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A Battery of Transcription Factors Involved in the Regulation of Secondary Cell Wall Biosynthesis in *Arabidopsis*

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SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1 (SND1) is a master transcriptional switch activating the developmental program of secondary wall biosynthesis. Here, we demonstrate that a battery of SND1-regulated transcription factors is required for normal secondary wall biosynthesis in *Arabidopsis thaliana*. The expression of 11 SND1-regulated transcription factors, namely, *SND2*, *SND3*, *MYB103*, *MYB85*, *MYB52*, *MYB54*, *MYB69*, *MYB42*, *MYB43*, *MYB20*, and *KNAT7* (a Knotted1-like homeodomain protein), was developmentally associated with cells undergoing secondary wall thickening. Of these, dominant repression of SND2, SND3, MYB103, MYB85, MYB52, MYB54, and KNAT7 significantly reduced secondary wall thickening in fiber cells. Overexpression of SND2, SND3, and MYB103 increased secondary wall thickening in fibers, and overexpression of MYB85 led to ectopic deposition of lignin in epidermal and cortical cells in stems. Furthermore, SND2, SND3, MYB103, MYB85, MYB52, and MYB54 were able to induce secondary wall biosynthetic genes. Direct target analysis using the estrogen-inducible system revealed that *MYB46*, *SND3*, *MYB103*, and *KNAT7* were direct targets of SND1 and also of its close homologs, NST1, NST2, and vessel-specific VND6 and VND7. Together, these results demonstrate that a transcriptional network consisting of SND1 and its downstream targets is involved in regulating secondary wall biosynthesis in fibers and that NST1, NST2, VND6, and VND7 are functional homologs of SND1 that regulate the same downstream targets in different cell types.

INTRODUCTION

Plant cells can be classified into three basic types (i.e., parenchyma, collenchyma, and sclerenchyma) based on their wall characteristics. While parenchyma and collenchyma cells have only primary walls, sclerenchyma cells contain both primary and secondary walls. Biosynthesis of secondary walls in sclerenchyma cells, an ability acquired when vascular plants emerged during the Silurian period, enabled vascular plants not only to build strong xylem conduits for transport of water and minerals but also to attain mechanical support for the plant body (Raven et al., 1999). Elucidation of the mechanisms that plants evolved to produce secondary walls is undoubtedly an important issue in plant biology and will help us understand both the evolution of vascular plants and the development of different cell types. The importance of studying the biosynthesis of secondary walls is further exemplified by the fact that wood, which is primarily composed of secondary walls, constitutes the most abundant biomass on earth and is widely used for pulping and papermaking, construction, and potentially also for biofuel production.

It was proposed that cells developmentally programmed to become sclerenchyma receive specific signals that are further transduced to activate the secondary wall biosynthetic pathways (Zhong and Ye, 2007). Although the secondary wall biosynthetic pathways have been characterized biochemically and genetically in great detail, little is known about the signals and transcriptional regulators that are responsible for turning on the secondary wall biosynthetic program. Recent studies on NAC and MYB transcription factors have provided a first glimpse into the complex process of transcriptional regulation of secondary wall biosynthesis (Zhong and Ye, 2007). Evidence indicates that a group of closely related *Arabidopsis thaliana* NAC domain proteins, including NAC SECONDARY WALL THICKENING PROMOTING FACTOR1 (NST1), NST2, SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1 (SND1; also called NST3), VASCULAR-RELATED NAC-DOMAIN6 (VND6), and VND7, are key transcriptional regulators of secondary wall biosynthesis in various sclerenchyma cell types (Kubo et al., 2005; Mitsuda et al., 2005, 2007; Zhong et al., 2006, 2007b; Ko et al., 2007). While SND1 and NST1 function redundantly in the activation of secondary wall biosynthesis in fibers (Zhong et al., 2006, 2007b; Mitsuda et al., 2007), VND6 and VND7 were proposed to regulate the differentiation of metaxylem and protoxylam, respectively, in primary roots (Kubo et al., 2005). NST1 and NST2 have been shown to function redundantly in regulating secondary wall thickening in endothecium cells of the anther (Mitsuda et al., 2005). Overexpression of all of these NAC genes leads to ectopic deposition of secondary walls in cells that are normally parenchymatous, and inhibition of their functions by knockout or dominant repression results in a reduction in secondary wall thickening (Kubo et al., 2005; Mitsuda et al., 2005, 2007; Zhong et al., 2006, 2007b). These studies demonstrate that these secondary wall NACs are master switches capable of turning on the entire biosynthetic pathways of cellulose, xylan, and lignin, which leads to the deposition of secondary walls.

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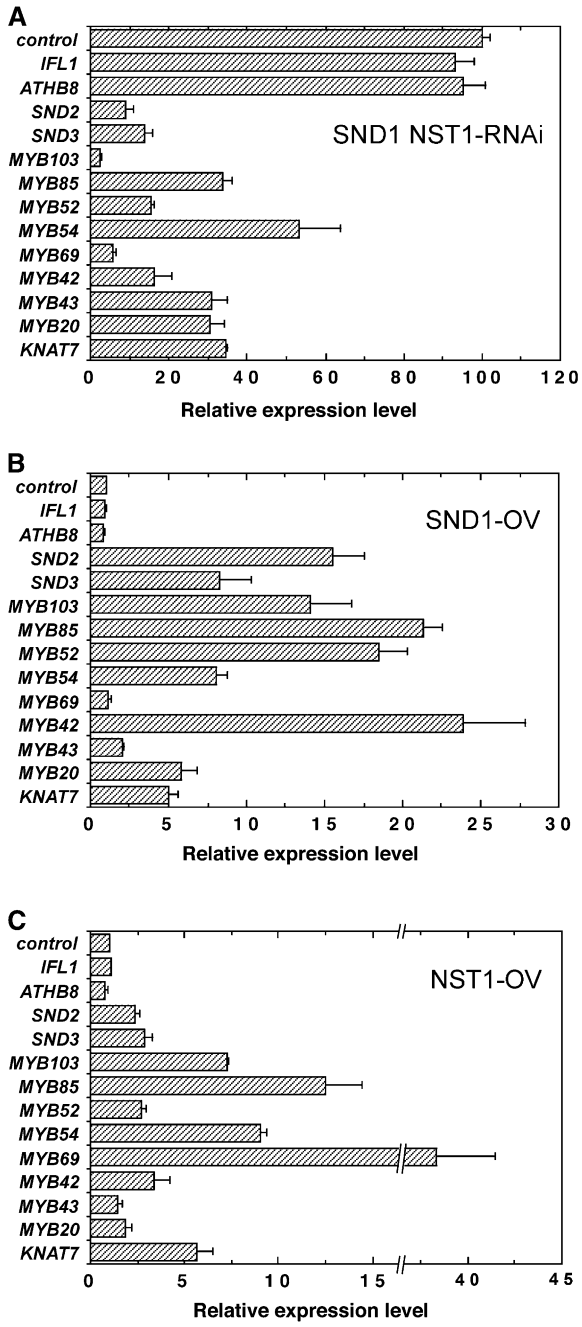

Figure 1. SND1 Regulates the Expression of a Set of Transcription Factor Genes.

Two genes, *IFL1* and *ATHB8*, which are not regulated by SND1 (Zhong et al., 2006), were included for comparison. Except for *IFL1* and *ATHB8*, the quantitative differences of expression of transcription factors between the wild type (control) and transgenic lines are statistically significant ($P < 0.001$). Error bars represent the SE of three biological replicates.

(A) Real-time quantitative PCR analysis showing a reduction in the expression of the transcription factor genes in the stems of *SND1* and *NST1* double RNAi plants (SND1 NST1-RNAi) relative to expression of these transcription factors in the wild type (control), which is set to 100.

(B) and **(C)** Real-time quantitative PCR analysis showing an induction in the expression of the transcription factors in the leaves of SND1 overexpressors (SND1-OV; **[B]**) and NST1 overexpressors (NST1-OV; **[C]**). The expression level of these transcription factors in the wild type (control) was set to 1.

In addition to NACs, several MYB transcription factors have also been shown to be important regulators of secondary wall biosynthesis in *Arabidopsis*. MYB46, a direct target of SND1, is able to turn on the biosynthetic pathways of cellulose, xylan, and lignin (Zhong et al., 2007a). Similarly, overexpression of MYB26, which was thought to act upstream of NST1 and NST2, results in ectopic deposition of secondary walls (Yang et al., 2007). Other *MYB* genes, such as pine (*Pinus taeda*) *MYB1* (Patzlaff et al., 2003b) and *MYB4* (Patzlaff et al., 2003a) and eucalyptus (*Eucalyptus grandis*) *MYB2* (Goicoechea et al., 2005), are able to bind to the AC elements present in the promoters of lignin biosynthetic genes and are proposed to regulate the biosynthesis of lignin.

It has been suggested that a network of transcription factors are involved in the regulation of secondary wall biosynthesis (Zhong and Ye, 2007). In this transcriptional network, the secondary wall NACs, including SND1, NST1, NST2, VND6, and VND7, are master switches turning on a subset of transcription factors, which in turn activate the secondary wall biosynthetic pathways. Previous studies have revealed that the expression of several transcription factors, including *SND2*, *SND3*, *MYB103*, *MYB85*, *MYB20*, and *KNAT7* (a Knotted1-like homeodomain protein), is regulated by SND1 (Zhong et al., 2006, 2007b).

