

in the OE lines but increased in the RS lines under salt and osmotic stresses (Supplemental Fig. S4A). Electrolyte leakage measurements were performed to further monitor cell death in whole plants. There was no significant difference in electrolyte leakage rates among all lines under normal conditions. However, electrolyte leakage in the wild-type plants was lower than in the RS lines but was higher in the OE lines under salt or osmotic stress conditions (Supplemental Fig. S4B). These results indicated that overexpression of *BpNAC012* can reduce cell death to improve salt and osmotic stress tolerance.

BpNAC012 Binds Directly to the Core Sequence CGT[A/G] in the Promoters of Abiotic Stress-Responsive Genes

NAC protein was demonstrated previously to bind specifically to the core sequence CGT[G/A], and this binding has been shown to be essential for the activation of gene expression in response to abiotic stresses (Tran et al., 2004; Olsen et al., 2005; Lindemose et al., 2014). To determine whether BpNAC012 can bind to this core sequence CGT[G/A], we first performed the yeast one-hybrid (Y1H) assay. Three tandem copies of the core sequence CGT[G/A] (Core-1 and Core-2) and its mutants (Mutant-1 and Mutant-2) were cloned separately into the pHIS2 vector (Clontech) and tested for their interaction with BpNAC012 that was harbored in the pGADT7-Rec2 vector (Clontech) by the Y1H assay (Fig. 4A). The Y1H assay showed that BpNAC012 could bind to the core sequence CGT[G/A] but failed to bind to its mutants (Fig. 4B). For further verification of the Y1H results, transactivation assays were carried out in tobacco (*Nicotiana tabacum*) leaves. The effector construct (35S:*BpNAC012*) was cotransformed into tobacco leaves with each of the reporter constructs containing the wild-type or mutated core element fused with the

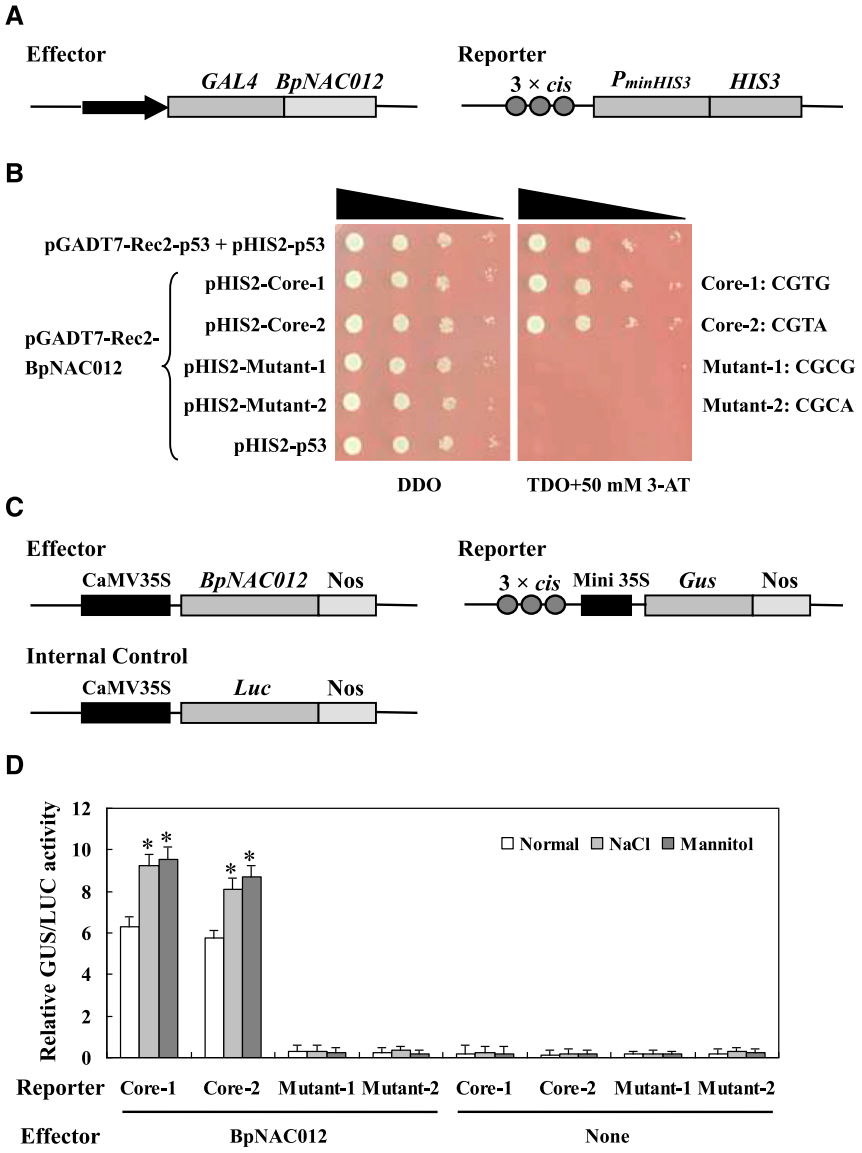

Figure 4. Analysis of the binding of BpNAC012 to the core sequence CGT[A/G]. A, Schematic diagram of the reporter and effector constructs used in the Y1H analysis. Three tandem copies of the core sequence CGT[A/G] or its mutants were inserted into pHIS2 as the reporter constructs. B, Y1H analysis of the binding of BpNAC012 to the core sequence CGT[A/G] and its mutants. The reporter constructs were cotransformed separately with the effector construct pGADT7-Rec2-BpNAC012 into Y187 cells using the Y1H method. The positive transformants were determined by spotting serial dilutions (1:1, 1:10, 1:100, and 1:1,000) of yeast onto Trp/-His/-Leu (TDO) plates supplemented with 3-amino-1, 2, 4-triazole (3-AT). DDO, SD/-Trp/-His. C, Schematic diagram of the reporter, effector, and internal control plasmids used in the transient transactivation assay. D, Confirmation of the binding of BpNAC012 to the core sequence CGT[A/G] in tobacco leaves by transient transformation. Each reporter was cotransformed with the effector and internal control plasmid. After transformation, a part of tobacco leaves was used for 200 mM NaCl or 300 mM mannitol treatment for 12 h. Transformations without the effector plasmid were used as negative controls. Error bars represent the SD of three technical replicates. Asterisks indicate significant differences of the binding affinity under salt or osmotic stress from that under normal conditions at the 0.05 level by one-way ANOVA.

46-bp minimal promoter to drive *GUS* expression. The 35S:firefly *luciferase* (*Luc*) construct was transformed together as an internal reference to normalize the transformation efficiency (Fig. 4C). GUS activity measurement confirmed that BpNAC012 was capable of binding to the core sequence but failed in binding to the mutants (Fig. 4D). Furthermore, we compared the binding affinities of BpNAC012 to the core sequence under normal conditions, salt, or osmotic stress conditions. The binding affinities of BpNAC012 to the core sequence can be induced significantly by salt and osmotic stresses (Fig. 4D). These results together suggested that BpNAC012 binds specifically to the core sequence CGT[G/A]. Importantly, the binding affinities of BpNAC012 to the CGT[G/A] sequence are in response to salt and osmotic stresses.

As the genes involved in Pro biosynthesis and ROS scavenging are found to be regulated by BpNAC012 (Figs. 2 and 3), ChIP assays were performed to determine whether BpNAC012 directly regulates these abiotic stress-responsive genes using 35S:*BpNAC012-GFP* transgenic birch plants under normal growth conditions, treated with 12 h of 200 mM NaCl or 300 mM mannitol. The promoter regions of BpNAC012 up-regulated genes containing the core sequence CGT[G/A] were selected for ChIP assays, as shown in Supplemental Figure S5. ChIP-qPCR analysis demonstrated the enrichment of the truncated promoters in chromatin samples immunoprecipitated with anti-GFP antibody compared with that in the negative controls (ChIP−). The results showed that, after immunoprecipitation with anti-GFP antibody, the promoters of *P5CS1*, *P5CS2*, *SODs*, and *PODs* were enriched significantly under normal growth, salt, and osmotic stress conditions (Fig. 5), indicating that BpNAC012 binds to the promoters of these genes to directly regulate their expression. Additionally, these promoters were much more highly enriched under salt and osmotic stress conditions compared with normal growth conditions (Fig. 5), suggesting that the binding affinities of BpNAC012 to the promoters of these studied genes are induced by salt and osmotic stresses. ChIP assays showing a lack of BpNAC012 binding to promoter regions not containing the core sequence CGT[G/A] demonstrated the specificity of the ChIP assays (Supplemental Fig. S6).