In this study, we report the functional characterization of a battery of SND1-regulated transcription factors and provide evidence that they are part of the transcriptional network regulating secondary wall biosynthesis. We show that the expression of these SND1-regulated transcription factors is specifically associated with cells undergoing secondary wall thickening. Using dominant repression and overexpression studies, we demonstrate their involvement in regulating secondary wall biosynthesis. We further reveal that, in addition to *MYB46*, which is a known SND1 direct target, *SND3*, *MYB103*, and *KNAT7* are also direct targets of SND1 and its close homologs, NST1, NST2, VND6, and VND7. Our study suggests that the secondary wall NACs, including SND1, NST1, NST2, VND6, and VND7, share common downstream targets in the activation of the secondary wall biosynthetic program.

RESULTS

Identification of Transcription Factors That Are Regulated by SND1

To unravel the transcriptional network regulating secondary wall biosynthesis, we searched for transcriptional factor genes that are regulated by SND1. We used interfascicular fiber cells laser-microdissected from *Arabidopsis* inflorescence stems to identify transcription factors that are associated with secondary wall thickening in fibers (Zhong et al., 2006) and then selected those

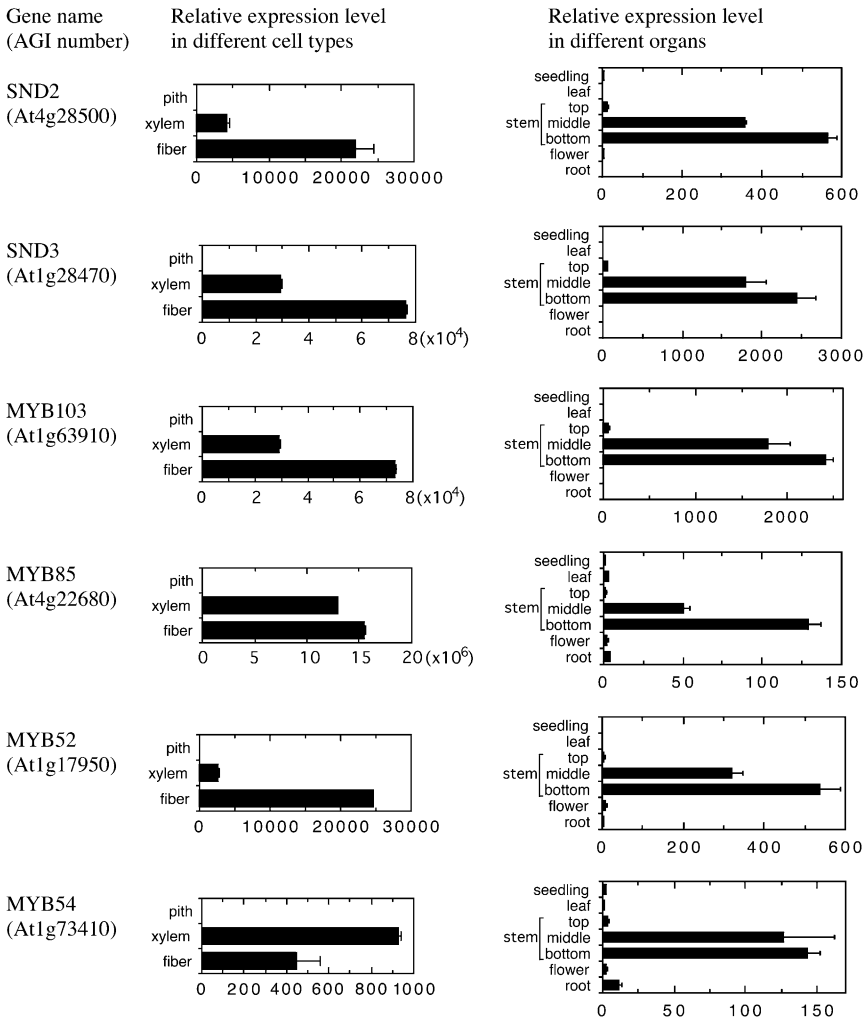

Figure 2. Quantitative Real-Time PCR Analysis of the Expression of *SND2*, *SND3*, *MYB103*, *MYB85*, *MYB52*, and *MYB54* Genes in Different Cell Types and Organs.

Pith, xylem, and interfascicular fiber cells were dissected from *Arabidopsis* inflorescence stems. The expression level of the transcription factor genes in pith cells was set to 1 for comparative expression analysis in different cell types. The expression level of the transcription factor genes in the organ with the lowest expression level was set to 1 for comparative expression analysis in different organs. Error bars represent the SE of three biological replicates.

that were downregulated by RNA interference (RNAi) inhibition of SND1 and NST1. In addition to *MYB46*, which has been shown to be a direct target of SND1 (Zhong et al., 2007a), we found 11 additional transcription factors whose expression is downregulated by RNAi inhibition of *SND1/NST1* (Figure 1A). These include two NACs (SND2 and SND3), eight MYBs (MYB103, MYB85, MYB52, MYB54, MYB69, MYB42, MYB43, and MYB20), and a homeodomain transcription factor (KNAT7). Among them, six transcription factors, including SND2, SND3, MYB103, MYB85, MYB20, and KNAT7, were previously shown to be regulated by SND1 (Zhong et al., 2006, 2007b); however, their roles in the regulation of secondary wall synthesis have not been characterized. Consistent with their downregulation of expression by *SND1/NST1* RNAi inhibition, the expression of all of these transcription factors except for MYB69 was significantly upregulated by SND1 overexpression (Figure 1B). The expression of these transcription factors including *MYB69* was also upregulated by NST1 overexpression (Figure 1C). These results indicate that these transcription factors are most likely involved in the SND1/NST1-mediated transcriptional regulation of secondary wall synthesis.

Expression Patterns of SND1-Regulated Transcription Factors

To investigate whether the expression of these SND1-regulated transcription factors is associated with secondary wall biosynthesis, we analyzed their expression in different cell types and organs by quantitative RT-PCR analysis. All 11 SND1-regulated transcription factors exhibited a high level of expression in

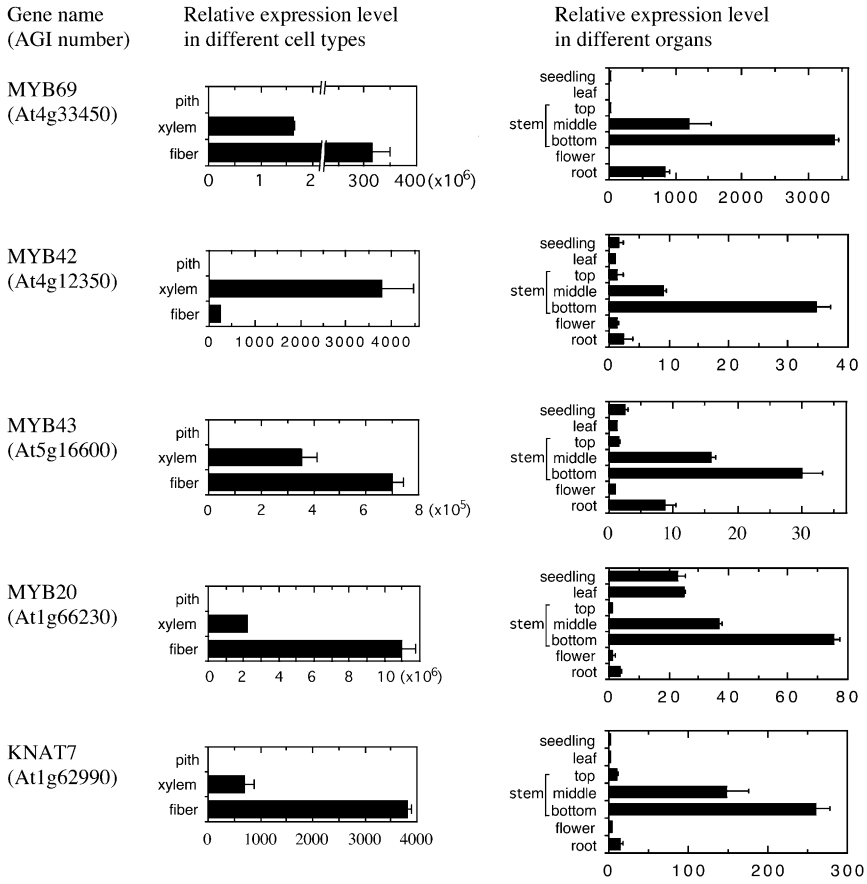

Figure 3. Quantitative Real-Time PCR Analysis of the Expression of *MYB69*, *MYB42*, *MYB43*, *MYB20*, and *KNAT7* Genes in Different Cell Types and Organs.

The expression level of the transcription factor genes in pith cells was set to 1 for comparative expression analysis in different cell types. The expression level of the transcription factor genes in the organ with the lowest expression level was set to 1 for comparative expression analysis in different organs. Error bars represent the SE of three biological replicates.

interfascicular fibers and xylem cells undergoing secondary wall thickening, but not in parenchymatous pith cells (Figures 2 and 3). At the organ level, they were preferentially expressed in inflorescence stems in which interfascicular fibers and xylem cells were abundantly developed (Figures 2 and 3).

We next used the β-glucuronidase (GUS) reporter gene and in situ mRNA hybridization to examine the developmental expression patterns of eight of these transcription factors, namely, SND2, SND3, MYB103, MYB85, MYB52, MYB54, MYB69, and KNAT7, which were found to cause secondary wall defects when their expression was dominantly repressed (see below). For the GUS reporter gene analysis, the entire exon and intron region of the transcription factor gene sequence, a 3-kb 5′ upstream sequence, and a 2-kb 3′ downstream sequence were included to ensure that the sequence used contains all of the *cis*-elements required for the endogenous gene expression. For in situ mRNA hybridization, gene-specific probes were used for detecting the gene expression patterns. In elongating stem internodes, all eight transcription factors were found to be expressed in developing protoxylem (Figures 4 and 5), the only secondary wall–containing cell type present (Ye et al., 2002). In addition to protoxylem, *SND2*, *SND3*, *MYB52*, and *MYB54* were also expressed in the elongating interfascicular fiber cells, although at this stage, they do not yet have visible secondary wall thickening. In nonelongating internodes, in which both metaxylem cells and interfascicular fibers are undergoing secondary wall thickening (Ye et al., 2002), a high level of expression of all eight transcription factors was evident in developing metaxylem cells and interfascicular fibers (Figures 4 and 5). In roots, these genes were predominantly expressed in developing secondary xylem, in which secondary wall thickening occurs (Figures 4 and 5). These results demonstrate that the expression of these SND1-regulated transcription factors is developmentally associated with secondary wall biosynthesis in vessels and fibers.

Consistent with their predicted function as transcription factors, yellow fluorescent protein (YFP)–tagged fusions of these transcription factors were found to be targeted to the nucleus when expressed in *Arabidopsis* leaf protoplasts (Figures 6A to 6L). Transcriptional activation analysis demonstrated that SND2, SND3, MYB103, MYB85, and MYB69 were able to activate the

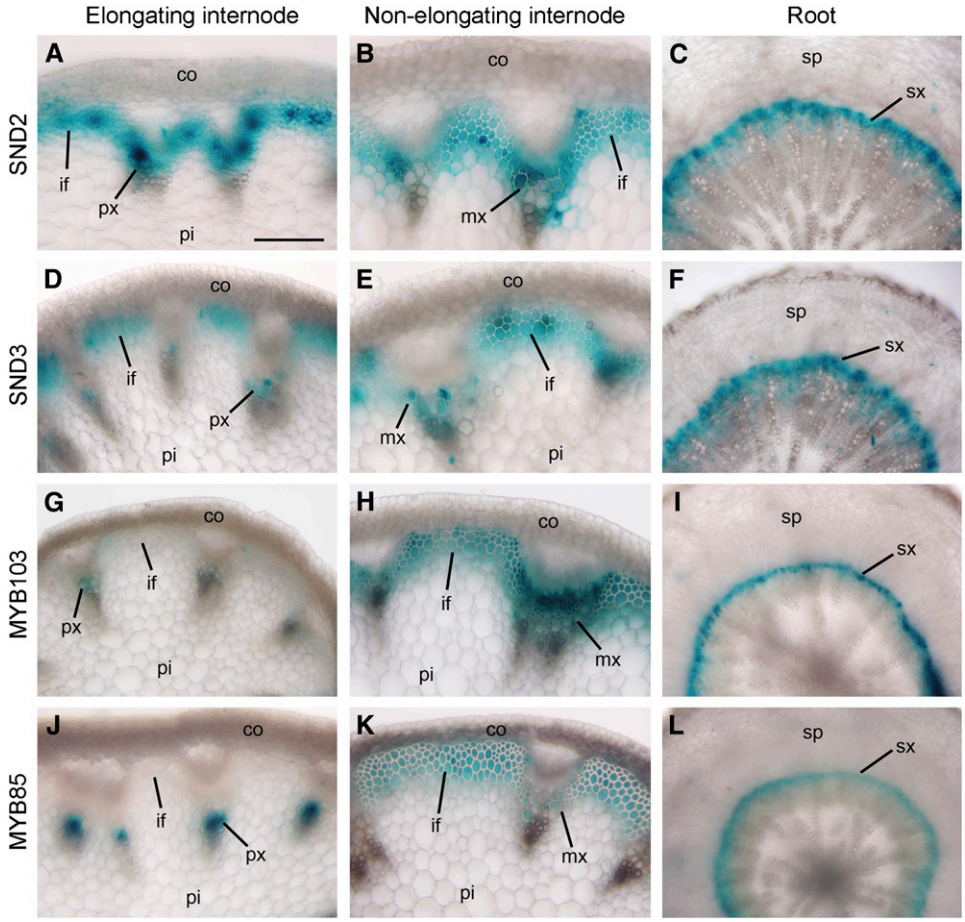

Figure 4. Expression Patterns of *SND2*, *SND3*, *MYB103*, and *MYB85* Genes in *Arabidopsis* Stems and Roots.

Transgenic *Arabidopsis* plants expressing the transcription factor-GUS reporter genes were examined for GUS activity. Cross sections of elongating internodes (**[A]**, **[D]**, **[G]**, and **[J]**) nonelongating internodes (**[B]**, **[E]**, **[H]**, and **[K]**), and roots (**[C]**, **[F]**, **[I]**, and **[L]**), showing the expression of *SND2* (**[A]** to **[C]**), *SND3* (**[D]** to **[F]**), *MYB103* (**[G]** to **[I]**), and *MYB85* (**[J]** to **[L]**) in developing vessels, xylary fibers, and interfascicular fibers in stems (**[A]**, **[B]**, **[D]**, **[E]**, **[G]**, **[H]**, **[J]**, and **[K]**), and in developing secondary xylem cells in the root (**[C]**, **[F]**, **[I]**, and **[L]**). co, cortex; if, interfascicular fiber; mx, metaxylem; pi, pith; px, protoxylem; sp, secondary phloem; sx, secondary xylem. Bar in **(A)** = 160 μm for **(A)** to **(L)**.

expression of the His3 and β-Gal reporter genes in yeast (Figure 6M), confirming that they are indeed transcriptional activators.

Dominant Repression of SND1-Regulated Transcription Factors Leads to Defects in Secondary Wall Thickening

Because the expression of the SND1-regulated transcription factors is closely associated with secondary wall thickening, we reasoned that these transcription factors might be involved in regulation of secondary wall biosynthesis. To elucidate their potential roles in the regulation of secondary wall biosynthesis, we first attempted to use the RNAi approach to study the effects of their downregulation on secondary wall thickening. The RNAi approach has been effectively used to study the redundant functions of SND1 and NST1 in regulation of secondary wall biosynthesis (Zhong et al., 2007b). However, no changes on secondary wall thickening were observed when these transcription factors were downregulated by RNAi inhibition, probably due to the functional redundancy of their homologs. We next resorted to the dominant repression approach that has been successfully applied to study transcription factors involved in secondary wall biosynthesis (Kubo et al., 2005; Mitsuda et al., 2005, 2007; Zhong et al., 2006, 2007a). The dominant repression approach was first developed by Hiratsu et al. (2004) to facilitate the analysis of functionally redundant transcription factors. It should be noted that transcription factors fused with the EAR (ethylene-responsive element binding factor-associated amphiphilic repression) dominant repression domain not only inhibit the function of transcription factors targeted for repression but also that of their homologs by competing with their binding to the same *cis*-elements or interacting proteins, thereby leading to dominant repression (Hiratsu et al., 2004).

To repress their functions, the genes encoding each of the SND1-regulated transcription factors were fused with the EAR repressor domain at the C terminus and expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter in