Secondary Wall Deposition in the Stems of *BpNAC012* Transgenic Birch

NST (NAC secondary wall thickening-promoting factor) clade transcription factors have been well documented as regulators of secondary cell wall formation (Mitsuda et al., 2005, 2007; Zhong et al., 2006). The high expression of *BpNAC012* in birch stems and its close homology to NST clade transcription factors suggested that it may play a role in wood formation. The basal parts of stems from the BpNAC012-OE (OE3 and OE8), BpNAC012-RS (RS1 and RS6), and wild-type plants were cross sectioned to examine the secondary wall deposition. Similar wood microscopy images were observed in the two OE lines or in the two RS lines, and representative images are shown in Figure 6. Wood from the BpNAC012-OE stems had xylem and phloem fibers with thick walls as did the wild type-plants and vessels with regular shapes (Fig. 6, A, B, D, E, and G–J). In contrast, the wall thickness of xylem and phloem fibers was reduced by 57% and 83%, respectively, in the stems of BpNAC012-RS transgenic plants compared with the wild type (Fig. 6, B, C, and E–H). In addition, a slight deformation of vessel walls was evident in the stems of BpNAC012-RS plants (Fig. 6K). These results demonstrated that RNAi-induced suppression of *BpNAC012* caused a significant inhibition of secondary wall thickening in stem fibers.

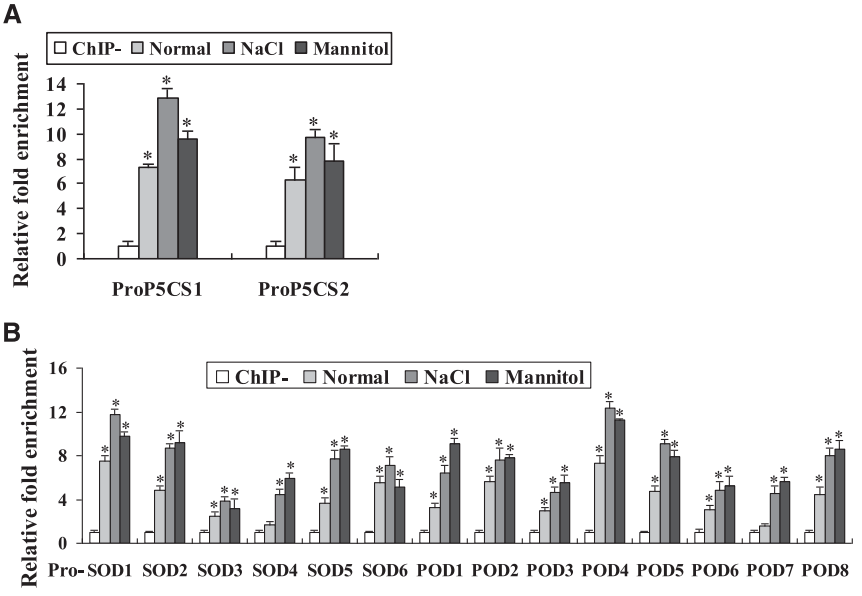

Figure 5. ChIP analysis of BpNAC012 binding affinity to the core sequence CGT[A/G]. The promoters of BpNAC012 stress-responsive downstream genes, including *P5CS1* and *P5CS2* (A) and *SODs* and *PODs* (B), were selected for ChIP-qPCR analysis. 35S:*BpNAC012-GFP* transgenic birch plants under normal growth conditions (Normal) or treated with 200 mM NaCl for 12 h (NaCl) or 300 mM mannitol for 12 h (Mannitol) were used for the ChIP assay. After normalization against *Ubiquitin*, the relative enrichment in ChIP− was designated as 1 to normalize that in chromatins immunoprecipitated with anti-GFP antibody (ChIP). ChIP−, Chromatins immunoprecipitated with hemagglutinin (HA) antibody (negative control). The data represent means of three independent experiments. Error bars represent the SD. Asterisks indicate statistically significant differences between the ChIP− and ChIP groups at the 0.05 level by one-way ANOVA.

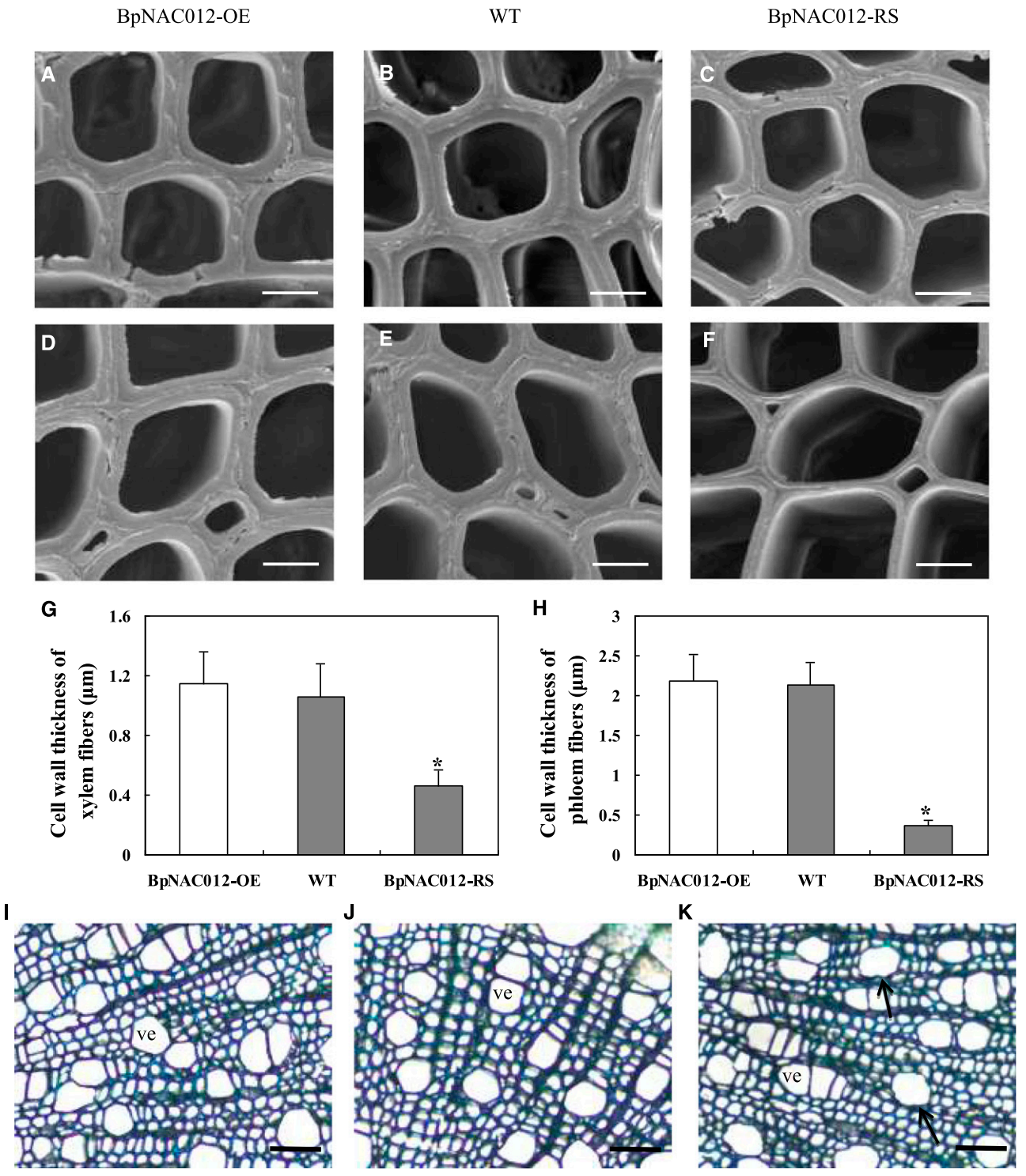

Figure 6. Wood microscopy in the stems of *BpNAC012* transgenic birch. A to C, Transmission electron micrographs of xylem fiber walls in BpNAC012-OE (A) and BpNAC012-RS plants (C) compared with the wild type (WT; B). D to F, Transmission electron micrographs of phloem fiber walls in BpNAC012-OE (D) and BpNAC012-RS plants (F) compared with the wild type (E). G and H, Cell wall thickness of xylem (G) and phloem (H) fibers. The data represent means of 40 cells. Error bars represent the SD. Asterisks indicate statistically significant differences between the wild type and the transgenic lines at the 0.05 level by one-way ANOVA. I to K, Toluidine Blue staining of stem sections of the BpNAC012-OE (I), wild-type (J), and BpNAC012-RS (K) plants. The arrows show a slight deformation of vessels. ve, Vessel. Bars = 2.5 μm (A–C), 4.5 μm (D–F), and 45 μm (I–K).

Next, we examined the presence of the three major secondary wall components, lignin, cellulose, and xylan, in the cross sections of stems. Lignin staining was observed only in the secondary xylem and phloem fibers in the stems of wild-type and BpNAC012-RS plants, and weaker staining of lignin was seen in the BpNAC012-RS stem sections compared with the wild type (Fig. 7, B and C). Similarly, staining of cellulose and xylan was less intense in the secondary xylem and phloem fibers of BpNAC012-RS stem sections compared with the wild type (Fig. 7, E, F, H, and I). In contrast, ectopic lignin deposition was evident in the stem epidermis of BpNAC012-OE plants in addition to the secondary xylem and phloem fibers (Fig. 7A). Ectopic deposition of secondary wall cellulose and xylan also was detected in the epidermis of the stems of BpNAC012-OE plants compared with the wild type (Fig. 7, D and G). These results revealed that overexpression of *BpNAC012*

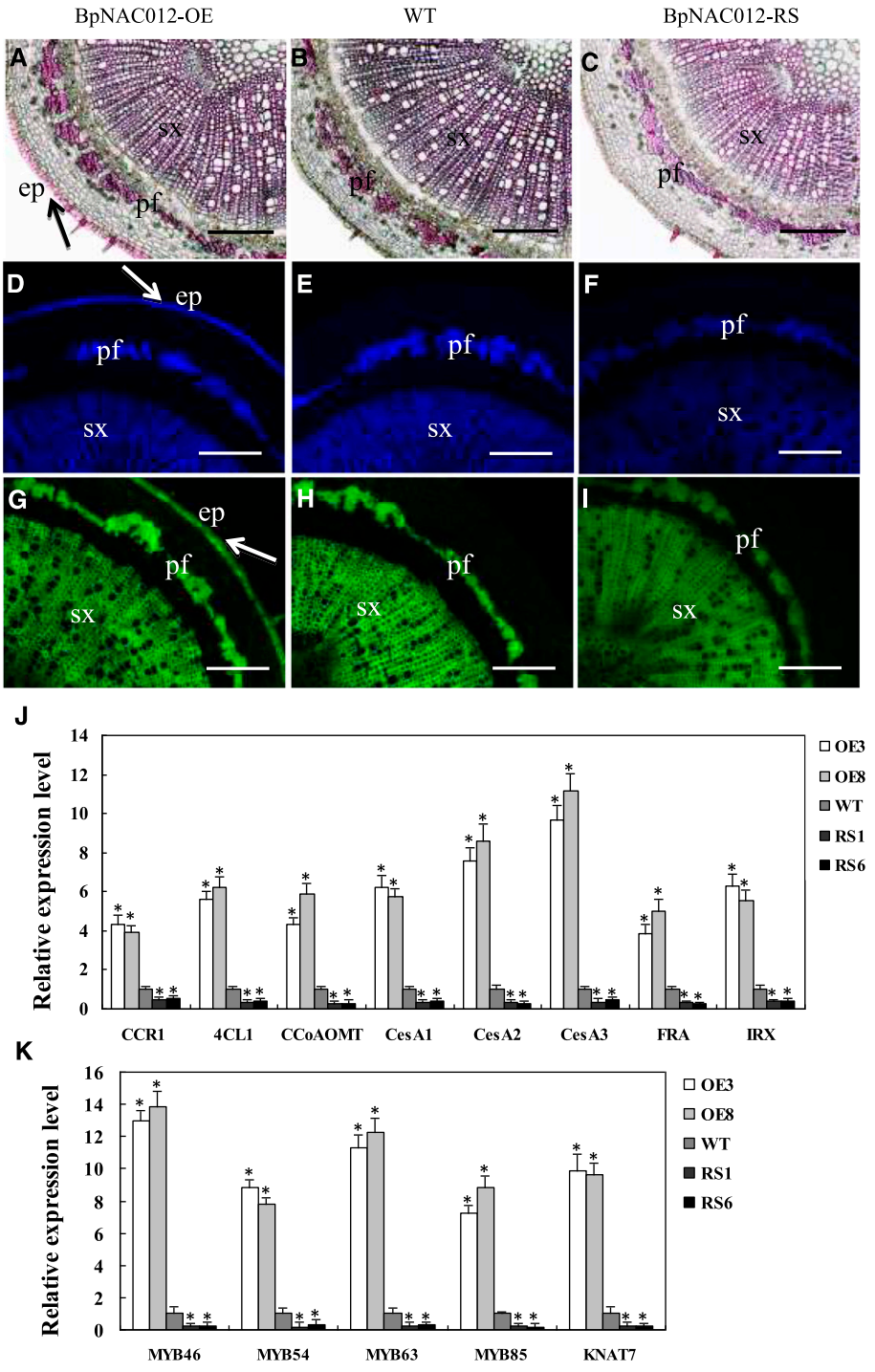

Figure 7. Detection of lignin, cellulose, and xylan in stem sections of *BpNAC012* transgenic birch. A to C, Phloroglucinol-HCl staining of lignin in stem sections of the BpNAC012-OE (A), wild-type (WT; B), and BpNAC012-RS (C) plants. The arrow shows intensive lignin staining in the walls of the epidermis. D to F, Calcofluor White staining of cellulose in stem sections of the BpNAC012-OE (D), wild-type (E), and BpNAC012-RS (F) plants. The arrow shows intensive cellulose staining in the walls of the epidermis. G to I, Detection of xylan in stem sections of the BpNAC012-OE (G), wild-type (H), and BpNAC012-RS (I) plants probed with the LM10 xylan monoclonal antibody. The arrow shows intensive xylan staining in the walls of the epidermis. ep, Epidermis; pf, phloem fiber; sx, secondary xylem. Bars = 100 μm. J, Expression analysis of secondary wall biosynthetic genes, including lignin biosynthetic genes (*CCR1*, *4CL1*, and *CCoAOMT*), cellulose synthases (*CesA1*, *CesA2*, and *CesA3*), and xylan biosynthetic genes (*FRA* and *IRX*), in the stems of BpNAC012-OE and BpNAC012-RS plants compared with the wild type. K, Expression analysis of secondary wall transcription factor genes (*MYB46*, *MYB54*, *MYB63*, *MYB85*, and *KNAT7*) in the stems of BpNAC012-OE and BpNAC012-RS plants compared with the wild type. The expression level of each gene in the wild type was set to 1. Error bars represent the SD of three biological replicates. Asterisks indicate statistically significant differences between the wild type and the transgenic lines at the 0.05 level by one-way ANOVA.