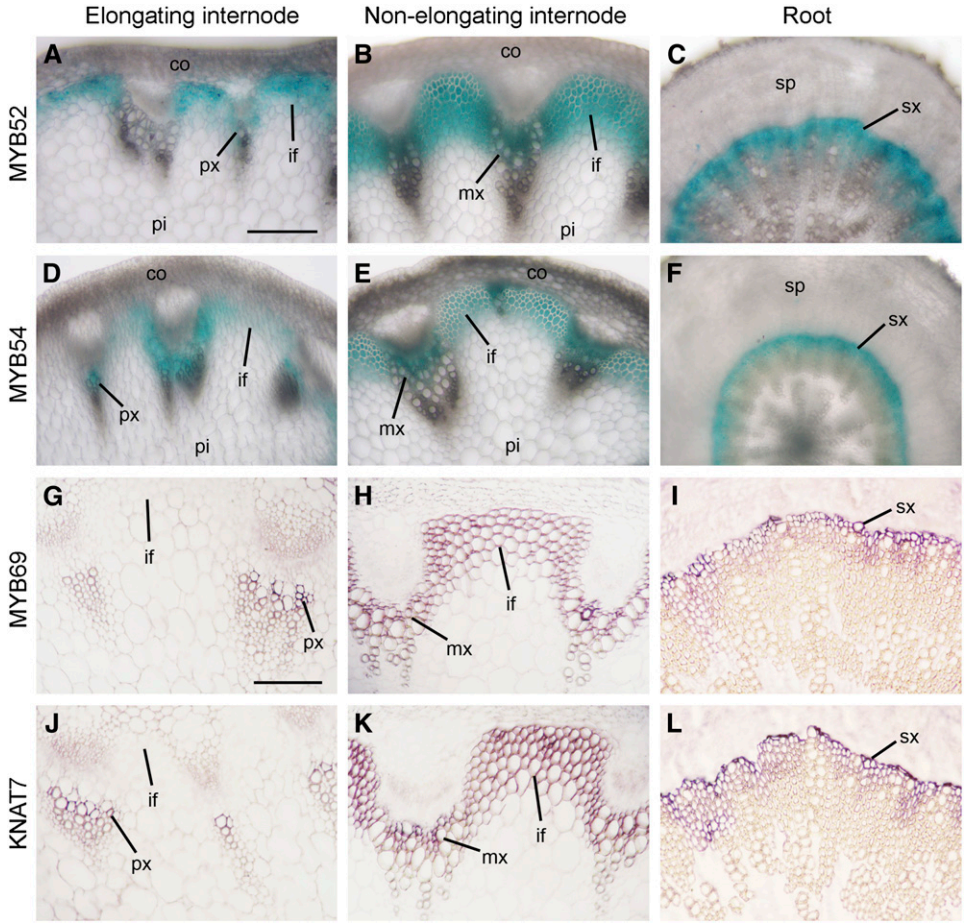

Figure 5. Expression Patterns of *MYB52*, *MYB54*, *MYB69*, and *KNAT7* Genes in *Arabidopsis* Stems and Roots.

The expression of the transcription factor genes was studied using the GUS reporter gene (**[A]** to **[F]**) or in situ mRNA hybridization (**[G]** to **[L]**). Cross sections of elongating internodes (**[A]**, **[D]**, **[G]**, and **[J]**), nonelongating internodes (**[B]**, **[E]**, **[H]**, and **[K]**), and roots (**[C]**, **[F]**, **[I]**, and **[L]**) showing the expression of *MYB52* (**[A]** to **[C]**), *MYB54* (**[D]** to **[F]**), *MYB69* (**[G]** to **[I]**), and *KNAT7* (**[J]** to **[L]**) in developing vessels, xylary fibers, and interfascicular fibers in stems (**[A]**, **[B]**, **[D]**, **[E]**, **[G]**, **[H]**, **[J]**, and **[K]**), and in developing secondary xylem cells in the root (**[C]**, **[F]**, **[I]**, and **[L]**). co, cortex; if, interfascicular fiber; mx, metaxylem; pi, pith; px, protoxylem; sp; secondary phloem; sx, secondary xylem. Bar in **(A)** = 160 μm for **(A)** to **(F)**, and bar in **(G)** = 78 μm for **(G)** to **(L)**.

transgenic *Arabidopsis* plants. For each transcription factor analyzed by dominant repression, at least 96 first generation transgenic plants were examined for the thickening of secondary walls. Among the 11 SND1-regulated transcription factors, dominant repression of eight of them, including SND2, SND3, MYB103, MYB85, MYB52, MYB54, MYB69, and KNAT7, caused a severe reduction in secondary wall thickening in both interfascicular fibers and xylary fibers in inflorescence stems (Figures 7 and 8, Table 1). In these repressor lines, 20 to 40% of transgenic plants showed significant reduction in secondary wall thickness, and the severity of reduction correlated with the amount of the corresponding repressor transcript. To examine the highest potential impact of dominant repression of these transcription factors on secondary wall thickening, transgenic plants with the most severe phenotypes were selected for analysis. The secondary wall thickening in vessels was not significantly affected in any of these repressor lines (Table 1). It was noted that dominant repression of MYB85 caused deformation of vessels (Figures 7Q and 7R), probably due to the weakening of the vessels' secondary walls. These results suggest that dominant repression of SND1-regulated transcription factors leads to a defect in secondary wall biosynthesis, which was further confirmed by a cell wall composition analysis, which showed that the amount of glucose and xylose, the main sugars in cellulose and xylan, respectively, was significantly reduced in these repressor lines (Table 2).

Effects of Overexpression of SND1-Regulated Transcription Factors on Secondary Wall Thickening

We next studied the effects of overexpression of the eight transcription factors, of which dominant repression resulted in secondary wall defects (Figure 9). These transcription factors were placed under the control of the CaMV 35S promoter and

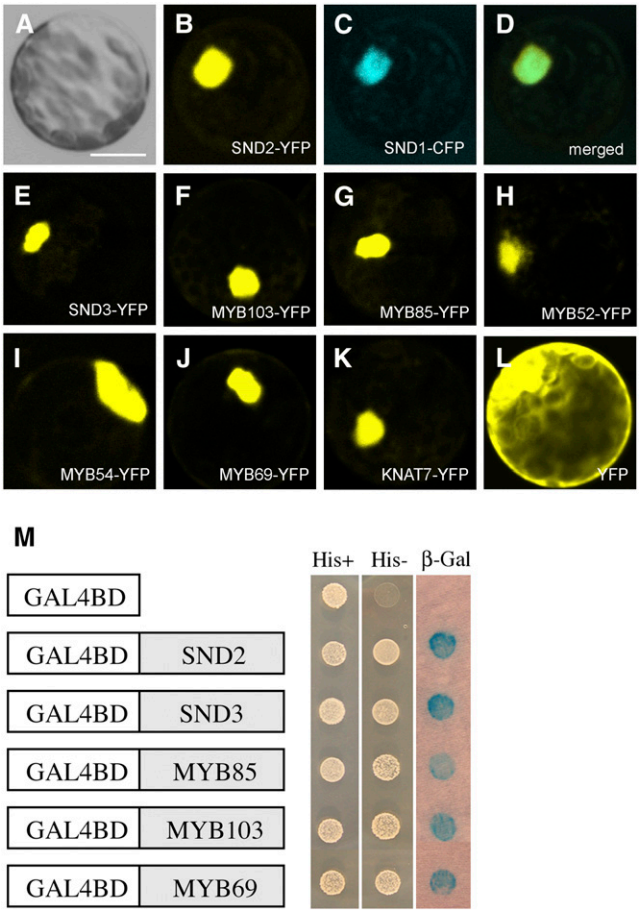

Figure 6. Subcellular Localization and Transcriptional Activation Analysis of the Secondary Wall–Associated Transcription Factors.

Subcellular localization was determined by expressing YFP-tagged transcription factors in *Arabidopsis* leaf protoplasts. For the transcriptional activation analysis, full-length cDNAs of transcription factors fused with the GAL4-DNA binding domain were expressed in yeast and tested for activation of expression of the His3 and β-Gal reporter genes. Bar in **(A)** = 25 μm for **(A)** to **(L)**.

(A) to **(D)** An *Arabidopsis* protoplast coexpressing SND2-YFP and SND1-CFP. The differential interference contrast image **(A)**, the SND2-YFP signal **(B)**, the SND1-cyan fluorescent protein (CFP) signal **(C)**, and the merged image **(D)** of **(B)** and **(C)** are shown. Note that SND2-YFP colocalizes with SND1-CFP in the nucleus.

(E) to **(K)** *Arabidopsis* protoplasts expressing SND3-YFP **(E)**, MYB103-YFP **(F)**, MYB85-YFP **(G)**, MYB52-YFP **(H)**, MYB54-YFP **(I)**, MYB69-YFP **(J)**, and KNAT7-YFP **(K)**, showing their nuclear localization.

(L) An *Arabidopsis* protoplast expressing YFP alone showing the fluorescent signal throughout the cytoplasm and in the nucleus.

(M) Transcriptional activation analysis of secondary wall–associated transcription factors fused with the GAL4 DNA binding domain (GAL4DB) in yeast. SND2, SND3, MYB85, MYB103, and MYB69 were able to activate the expression of the His3 and β-Gal reporter genes.

expressed in wild-type *Arabidopsis* plants. Examination of leaves, stems, and roots of transgenic plants did not reveal any ectopic deposition of secondary walls. The only exception was that plants overexpressing MYB85 had ectopic deposition of lignin in the epidermal and cortical cells of stems (Figures 9Q and 9R). No ectopic deposition of cellulose and xylan was observed in these lignified cells by Calcoflour White staining and xylan antibody immunolocalization, indicating that overexpression of MYB85 specifically induces the lignin biosynthetic pathway. Although no ectopic deposition of secondary walls was seen in transgenic plants overexpressing the other transcription factors, it was found that overexpression of three of them, SND2, SND3, and MYB103, resulted in a noticeable increase in the secondary wall thickness of both xylary fibers and interfascicular fibers (Figures 9A to 9P). Quantitative analysis showed that the wall thickness of xylary fibers and interfascicular fibers was increased by at least 32 and 58%, respectively, in these overexpressors compared with the wild type (Table 1). No changes were observed in the secondary wall thickness of fibers and vessels in transgenic plants overexpressing MYB52, MYB54, MYB69, and KNAT7.

To test whether any of these transcription factors has the ability to induce the expression of secondary wall biosynthetic genes, we engineered reporter constructs by ligating the promoters of a cellulose synthase gene (*CesA8*; Taylor et al., 2004), a xylan biosynthetic gene (*IRX9*; Pena et al., 2007), and a lignin biosynthetic gene (*4CL1*; Boerjan et al., 2003) in front of the GUS reporter gene. These reporter constructs were then cotransfected with the transcription factor overexpression constructs into *Arabidopsis* leaf protoplasts for transactivation analysis. We found that SND2, SND3, and MYB103 significantly induced the GUS reporter gene expression driven by the *CesA8* promoter, whereas MYB85 specifically induced GUS expression driven by the *4CL1* promoter (Figure 10). MYB52 and MYB54 appeared to induce GUS expression driven by all three promoters, albeit to various levels (Figure 10). No significant induction in GUS expression was observed for MYB69 and KNAT7. We confirmed that all of the transcription factor genes were highly expressed in the transfected protoplasts (Figure 10C). These results suggest that some of these SND1-regulated transcription factors have the ability to induce the expression of certain secondary wall biosynthetic genes.

Expression of *SND3*, *MYB103*, and *KNAT7* Is Directly Activated by SND1

Our findings that the SND1-regulated transcription factors are expressed in cells undergoing secondary wall thickening and are involved in regulation of secondary wall synthesis suggest that they are downstream targets of SND1. To investigate whether any of them are direct targets of SND1, we set out to test whether SND1 can directly activate the expression of these transcription factors using the steroid receptor-based inducible system. In this system, a transcription factor is fused with a steroid binding domain, which renders the transcription factor inactive in the cell, as it is sequestered in the cytoplasm via binding to a cytoplasmic complex. Binding of the steroid to the steroid binding domain disrupts the complex and thus allows the transcription factor to enter the nucleus and regulate the expression of downstream target genes. In the presence of a protein synthesis inhibitor, activation of the transcription factor by a steroid treatment should still induce the expression of the direct target genes, whereas the induction of further downstream genes is inhibited

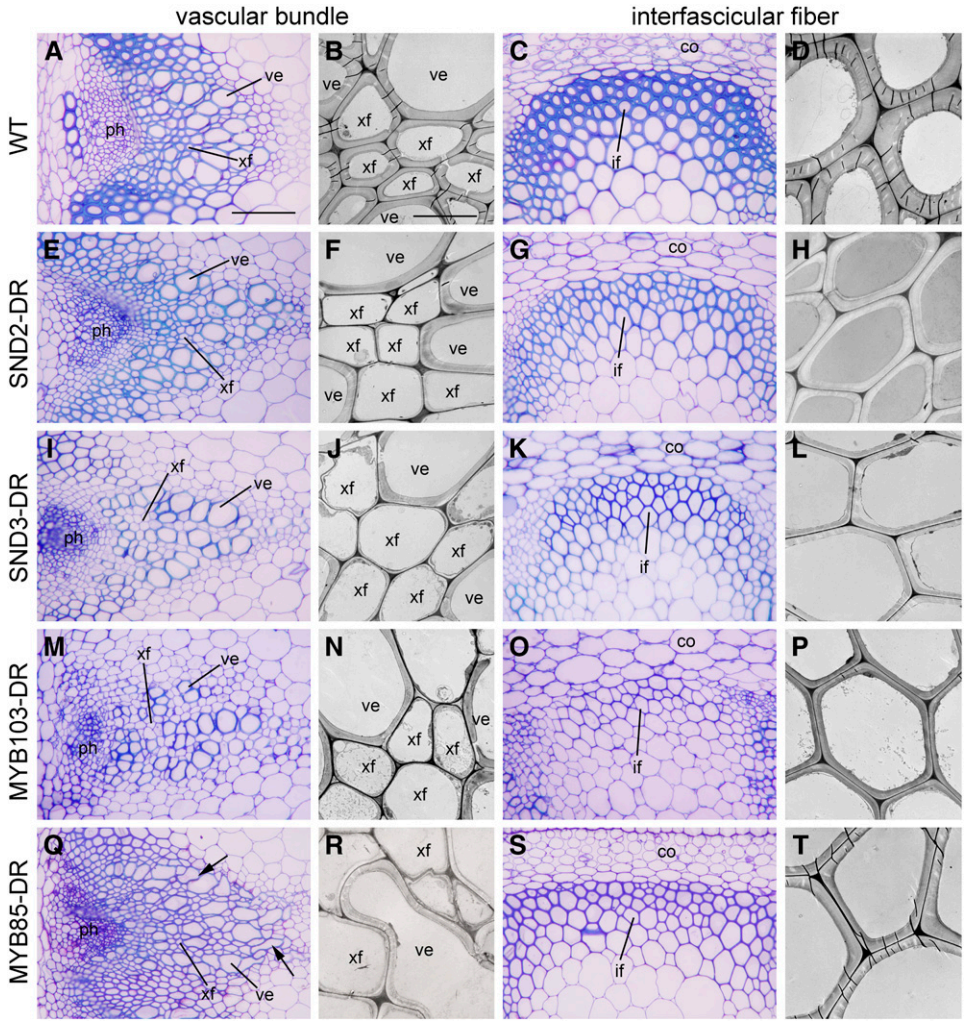

Figure 7. Effects of Dominant Repression of SND2, SND3, MYB103, and MYB85 on Secondary Wall Thickening in Fibers.

The full-length cDNAs of *SND2*, *SND3*, *MYB103*, and *MYB85* were fused in frame with the dominant EAR repression sequence (DR) and expressed in *Arabidopsis* plants. The bottom internodes of 8-week-old transgenic plants were examined for secondary wall thickening in fibers and vessels. co, cortex; if, interfascicular fiber; ph, phloem; ve, vessel; xf, xylary fiber. Bar in **(A)** = 78 μm for the light micrographs (**[A]**, **[C]**, **[E]**, **[G]**, **[I]**, **[K]**, **[M]**, **[O]**, **[Q]**, and **[S]**), and bar in **(B)** = 8.1 μm for the transmission electron micrographs (**[B]**, **[D]**, **[F]**, **[H]**, **[J]**, **[L]**, **[N]**, **[P]**, **[R]**, and **[T]**).

(A) to **(D)** Cross sections of wild-type stems showing secondary wall thickening in xylary fibers and vessels (**[A]** and **[B]**) and in interfascicular fibers (**[C]** and **[D]**).

(E) to **(T)** Cross sections of SND2-DR (**[E]** to **[H]**), SND3-DR (**[I]** to **[L]**), MYB103-DR (**[M]** to **[P]**), and MYB85-DR (**[Q]** to **[T]**) stems showing reduced secondary wall thickening in xylary fibers (**[E]**, **[F]**, **[I]**, **[J]**, **[M]**, **[N]**, **[Q]**, and **[R]**) and in interfascicular fibers (**[G]**, **[H]**, **[K]**, **[L]**, **[O]**, **[P]**, **[S]**, and **[T]**).

since it requires new protein synthesis. Therefore, the steroid receptor-based inducible system can be employed to search for direct targets of transcription factors and has been successfully applied to finding direct targets of transcription factors in plants (Sablowski and Meyerowitz, 1998; Wagner et al., 1999; Baudry et al., 2004).

To identify direct targets of SND1, we fused SND1 with the regulatory region of the human estrogen receptor (HER) (Figure 11A; Zuo et al., 2000) and constitutively expressed the SND1-HER fusion protein under the control of the CaMV 35S promoter in *Arabidopsis* leaf protoplasts. We first tested whether the SND1-HER chimeric protein is functional using protoplasts cotransfected with 35S:SND1-HER and MYB46P:GUS (Figure 11A). Activation of SND1 by estradiol treatment drastically induced the GUS reporter activity driven by the *MYB46* promoter (Figure 11B), demonstrating that SND1-HER is functional in the induction of downstream genes. We next examined the effect of various concentrations of cycloheximide on the inhibition of new protein synthesis. The GUS activity induced by estradiol-activated SND1-HER was completely abolished when as little as 2 μM cycloheximide was added (Figure 11B), indicating that new protein synthesis is completely inhibited by cycloheximide.