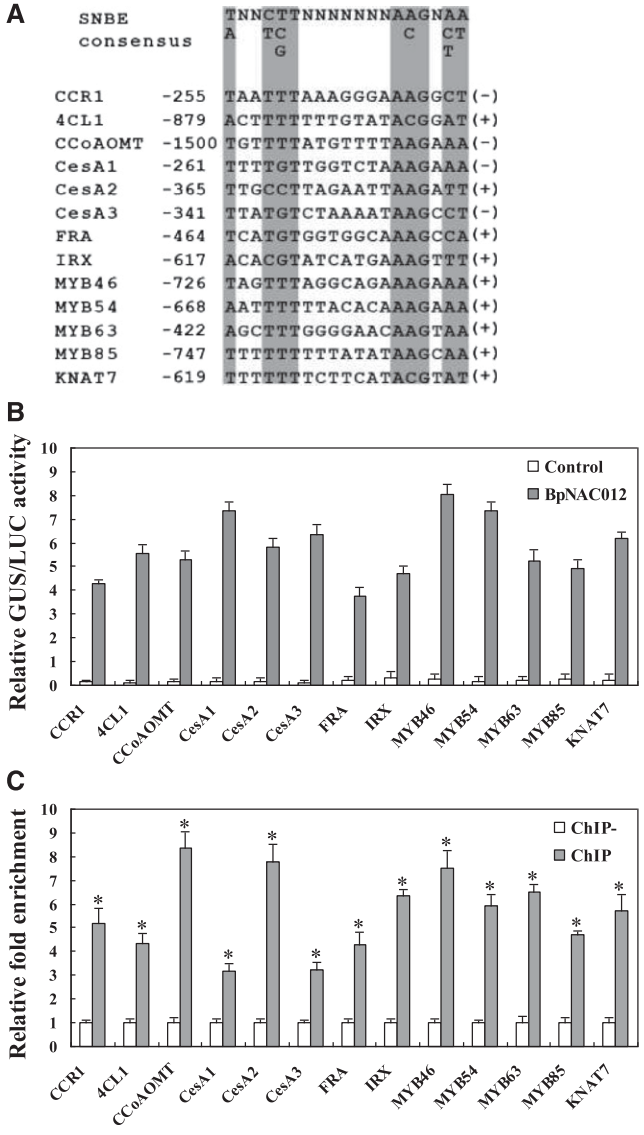

Figure 8. BpNAC012 is able to activate the SNBE sites in the promoters of the secondary wall-associated genes. A, SNBE sequences from the promoters of lignin biosynthetic genes (*CCR1*, *4CL1*, and *CCoAOMT*), cellulose synthase genes (*CesA1*, *CesA2*, and *CesA3*), xylan biosynthetic genes (*FRA* and *IRX*), and five secondary wall transcription factor genes (*MYB46*, *MYB54*, *MYB63*, *MYB85*, and *KNAT7*). The number represents the position of the first nucleotide relative to the start codon. The plus or minus symbol at the right indicates the SNBE sequence from the forward or reverse strand of DNA, respectively. The critical nucleotides in the SNBE sequences are shaded. B, Transactivation analysis showing the activation of the SNBE-driven GUS reporter gene by BpNAC012. Two tandem copies of the representative SNBE sequence were fused with the 35S *Cauliflower mosaic virus* (CaMV) minimal promoter (−46 to +1) to drive the *GUS* gene as reporters. The reporter construct was cotransformed with the effector construct (35S:*BpNAC012*) into tobacco leaves. Transformations with the reporter construct alone were used as negative controls. 35S:*Luc* was cotransformed to normalize the transformation efficiency. Error bars represent the SD of three technical replicates. C, ChIP-qPCR analysis of the binding of BpNAC012 to the promoters of the secondary wall-associated genes containing SNBE sites. After normalization against *Ubiquitin*, the relative enrichment in ChIP− was designated as 1 to normalize that in chromatins immunoprecipitated with anti-GFP antibody (ChIP). ChIP−, Chromatins immunoprecipitated with HA antibody (negative control). The data represent means of three independent experiments. Error bars represent the SD. Asterisks indicate statistically significant differences between the ChIP− and ChIP groups at the 0.05 level by one-way ANOVA.

resulted in ectopic secondary wall deposition during wood formation in birch.

Overexpression of *BpNAC012* Induces the Expression of Secondary Wall-Associated Genes

The ectopic deposition of secondary wall components promoted us to further investigate the expression of the lignin biosynthetic genes *CCR1* (for *cinnamoyl-CoA reductase*), *4CL1* (for *4-coumarate:CoA ligase*), and *CCoAOMT* (for *caffeoyl-CoA 3-O-methyltransferase*), the cellulose synthases *CesA1/CesA2/CesA3* (which are *BplCESA8/BplCESA7/BplCESA4*, respectively; Liu et al., 2012), and the xylan biosynthesis-related genes *FRAGILE FIBER* (*FRA*) and *IRREGULAR XYLEM* (*IRX*), which are close homologs of Arabidopsis *FRA8* (AT2G28110) and *IRX9* (AT2G37090), respectively. RT-qPCR analysis showed that transcript levels of these secondary wall biosynthetic genes were all increased significantly in BpNAC012-OE plants but obviously reduced in BpNAC012-RS plants (Fig. 7J). We also found significantly increased expression levels of several secondary wall transcription factor genes in BpNAC012-OE plants, including *MYB46*, *MYB54*, *MYB63*, *MYB85*, and *KNAT7*, which are homologs of Arabidopsis SND1-regulated downstream targets (Fig. 7K). Taken together, these results demonstrated that *BpNAC012* overexpression is able to activate the secondary wall biosynthetic pathways in birch, which, in turn, leads to the ectopic deposition of secondary walls during wood formation.

BpNAC012 Activates the Secondary Wall-Associated Genes by Binding to the SNBE Sites in Their Promoters

Previous studies revealed that SND1 binds to the SNBE, an imperfect palindromic 19-bp consensus sequence, (T/A)NN(C/T)(T/C/G)TNNNNNNNA(A/C)GN(A/C/T)(A/T), and this binding has been shown to be essential for the activation of SND1 direct target genes involved in secondary cell wall formation (Zhong et al., 2010b). Here, we searched for SNBE sites in the 1.5-kb promoter sequences of the secondary wall-associated genes studied in Figure 7, J and K, and then detected the binding of BpNAC012 to these SNBE sites. The sequences of these SNBE sites used for transactivation analysis are shown in Figure 8A. The effector construct (35S:*BpNAC012*) was cotransformed with the reporter construct containing various SNBE sites fused with the 46-bp minimal promoter to drive the expression of the GUS reporter gene in tobacco leaves. GUS activity was

normalized against the Luc activity. GUS activity measurements showed that BpNAC012 can bind to SNBE sites from various promoters of the studied genes. In addition, the activation strength varied among different SNBE sequences, suggesting that BpNAC012 exhibits differential binding affinity toward various SNBE sequences (Fig. 8B).

To further investigate whether BpNAC012 activates the SNBE sites in the promoters of the secondary wall-associated genes in birch, we performed ChIP assays. ChIP-qPCR analysis showed that the studied promoters containing the representative SNBE sites shown in Figure 8A were enriched significantly compared with the ChIP− control, indicating that BpNAC012 can bind directly to these promoters (Fig. 8C). Taken together, these results indicated that BpNAC012 activates the expression of the genes involved in secondary wall formation through binding directly to the SNBE sites in their promoters.

The Binding Preferences of BpNAC012 to the Core Sequence CGT[A/G] and the SNBE Site

Among the BpNAC012 downstream genes including the abiotic stress-responsive genes and the secondary wall-associated genes, some have both the core sequence CGT[G/A] and the SNBE site in their promoters. To investigate the BpNAC012 binding preferences of the core sequence CGT[G/A] and the SNBE site present in the same promoter, primers were designed to specifically amplify the core sequence CGT[G/A] and the SNBE site from the promoters of *SOD2*, *POD1*, *POD5*, *4CL1*, *CesA2*, and *MYB46*. The specific promoter regions containing the SNBE site or the core sequence CGT[G/A] were used for ChIP-qPCR assays, as shown in Supplemental Figure S7. ChIP assays showed that BpNAC012 specifically bound the core sequence CGT[G/A] in the promoters of *SOD2*, *POD1*, and *POD5* genes (Fig. 9A). Conversely, BpNAC012 specifically bound the SNBE site in the promoters of *4CL1*, *CesA2*, and *MYB46* genes (Fig. 9B). These results indicated that BpNAC012 specifically activates the core sequence CGT[G/A] and the SNBE site in the promoters of abiotic stress-responsive genes and secondary wall-associated genes, respectively.

Overexpression of *BpNAC012* Induces Increased Lignification in Response to Abiotic Stress

BpNAC012-OE lines showed higher levels of lignin accumulation, whereas lower levels of lignin were

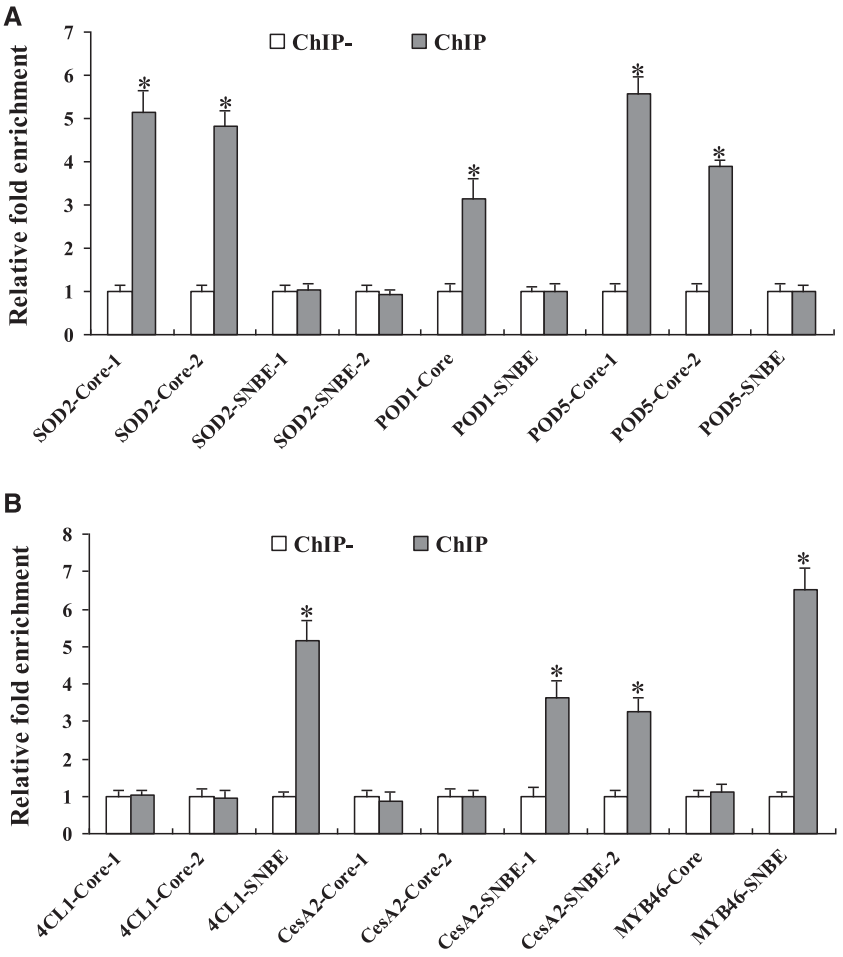

Figure 9. BpNAC012 binding preferences to the core sequence CGT[A/G] and the SNBE site. The promoters of BpNAC012 downstream genes, including the abiotic stress-responsive genes (A) and the secondary wall-associated genes (B), were selected for ChIP-qPCR analysis. The relative enrichment in ChIP− was designated as 1 to normalize that in chromatins immunoprecipitated with anti-GFP antibody (ChIP) after normalization against *Ubiquitin*. ChIP−, Chromatins immunoprecipitated with HA antibody (negative control). The data represent means of three independent experiments. Error bars represent the SD. Asterisks indicate statistically significant differences between the ChIP− and ChIP groups at the 0.05 level by one-way ANOVA.

detected in BpNAC012-RS lines, compared with the wild type (Fig. 7, A–C). The total lignin contents in stems of BpNAC012-OE, BpNAC012-RS, and wild-type plants were examined further. As expected, BpNAC012-OE lines showed higher levels of lignin content, whereas lower levels of lignin were detected in BpNAC012-RS lines, compared with the wild type (Fig. 10A). It has been reported that lignin production provides a defense mechanism against abiotic stresses such as low temperature, drought, and high light (Lee et al., 2007; Moura et al., 2010). To determine whether lignification is responsible for abiotic stress resistance in *BpNAC012* transgenic plants, lignin biosynthesis under stress conditions was examined. Under NaCl and mannitol treatments, substantially increased expression of *BpNAC012* and lignin biosynthetic genes was observed in BpNAC012-OE lines but not in BpNAC012-RS and wild-type plants (Fig. 10B). These results suggest that overexpression of *BpNAC012* induces elevated expression of lignin biosynthetic genes in response to abiotic stress treatments.

To further investigate the relationship between abiotic stress response and secondary wall formation, stems from wild-type and BpNAC012-OE transgenic plants exposed to NaCl or mannitol treatment for 24 h were used to examine the lignin accumulation. Ectopic lignin deposition was evident in the epidermis of BpNAC012-OE stems even under normal growth conditions, and enhanced lignin staining was seen specifically in the secondary xylem and phloem fibers of BpNAC012-OE stems under abiotic stress conditions (Fig. 10C), whereas wild-type stems showed lignin deposition only in the secondary xylem and phloem fibers, and no substantial difference was observed in the lignin staining among normal growth and abiotic stress conditions (Fig. 10C). Consistent with the histological staining, significantly increased lignin content was observed in BpNAC012-OE plants but not in wild-type plants under stress conditions (Fig. 10D). The lignin content was increased by 30% in BpNAC012-OE plants compared with the wild type under normal growth conditions, while, under salt and osmotic stress conditions, the lignin content of BpNAC012-OE plants was increased by 58% and 53% compared with the wild type, respectively, indicating enhanced lignification in BpNAC012-OE plants under abiotic stress conditions (Fig. 10D). Taken together, these results demonstrated that overexpression of *BpNAC012* induces increased lignification as a mechanical barrier against abiotic stresses.

DISCUSSION

In this study, we characterized the *BpNAC012* gene, which is closely related to Arabidopsis *SND1/NST3/ANAC012*. SND1/NST3/ANAC012 is a master switch for secondary cell wall formation (Zhong et al., 2006). However, little was known about the functions of NST clade transcription factors in abiotic stress responses. Additionally, we wonder whether there is a connection between secondary cell wall formation and abiotic stress responses. New functions of SND1 in abiotic stress signaling were revealed recently (Jeong et al., 2018). The expression of *SND1* was induced significantly by salt or osmotic treatment, and the *snd1* knockout mutant exhibited low tolerance to salt stress. SND1 can inhibit ABA biosynthesis via directly repressing the *ABI4* transcript under salt stress conditions. Several observations presented here shed light on the roles of BpNAC012. The expression of *BpNAC012* can be induced by abiotic stress treatments, and *BpNAC012-OE* transgenic plants were more tolerant to salt and osmotic stresses. Moreover, we found that BpNAC012 is essential for secondary wall formation in white birch. We discovered a function of BpNAC012 in the positive regulation of both abiotic stress responses and secondary cell wall deposition by binding specifically to the core sequence CGT[G/A] and the SNBE site.

BpNAC012 Binds Specifically to the Core Sequence CGT[A/G] and the SNBE Site

Transcription factors bind to specific cis-acting elements to regulate the expression of genes. Therefore, the determination of motifs recognized by a transcription factor is important to reveal its function. Previous reports have shown that NAC family proteins bind to various cis-DNA elements (Tran et al., 2004; Olsen et al., 2005; Jensen et al., 2010; Zhong et al., 2010a, 2010b; Xu et al., 2013; Lindemose et al., 2014). Arabidopsis secondary wall NACs, including SND1, NST1, NST2, VND6, and VND7, directly regulate the expression of a set of genes involved in secondary wall biosynthesis through binding to the SNBE sites in their promoters (Zhong et al., 2010b). SND1 was newly found to bind to the *ABI4* promoter, especially to the promoter fragment including the ABA-responsive element, and thus to regulate the expression of *ABI4* and maintain a low ABA level (Jeong et al., 2018). Here, using ChIP assays, we demonstrated that BpNAC012 binds specifically to the core sequence CGT[G/A] and the SNBE site. Interestingly, BpNAC012 binds specifically to the core sequence CGT[G/A] in the promoters of abiotic stress-responsive genes but binds specifically to the SNBE site in the promoters of secondary wall-associated genes. Moreover, the binding affinity of BpNAC012 to the core sequence CGT[G/A] is induced significantly in response to salt and osmotic stresses. These findings indicated that BpNAC012 plays distinct roles in abiotic stress responses and secondary cell wall biosynthesis.

BpNAC012 Reduces ROS Accumulation to Enhance Stress Tolerance

The modulation of ROS is critical for stress tolerance of plants, because ROS can act as signaling molecules at a low level and also as damaging agents at higher levels (Zhang et al., 2011). Given the importance of ROS scavenging, we determined whether BpNAC012