To investigate whether the SND1-HER inducible system could be used to search for direct targets of SND1, we tested whether

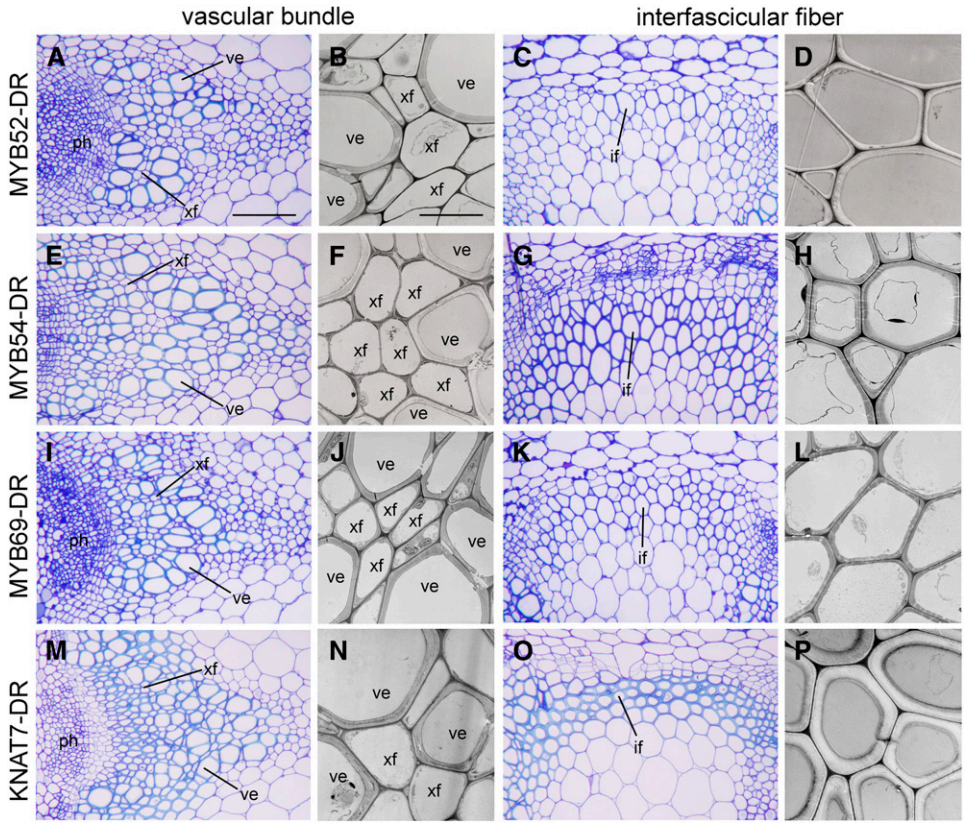

Figure 8. Effects of Dominant Repression of MYB52, MYB54, MYB69, and KNAT7 on Secondary Wall Thickening in Fibers.

The full-length cDNAs of *MYB52*, *MYB54*, *MYB69*, and *KNAT7* were fused in frame with the dominant EAR repression sequence (DR) and expressed in *Arabidopsis* plants. The bottom internodes of 8-week-old transgenic plants were examined for the secondary wall thickening in fibers and vessels. Cross sections of MYB52-DR (**[A]** to **[D]**), MYB54-DR (**[E]** to **[H]**), MYB69-DR (**[I]** to **[L]**), and KNAT7-DR (**[M]** to **[P]**) stems showing reduced secondary wall thickening in xylary fibers (**[A]**, **[B]**, **[E]**, **[F]**, **[I]**, **[J]**, **[M]**, and **[N]**) and in interfascicular fibers (**[C]**, **[D]**, **[G]**, **[H]**, **[K]**, **[L]**, **[O]**, and **[P]**). co, cortex; if, interfascicular fiber; ph, phloem; ve, vessel; xf, xylary fiber. Bar in **(A)** = 78 μm for the light micrographs (**[A]**, **[C]**, **[E]**, **[G]**, **[I]**, **[K]**, **[M]**, and **[O]**), and bar in **(B)** = 8.1 μm for the transmission electron micrographs (**[B]**, **[D]**, **[F]**, **[H]**, **[J]**, **[L]**, **[N]**, and **[P]**).

SND1-HER could directly activate the expression of the *MYB46* gene, which was previously shown to be a direct target of SND1 (Zhong et al., 2007a). The expression of the *MYB46* gene was drastically induced by estradiol-activated SND1-HER, and this induction still occurred when new protein synthesis was completely inhibited by cycloheximide (Figure 11C), indicating that the induction of *MYB46* expression by SND1 does not require new protein synthesis and is thus direct. It should be noted that the induction level of *MYB46* by SND1-HER in the presence of cycloheximide was lower than that without cycloheximide, which was most likely due to cycloheximide inhibition of the overall protein synthesis, including that of SND1-HER. These results established that it is feasible to use the SND1-HER–inducible system to identify additional direct targets of SND1.

We next investigated the expression of SND1-regulated transcription factors in the SND1-HER–inducible system. Estradiol activation of SND1 resulted in a substantial induction in the expression of these transcription factors (Figure 11D). To find out whether any of them are direct targets of SND1, we applied the protein synthesis inhibitor cycloheximide before addition of estradiol and examined their induction by SND1. We found that the expression of *SND3*, *MYB103*, and *KNAT7* was still highly induced upon estradiol activation of SND1 even when new protein synthesis was completely inhibited (Figure 11E), indicating that their induction by SND1 occurred without new protein synthesis and is therefore direct. By contrast, the expression of other transcription factors was not induced under the same conditions. Consistent with the finding that the expression of *SND3*, *MYB103*, and *KNAT7* is directly activated by SND1, electrophoretic mobility shift assay (EMSA) showed that SND1 was able to bind to their promoter fragments and caused a mobility shift (Figure 11F). These results demonstrate that, besides MYB46, the SND3, MYB103, and KNAT7 transcription factors are additional direct targets of SND1.

Direct Induction of *MYB46*, *SND3*, *MYB103*, and *KNAT7* by the SND1 Homologs NST1, NST2, VND6, and VND7

We previously showed that, like SND1, close homologs of SND1, namely, NST1, NST2, VND6, and VND7, were able to activate the

Table 1. Wall Thickness of Vessels and Fibers in the Stems of Wild-Type Plants and in Dominant Repressors and Overexpressors of SND1-Regulated Transcription Factors

Sample	Relative Expression Level of the Transgene[b]	Wall Thickness (μm)[a]		
		Interfascicular Fibers	Vessels	Xylary Fibers
Wild type	NA	1.89 ± 0.45	1.06 ± 0.15	0.82 ± 0.15
SND2 repressors	11.2 ± 1.3	0.94 ± 0.16	0.99 ± 0.16	0.27 ± 0.09
SND3 repressors	16.4 ± 2.2	0.65 ± 0.10	0.77 ± 0.12	0.13 ± 0.04
MYB103 repressors	12.2 ± 2.4	0.86 ± 0.17	0.79 ± 0.11	0.13 ± 0.03
MYB85 repressors	10.8 ± 1.2	1.21 ± 0.21	0.99 ± 0.14	0.44 ± 0.08
MYB52 repressors	20.6 ± 3.1	0.63 ± 0.17	0.79 ± 0.12	0.15 ± 0.03
MYB54 repressors	18.4 ± 2.8	0.58 ± 0.12	0.71 ± 0.16	0.14 ± 0.04
MYB69 repressors	18.2 ± 2.5	0.48 ± 0.08	0.71 ± 0.11	0.18 ± 0.05
KNAT7 repressors	12.6 ± 1.8	1.36 ± 0.25	0.78 ± 0.14	0.16 ± 0.04
SND2 overexpressors	15.9 ± 2.5	2.96 ± 0.46	0.89 ± 0.17	1.21 ± 0.28
SND3 overexpressors	12.4 ± 3.6	3.03 ± 0.35	0.92 ± 0.12	1.09 ± 0.27
MYB103 overexpressors	11.8 ± 1.4	3.00 ± 0.36	1.01 ± 0.15	1.08 ± 0.19

[a] Measured from transmission electron micrographs of fibers and vessels, and the data are means (μm) ± SE from 25 cells.
[b] Determined by real-time quantitative PCR analysis, and the data are shown as a multiple (±SE from three biological replicates) of the expression level of each transgene relative to that of the corresponding endogenous gene in stems. NA, not applicable.

expression of *MYB46*, suggesting that these secondary wall NACs might regulate common target genes involved in secondary wall synthesis (Zhong et al., 2007a). To further substantiate this hypothesis, we investigated whether these SND1 homologs were also able to directly activate the expression of other direct target genes of SND1. To do this, NST1, NST2, VND6, and VND7 were fused with the regulatory region of the human estrogen receptor and expressed under the control of the CaMV 35S promoter in *Arabidopsis* protoplasts (Figure 12A). Estradiol activation of these transcription factors greatly induced expression of *MYB46*, *SND3*, *MYB103*, and *KNAT7* (Figures 12B to 12E). To test whether this induction was direct, new protein synthesis was inhibited by addition of cyclohexmide before addition of estradiol. We found that NST1, NST2, VND6, and VND7 still effectively activated the expression of *MYB46*, *SND3*, *MYB103*, and *KNAT7*, while expression of *SND3* was no longer induced by NST1 (Figures 12B to 12E). The induction level of the target genes by NST1, NST2, VND6, and VND7 in the presence of cycloheximide was lower than that in the absence of cycloheximide, which is most likely due to the overall inhibition of protein synthesis by cycloheximide. This result suggests that SND1 and its close homologs, NST1, NST2, VND6, and VND7, activate the same set of direct targets, thereby regulating secondary wall biosynthesis.