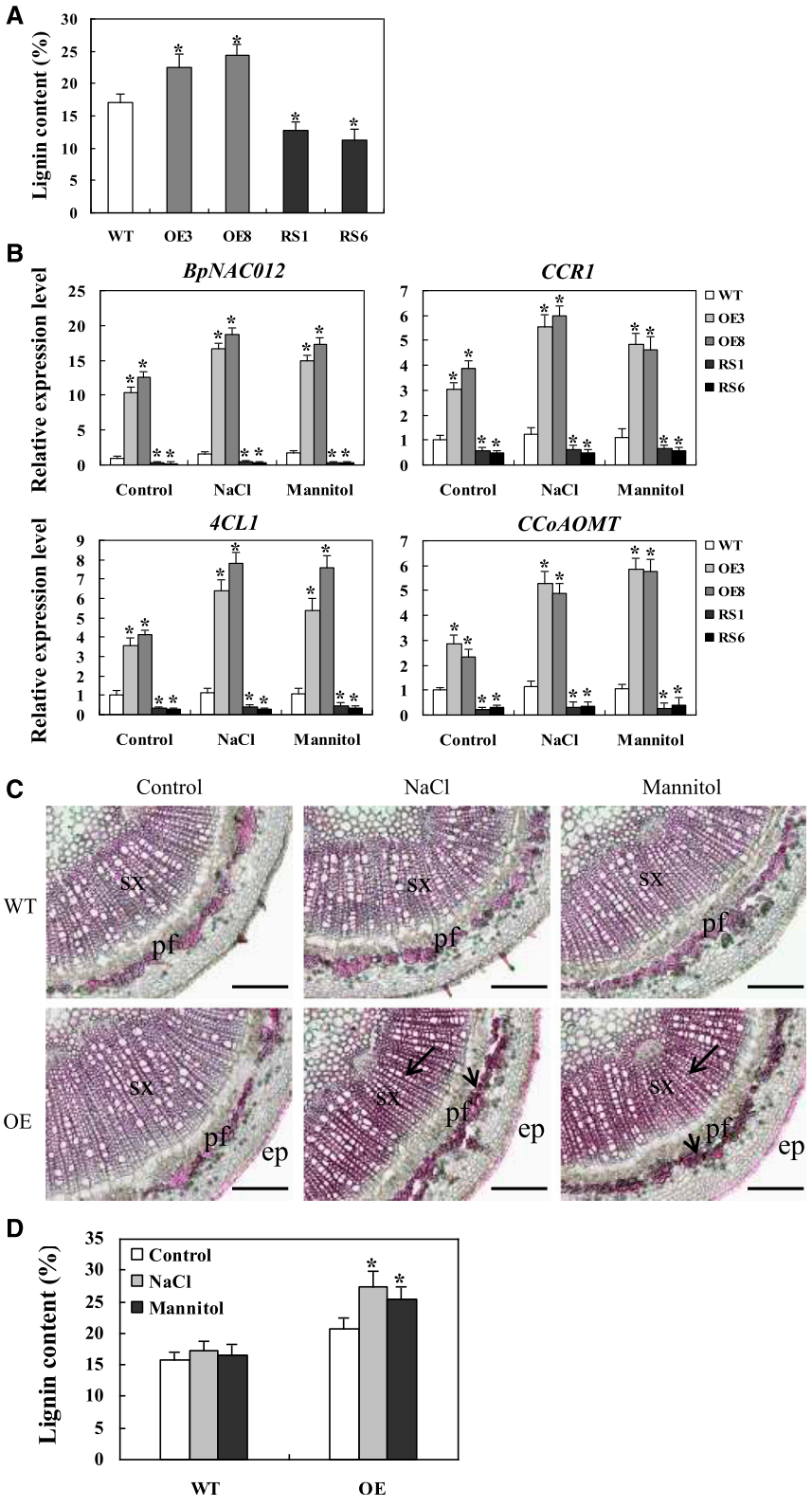

Figure 10. Increased lignification induced by BpNAC012 against salt and osmotic stresses. A, Quantitative analysis of lignin content in wild-type (WT), BpNAC012-OE, and BpNAC012-RS stems. Error bars represent the SD of three technical replicates. Asterisks indicate statistically significant differences between the wild type and transgenic lines at the 0.05 level by one-way ANOVA. B, Expression patterns of *BpNAC012* and lignin biosynthetic genes (*CCR1*, *4CL1*, and *CCoAOMT*) in BpNAC012-OE, wild-type, and BpNAC012-RS plants under normal growth conditions, treated with 200 mM NaCl or 300 mM mannitol for 24 h. The expression of each gene in the wild type under normal conditions (Control) was used to normalize the expression under the stress treatment. Error bars represent the SD of three biological replicates. Asterisks indicate statistically significant differences between the wild type and the transgenic lines at the 0.05 level by one-way ANOVA. C, Phloroglucinol-HCl staining of lignin in stem sections of the wild-type and BpNAC012-OE plants under normal growth or stress conditions. The arrows show enhanced lignin staining in secondary xylem and phloem fiber cells. ep, Epidermis; pf, phloem fiber; sx, secondary xylem. Bars = 100 μm. D, Lignin content in the stems of wild-type and BpNAC012-OE plants under normal growth or stress conditions. Error bars represent the SD of three technical replicates. Asterisks indicate statistically significant differences between the normal growth conditions and stress conditions at the 0.05 level by one-way ANOVA.

mediates ROS scavenging. qPCR and ChIP assays together showed that BpNAC012 can bind to the promoters of *SOD* and *POD* genes, especially under salt and osmotic stress conditions, to induce their expression. Therefore, BpNAC012 regulates the expression of *SODs* and *PODs* to increase SOD and POD activities,

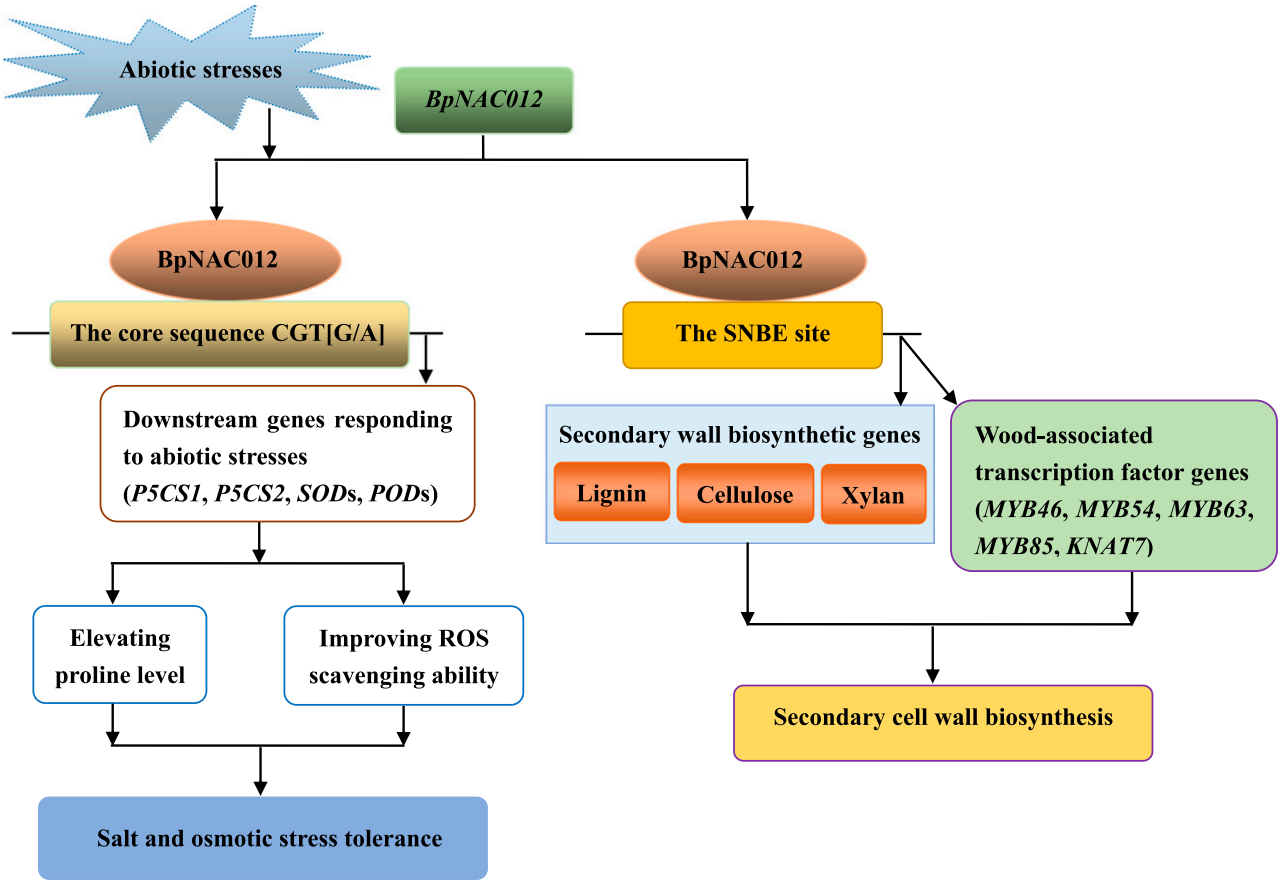

Figure 11. Model of the BpNAC012 regulatory network involved in abiotic stress responses and secondary wall biosynthesis. Salt and osmotic stresses highly induce the expression of *BpNAC012* in birch leaves. BpNAC012 binds directly to the core sequence CGT[G/A] to regulate the expression of abiotic stress-responsive downstream genes, including *P5CS1*, *P5CS2*, *SODs*, and *PODs*, resulting in elevated Pro level and decreased ROS accumulation, thus improving salt and osmotic stress tolerance. BpNAC012 also is involved in secondary cell wall formation. BpNAC012 is able to activate the expression of secondary wall biosynthetic genes and wood-associated transcription factor genes by binding directly to the SNBE sites in their promoters, controlling secondary wall biosynthesis during wood formation in birch.

which elevates the ROS-scavenging capability to enhance salt and osmotic stress tolerance.

BpNAC012 Controls the Biosynthesis of Pro

In plants, Pro can serve as both a radical scavenger and an osmotic agent to protect cells from damage caused by stress, and the stored Pro is degraded to provide a supply of energy for growth when stress is relieved. Pro plays a role in maintaining sustainable growth under long-term stress, and the homeostasis of Pro is necessary for actively dividing cells (Kavi Kishor and Sreenivasulu, 2013). In this study, the binding of BpNAC012 to the promoters of Pro biosynthesis genes, *P5CS1* and *P5CS2*, is highly induced by salt and osmotic stresses, which leads to the elevated expression of *P5CS* genes to facilitate Pro biosynthesis, resulting in highly increased Pro content under salt and osmotic stress conditions to improve stress tolerance.

BpNAC012 Facilitates the Secondary Wall Biosynthesis

Secondary cell walls, consisting of lignin, cellulose, and xylan, constitute the bulk part of wood biomass, which is widely used for pulping, construction, paper making, and biofuel production (Mellerowicz and Sundberg, 2008). Therefore, dissection of the molecular mechanisms underlying the regulation of secondary wall biosynthesis will be instrumental to unravel the process of wood formation and have important biotechnological implications in tree species. In Arabidopsis, NAC domain transcription factors, including SND1, NSTs and VNDs, have been shown to be master switches activating secondary wall biosynthetic pathways during wood formation (Mitsuda et al., 2005, 2007; Zhong et al., 2006, 2007). In this study, we have demonstrated that RNAi-induced suppression of *BpNAC012* dramatically inhibits secondary wall deposition in fibers of stems, suggesting that BpNAC012 is essential for secondary wall deposition in birch. We have further revealed that BpNAC012 is capable of inducing the ectopic deposition of secondary walls in the epidermis of

stems when overexpressed in birch. This is consistent with the data showing that BpNAC012 can significantly induce the expression of lignin biosynthetic genes, cellulose synthases, and xylan biosynthetic genes. Our results suggest that BpNAC012 is a master regulator of secondary wall biosynthesis during wood formation in birch.

To make lignified secondary walls during wood formation, genes involved in the biosynthesis of lignin, cellulose, and xylan need to be activated coordinately. Therefore, it is conceivable that transcriptional switches must be required to regulate the secondary wall biosynthetic program. It has been shown that the PtrWND master switches together with a cascade of downstream transcription factors form a transcriptional network controlling secondary wall biosynthesis during wood formation in poplar (Zhong et al., 2010a, 2011). This study has revealed that BpNAC012 up-regulates the expression of not only a number of genes involved in secondary wall biosynthesis but also some secondary wall transcription factors, which are homologs of Arabidopsis SND1-regulated downstream targets. Further deciphering the functional roles of the BpNAC012-regulated downstream transcription factors in secondary wall biosynthesis will provide foundational knowledge for unraveling the transcriptional network underlying wood formation in birch.

Overexpression of *BpNAC012* Induces Enhanced Lignification against Salt and Osmotic Stresses

Several studies have shown that lignin accumulation and high levels of lignin biosynthetic gene expression are important for abiotic stress tolerance (Hu et al., 2009; Shafi et al., 2015; Srivastava et al., 2015). In this study, heavier deposition of lignin was observed specifically in the secondary xylem and phloem fibers of BpNAC012-OE transgenic plants under salt and osmotic stress conditions, which was consistent with the results that BpNAC012 highly induced the expression of lignin biosynthetic genes in response to abiotic stress treatments. Therefore, it can be concluded that the enhanced lignification mediated by BpNAC012 provides an extra defense mechanism in BpNAC012-OE transgenic plants against salt and osmotic stresses.

CONCLUSION

In summary, we identified the BpNAC012 protein as a transcription factor that modulates abiotic stress responses and secondary wall biosynthesis (Fig. 11). The expression of *BpNAC012* is highly induced in birch leaves under salt and osmotic stress treatments. BpNAC012 activates the expression of abiotic stress-responsive downstream genes, resulting in an elevated Pro level and enhanced ROS-scavenging ability to improve salt and osmotic stress tolerance. We also demonstrate that BpNAC012 is essential for secondary wall thickening in stem fibers and that *BpNAC012* overexpression is able to activate secondary wall biosynthetic pathways. ChIP assays revealed a novel mechanism whereby BpNAC012 specifically binds to the core sequence CGT[A/G] and the SNBE site in the promoters of abiotic stress-responsive downstream genes and secondary wall-associated downstream genes, respectively. We believe that the identification of BpNAC012 as a transcriptional regulator of both abiotic stress responses and secondary wall biosynthesis has significant biotechnological implications in birch.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Three-month-old white birch (*Betula platyphylla*) seedlings grown in soil were used for tissue-specific expression analysis of *BpNAC012*. Young leaves were harvested by collecting the leaves at the first to third internodes, and mature leaves were harvested by collecting the sixth internodes. Young stems from the third internodes and mature stems from the sixth internodes also were harvested. Three-month-old birch plants grown in soil were watered with 200 mM NaCl or 300 mM mannitol solution in the roots. For ABA treatment, 100 μM ABA solution was watered on the roots and also sprayed on the leaves. Plants were treated for 3, 6, 9, 12, 24, and 48 h; after this, the plant leaves were harvested and pooled (three birch trees in each pool) for RT-qPCR. Plants watered with fresh water were harvested at the corresponding time points as controls. For the drought treatment, birch plants were grown in soil without water for 3, 7, 10, and 14 d, and leaves were harvested for RT-qPCR.

Sequence Analysis

Arabidopsis (*Arabidopsis thaliana*) NAC sequences were retrieved from The Arabidopsis Information Resource (http://www.arabidopsis.org/). The deduced protein sequences were aligned using the Clustal Omega method (https://www.ebi.ac.uk/Tools/msa/clustalo/). The unrooted phylogenetic tree was constructed for these NAC genes by the neighbor-joining method using MEGA 5.1 with 1,000 replicates for bootstrap assessment.

Cloning of *BpNAC012* and the Generation of Transgenic Birch Plants

Previously, we constructed birch transcriptomes (Wang et al., 2014), and a NAC homolog (*BpNAC012*), which is highly expressed in the stem, was identified. The CDS of *BpNAC012* was cloned into the pROK2 vector under the control of the 35S CaMV promoter for overexpression of *BpNAC012* (OE). A truncated *BpNAC012* promoter (207 bp) was forwardly and reversely inserted into pFGC5941 to form an inverted repeat promoter for RNAi-induced suppression of *BpNAC012* (RS). The CDS of *BpNAC012*, without the stop codon, was ligated in-frame to the N terminus of the GFP gene to generate the *BpNAC012-GFP* fusion gene under the control of the CaMV 35S promoter (35S:*BpNAC012-GFP*). The primers used to construct these vectors are shown in Supplemental Table S1. For birch transformation, the birch explants, including stem cuts (about 1 cm in length) or leaves (with the base of the leaves cut off), were soaked in *Agrobacterium tumefaciens* cells with gentle shaking for 10 min and then were cultured on a coculture medium (woody plant medium [WPM] containing 3% [w/v] Suc + 1 mg L^{-1} 6-benzylaminopurine + 150 μM acetosyringone, pH 5.8) for 3 d. After coculture, the explants were grown on callus induction and selective medium (WPM + 1 mg L^{-1} 6-BA + 50 mg L^{-1} kanamycin or 2 mg L^{-1} glyphosate + 600 mg L^{-1} carbenicillin, pH 5.8) to induce antibiotic-resistant calli (kanamycin was used to select BpNAC012-OE and 35S:*BpNAC012-GFP* transformants, and glyphosate was used to select BpNAC012-RS transformants). The antibiotic-resistant calli were transferred onto shoot-regeneration medium (WPM + 1 mg L^{-1} 6-benzylaminopurine + 50 mg L^{-1} kanamycin or 2 mg L^{-1} glyphosate) for shoot generation. The regenerated shoots from the calli were transferred to rooting medium (WPM + 0.2 mg L^{-1} naphthylacetic acid + 50 mg L^{-1} kanamycin or 2 mg L^{-1} glyphosate) to generate roots. The expression levels of *BpNAC012* in the transgenic lines were studied using RT-qPCR.