VND6 and *VND7* Genes Are Expressed in Vessels of Stems

SND1 is specifically expressed in fibers but not in vessels in *Arabidopsis* inflorescence stems, and it functions redundantly with NST1 to regulate secondary wall biosynthesis in fibers, suggesting that other SND1 homologs are involved in regulation of secondary wall biosynthesis in the vessels of stems (Zhong et al., 2006). RT-PCR analysis of the expression of SND1 homologs in laser-dissected interfascicular fibers, xylem, and pith cells from inflorescence stems revealed that *VND6* and *VND7* were expressed in the xylem but not in interfascicular fibers or pith cells in *Arabidopsis* inflorescence stems (Figure 13A). Further gene expression analysis in transgenic plants expressing the

Table 2. Monosaccharide Composition of Cell Walls from the Stems of Wild Type Plants and of Dominant Repressors of SND1-Regulated Transcription Factors

Sample	Glucose	Xylose	Mannose	Galactose	Arabinose	Rhamnose
Wild type	403 ± 8	100 ± 6	18.4 ± 1.2	20.7 ± 3.0	7.8 ± 0.5	10.1 ± 0.3
SND2 repressors	205 ± 2	39 ± 1	11.6 ± 3.2	28.9 ± 1.1	19.8 ± 0.8	12.2 ± 0.3
SND3 repressors	161 ± 6	21 ± 1	9.5 ± 0.6	22.4 ± 1.3	15.2 ± 0.2	11.7 ± 0.1
MYB103 repressors	216 ± 12	35 ± 4	9.3 ± 1.5	33.5 ± 4.5	16.5 ± 1.6	12.2 ± 0.4
MYB85 repressors	334 ± 10	74 ± 5	15.7 ± 1.7	16.4 ± 0.5	7.6 ± 0.3	11.7 ± 0.4
MYB52 repressors	276 ± 20	47 ± 2	12.8 ± 0.5	25.6 ± 2.3	16.9 ± 0.6	12.4 ± 0.8
MYB54 repressors	226 ± 23	34 ± 5	6.9 ± 1.7	25.5 ± 3.0	13.2 ± 1.8	9.2 ± 1.1
MYB69 repressors	287 ± 28	58 ± 6	14.4 ± 1.5	26.0 ± 3.7	15.8 ± 0.2	13.1 ± 0.8
KNAT7 repressors	321 ± 14	80 ± 2.8	12.8 ± 1.2	15.3 ± 1.3	9.2 ± 0.5	7.3 ± 0.0

Cell wall residues used for monosaccharide composition analysis were prepared from stems of 8-week-old plants. Data are means (mg/g dry cell wall) ± SE of duplicate assays.

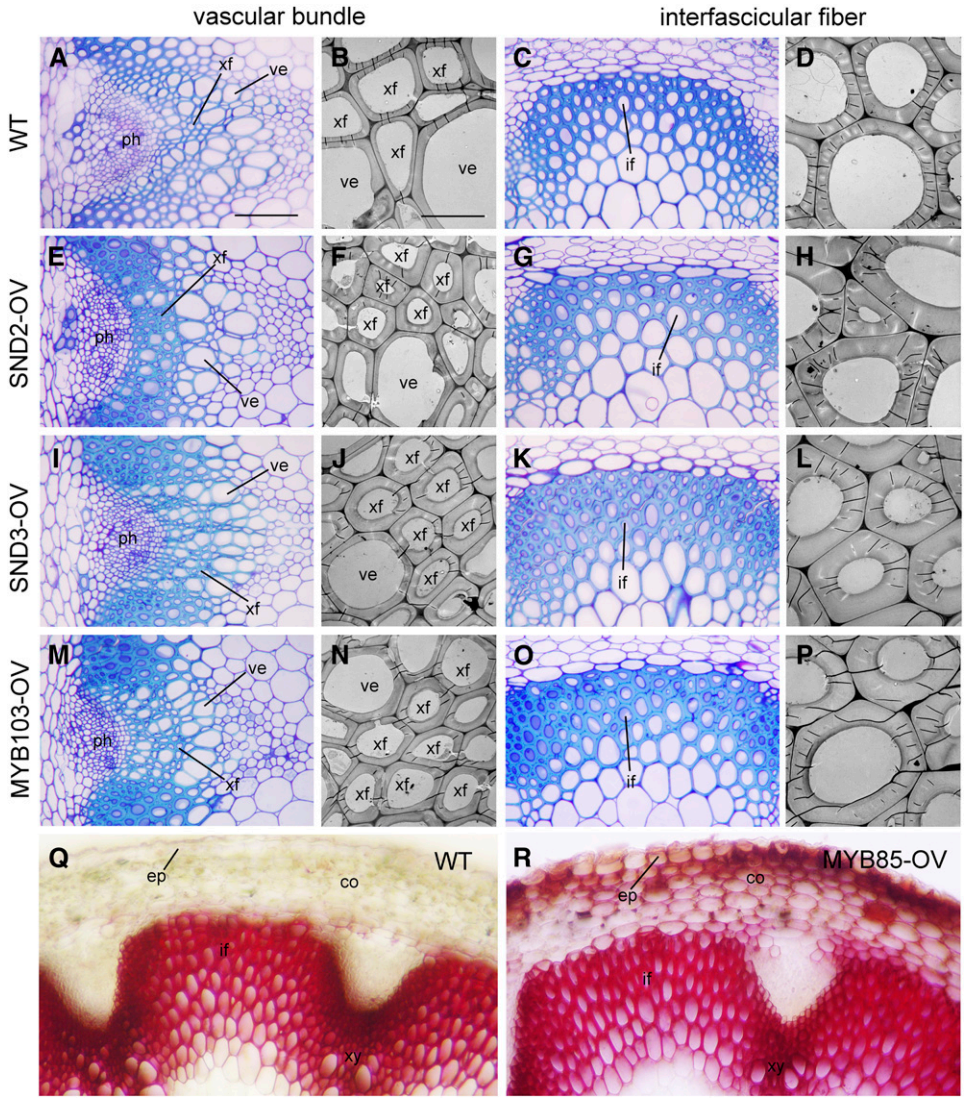

Figure 9. Effects of Overexpression of SND2, SND3, MYB103, and MYB85 on Secondary Wall Deposition.

The full-length cDNAs of *SND2*, *SND3*, *MYB103*, and *MYB85* driven by the CaMV 35S promoter were overexpressed (OV) in *Arabidopsis* plants. The bottom internodes of 8-week-old transgenic plants were examined for alterations in secondary wall deposition. co, cortex; if, interfascicular fiber; ph, phloem; ve, vessel; xf, xylary fiber; xy, xylem. Bar in **(A)** = 78 μm for the light micrographs (**[A]**, **[C]**, **[E]**, **[G]**, **[I]**, **[K]**, **[M]**, **[O]**, **[Q]**, and **[R]**), and bar in **(B)** = 8.1 μm for the transmission electron micrographs (**[B]**, **[D]**, **[F]**, **[H]**, **[J]**, **[L]**, **[N]**, and **[P]**).

(A) to **(D)** Cross sections of wild-type stems, showing secondary wall thickening in xylary fibers and vessels (**[A]** and **[B]**) and in interfascicular fibers (**[C]** and **[D]**).

(E) to **(P)** Cross sections of SND2-OV (**[E]** to **[H]**), SND3-OV (**[I]** to **[L]**), and MYB103-OV (**[M]** to **[P]**) stems, showing increased secondary wall thickening in xylary fibers (**[E]**, **[F]**, **[I]**, **[J]**, **[M]**, and **[N]**) and in interfascicular fibers (**[G]**, **[H]**, **[K]**, **[L]**, **[O]**, and **[P]**).

(Q) and **(R)** Cross sections of stems, showing ectopic lignin deposition in the epidermal and cortical cell walls of MYB85-OV **(R)** compared with the wild type **(Q)**. Lignified walls were stained (red) with phloroglucinol-HCl.

GUS reporter gene fused with the *VND6* and *VND7* genes, including a 3-kb 5′ upstream sequence, the entire exon and intron region, and a 2-kb 3′ downstream sequence, confirmed their expression in xylem but not in interfascicular fibers of inflorescence stems. Detailed examination of their expression in the xylem of stems revealed that while no expression of *VND6* and *VND7* was detected in xylary fibers, *VND6* was specifically expressed in the vessels of metaxylem (Figures 13B and 13C), and *VND7* was expressed in the vessels of both protoxylem and metaxylem (Figures 13E and 13F). It was previously reported that *VND6* and *VND7* were expressed in metaxylem and protoxylem, respectively, in primary roots (Kubo et al., 2005). *VND7* was also found to be expressed in the developing vessels of the secondary xylem in roots undergoing secondary growth (Figure 13D).