RT-qPCR

Total RNA was isolated from birch using the cetyltrimethylammonium bromide method and treated with RNase-free DNase I (TaKaRa). Total RNA was reverse transcribed into cDNA using a PrimeScript RT Reagent Kit (TaKaRa) with oligo(dT) as primers in a 10-μL volume. The synthesized cDNA was diluted to 100 μL as a PCR template. qPCR was performed in a 20-μL reaction containing 0.5 μM of each forward or reverse primer, 10 μL of SYBR Premix Ex Taq (TaKaRa), and 2 μL of cDNA template on an MJ Research Opticon instrument (Bio-Rad). The thermal profile was 94°C for 30 s, followed by 45 cycles of 94°C for 12 s, 58°C for 30 s, 72°C for 45 s, and 79°C for 1 s for plate reading. The *Ubiquitin* and *Tubulin* genes were used as internal controls. The GenBank accession numbers of all the studied genes and the primers used for RT-qPCR are shown in Supplemental Table S2. The relative expression levels were calculated according to the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (2001). Three biological replications were performed.

Analysis of Transcriptional Activity in Yeast

The complete CDS of *BpNAC012* and different truncated CDSs of *BpNAC012* were fused separately to the GAL4 DNA-binding domain in pGBKT7 (Clontech) to create fusion constructs (primers are shown in Supplemental Table S3) and were transformed into yeast strain Y2H following the Matchmaker Gold Yeast Two-Hybrid System User Manual (Clontech). Yeast transformants were dropped onto synthetic dextrose (SD)/-Trp plates or SD/-Trp/-Ade/-His plates in the presence of 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside acid and incubated at 30°C for 3 d to assay transcriptional activity.

Abiotic Stress Treatments

Birch plantlets of similar size (with roots removed) were transferred to 1/2MS solid medium containing 80 mM NaCl or 100 mM mannitol and grown for 21 d, and plantlets grown in normal 1/2MS solid medium were used as controls. The plant height and root length were measured after 21 d of NaCl and mannitol stresses.

Detection of ROS and Cell Death

The detached leaves from birch plantlets treated with 200 mM NaCl or 300 mM mannitol for 0, 6, and 12 h were used for histochemical staining analysis. Infiltration of leaves with DAB or NBT, which allowed the detection of H_2O_2 or O^{2-}, respectively, was performed following the method of Fryer et al. (2002). Cell death was detected by Evans Blue staining, as described by Zhang et al. (2011). Electrolyte leakage was measured according to Lutts et al. (1996).

Physiological and Gene Expression Analyses

Similar-sized birch plantlets grown in soil were irrigated with 200 mM NaCl or 300 mM mannitol for 3 d. SOD and POD activities were determined according to the method of Wang et al. (2010). Pro content was determined using the method of Bates et al. (1973). H_2O_2 levels were measured according to Jin et al. (2008). Each sample contained at least 10 plantlets. To determine the expression of *BpNAC012*, *SOD*, *POD*, *P5CS*, *CCR1*, *4CL1*, and *CCoAOMT* genes under stress conditions, birch plantlets were treated with 200 mM NaCl or 300 mM mannitol for 24 h and harvested for RT-qPCR analysis. Three independent biological replicates were performed in the experiments.

Analysis of BpNAC012 Binding to the Core Sequence CGT[G/A] in Yeast

Three tandem copies of the core sequence CGT[G/A] and its mutants were inserted into pHIS2 (Clontech) separately, upstream of the reporter gene HIS3. *BpNAC012* was cloned into pGADT7-Rec2 (pGADT7-Rec2-BpNAC012) as the effector. Primers used for the relative vector construction are shown in Supplemental Table S4. The binding of BpNAC012 to the core sequence CGT[G/A] and its mutants was studied using the Y1H method (Clontech). Transformation of pHIS2-p53 with pGADT7-Rec2-p53 was used as a positive control. Transformation of pHIS2-p53 with pGADT7-Rec2-BpNAC012 was used as a negative control. Yeast transformants were spotted onto SD/-Trp/-His plates or SD/-Trp/-His/-Leu plates supplemented with 50 mM 3-amino-1, 2, 4-triazole.

Transactivation Assay in Tobacco

Three tandem copies of the CGT[G/A] and its mutants or two tandem copies of the representative SNBE sequence were fused separately with the 35S CaMV minimal promoter (−46 to +1) to drive the *GUS* gene in a reformed pCAMBIA1301 (where the 35S:*Hygromycin* region was deleted) as reporters. The effector (35S:*BpNAC012*) was cotransformed with each reporter into tobacco (*Nicotiana tabacum*) leaves by *A. tumefaciens*-mediated transient expression transformation (Ji et al., 2014). Primers used to construct the relative vectors are shown in Supplemental Table S5. The *Luc* gene driven by the CaMV 35S promoter (35S:*Luc*) was cotransformed to normalize the transformation efficiency. Three biological replicates were performed. GUS activity was determined according to the method of Jefferson et al. (1987).

ChIP Assay

For ChIP assays, 35S:*BpNAC012-GFP* transgenic birch plants under normal growth conditions, treated with 12 h of 200 mM NaCl or 300 mM mannitol, were used. The ChIP procedure was performed according to Kim et al. (2013) with minor modification. The sonicated chromatin was immunoprecipitated with anti-GFP antibody (ChIP) or a rabbit anti-HA antibody as a negative control (ChIP−). RT-qPCR was performed to study the enriched fold of the studied promoters with the following parameters: 94°C for 1 min, followed by 45 cycles of 94°C for 12 s, 56°C for 30 s, 72°C for 45 s, and 79°C for 1 s for plate reading. Melting curves were generated for each reaction to evaluate the amplification specificity. The DNA sequence of *Ubiquitin* was used as the internal control. The primers used for ChIP-qPCR are shown in Supplemental Table S6.

Microscopy

The basal part of the stems from 3-month-old soil-grown birch plants was selected for secondary wall thickness analysis. The secondary wall microstructures of the stem cross sections were detected by a scanning electron microscope (S-4800; Hitachi). Wall thickness was measured from transmission electron micrographs of fibers and vessels using the software ImageJ (http://rsbweb.nih.gov/ij). Data from 40 cells in each plant were collected and analyzed.

Histological Analysis

Stem segments were fixed in FAA solution (70% [v/v] ethanol:glacial acetic acid:formaldehyde, 90:5:5, v/v) and embedded in low-viscosity (Spurr's) resin (Electron Microscopy Sciences) as described (Burk et al., 2006). Then, 1-mm-thick stem sections were cut with a microtome and stained with Toluidine Blue. The presence of lignin was visualized by staining the stem sections with phloroglucinol-HCl using light microscopy (Zhong et al., 2006). For cellulose staining, the stem sections were incubated with 0.01% (v/v) Calcofluor White and observed using a UV fluorescence microscope as described (Hughes and McCully, 1975). Xylan was detected by incubating the stem sections with the LM10 monoclonal xylan antibody and fluorescein isothiocyanate-conjugated secondary antibodies according to McCartney et al. (2005), and the fluorescence signals were observed using a confocal microscope.

Quantitative Analysis of Total Lignin Content

Stem explants of 3-month-old soil-grown birch plants were used for the determination of total lignin contents using Automatic Fiber Analytical Instruments (Ankom 2000i; Ankom). Lignin was expressed as a percentage of the original dry weight of stems. Ten independent biological replicates were performed.

Statistical Analysis

Data were analyzed by one-way ANOVA. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS 18), and statistical significance was set at $P < 0.05$.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the accession numbers listed in Supplemental Table S2.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Phylogenetic analysis of *BpNAC012* with Arabidopsis NAC genes.

Supplemental Figure S2. Transactivation activity of BpNAC012.

Supplemental Figure S3. Relative expression levels of *BpNAC012* in overexpressing or RNAi-suppressing *BpNAC012* transgenic birch lines.

Supplemental Figure S4. Detection of cell death and electrolyte leakage rate under NaCl and mannitol stress treatments.

Supplemental Figure S5. Schematic diagrams of promoter regions of BpNAC012 downstream abiotic stress-responsive genes used for ChIP-qPCR analysis.

Supplemental Figure S6. ChIP analysis of BpNAC012 binding affinity to promoter regions not containing the core sequence CGT[A/G].

Supplemental Figure S7. Schematic diagrams of promoter regions containing the SNBE site or the core sequence CGT[G/A].

Supplemental Table S1. Primers used in this study for vector construction.

Supplemental Table S2. GenBank accession numbers of all studied genes and primers used in RT-qPCR.

Supplemental Table S3. Primers used for transcriptional activity analysis.

Supplemental Table S4. Primers used for Y1H analysis.

Supplemental Table S5. Primers used for in vivo binding assay in tobacco.

Supplemental Table S6. Primers used for ChIP-qPCR analysis.

Received October 16, 2018; accepted November 29, 2018; published December 10, 2018.

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