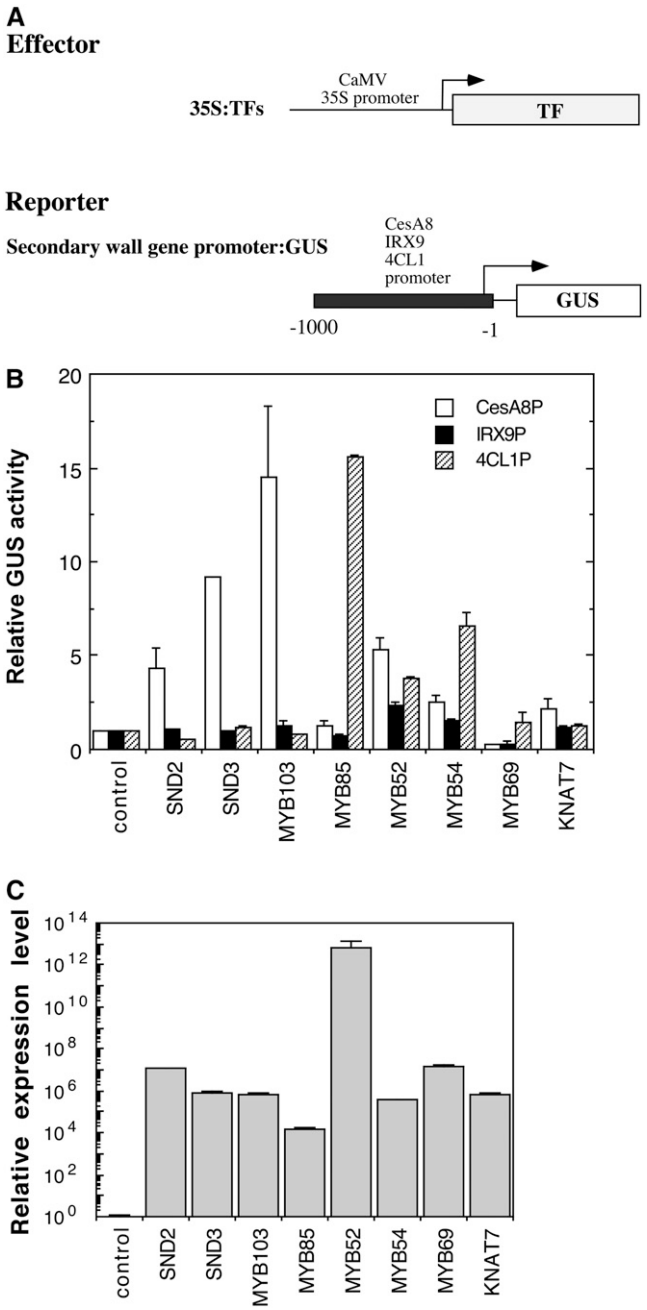

Figure 10. Induction of Secondary Wall Biosynthetic Genes by Secondary Wall–Associated Transcription Factors.

The secondary wall–associated transcription factors were coexpressed in *Arabidopsis* leaf protoplasts with the GUS reporter gene driven by the *CesA8*, *IRX9*, or *4CL1* promoter. The induction of GUS gene expression by the transcription factors was measured by assaying the GUS activity.

(A) Diagrams of the effector and reporter constructs used for the expression analysis. The effector constructs contain the secondary wall–associated transcription factors (TFs) driven by the CaMV 35S promoter. The reporter constructs consist of the GUS reporter gene driven by the promoters of three representative secondary wall biosynthetic genes, *CesA8*, *IRX9*, and *4CL1*.

VND6 expression was not detected in the secondary xylem of roots. These results suggest that, in *Arabidopsis* inflorescence stems, SND1 and NST1 are master switches activating secondary wall biosynthesis in fibers, whereas VND6 and VND7 are responsible for activating secondary wall biosynthesis in vessels.

DISCUSSION

The developmental program of secondary wall biosynthesis is activated at the end of cell elongation in some specialized cells, such as fibers and tracheary elements (Ye et al., 2002). Because fibers and tracheary elements are the principal components of wood, which is an important raw material for our daily lives, it is imperative to investigate the molecular mechanisms underlying the activation of the secondary wall biosynthetic program during wood formation. Previous studies have discovered that the secondary wall–associated *NAC* genes, such as *SND1*, *NST1*, *NST2*, *VND6*, and *VND7*, are master switches activating secondary wall biosynthesis, which opens an unprecedented avenue to uncover the transcriptional network regulating the secondary wall biosynthetic program (Zhong and Ye, 2007). Our findings that the SND1-regulated transcription factors are required for secondary wall biosynthesis and that three of them are direct targets of SND1 and its close homologs mark another important step toward our dissection of the transcriptional network regulating secondary wall biosynthesis.

SND3, *MYB103*, and *KNAT7* Are Direct Targets of SND1

We have found that SND1 is able to directly activate the expression of *SND3*, *MYB103*, and *KNAT7* without new protein synthesis, indicating that these three transcription factors are direct targets of SND1. This finding is consistent with the expression data, showing that their expression is regulated by SND1 and they are expressed in the same cell types as SND1. In addition, dominant repression of SND3, MYB103, and KNAT7 leads to a reduction in the secondary wall thickening of fibers. These results provide direct evidence that SND3, MYB103, and KNAT7, together with the previously characterized MYB46, are part of the SND1-mediated transcriptional network regulating secondary wall biosynthesis.

Although overexpression of MYB46 leads to activation of the entire secondary wall biosynthetic program, including the biosynthesis of cellulose, xylan, and lignin (Zhong et al., 2007a),

(B) Transactivation analysis showing the effects of secondary wall–associated transcription factors on the induction of the GUS reporter gene driven by the promoter of *CesA8*, *IRX9*, or *4CL1*. The expression level of the GUS reporter gene in protoplasts transfected with the reporter construct alone was used as a control and was set to 1. Error bars represent SE of three biological replicates.

(C) Real-time quantitative PCR analysis showing that the effector genes were highly expressed in the protoplasts cotransfected with the reporter and the individual effector constructs. The expression level of these transcription factors in protoplasts transfected with the reporter construct alone was used as a control and was set to 1. Error bars represent the SE of three biological replicates.

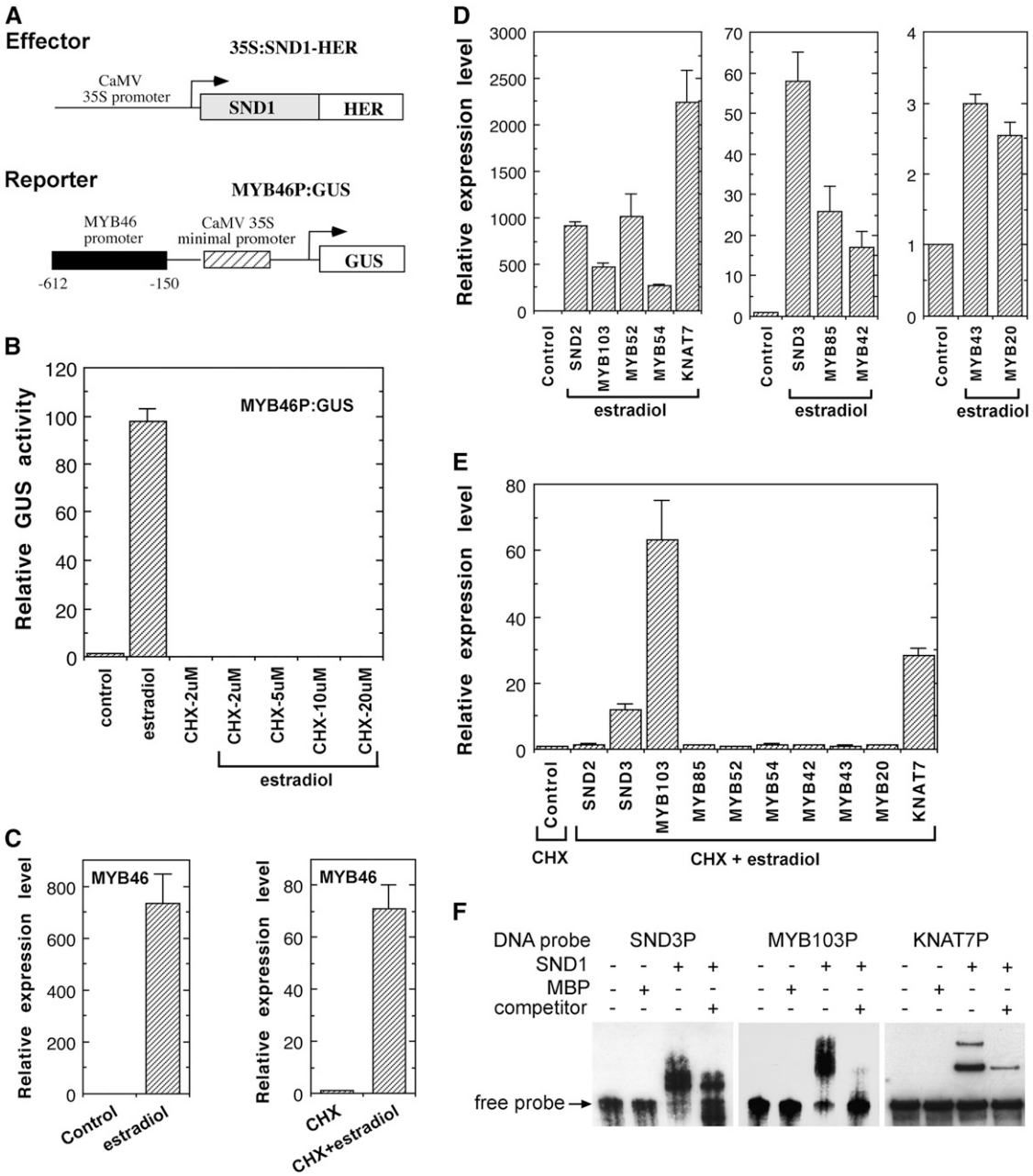

Figure 11. SND1 Directly Activates the Expression of the *SND3*, *MYB103*, and *KNAT7* Genes.

SND1 fused with the regulatory region of HER was expressed under the control of the CaMV 35S promoter in *Arabidopsis* leaf protoplasts. The protoplasts were treated with estradiol, cycloheximide (CHX), or CHX plus estradiol. Without estradiol treatment, the SND1-HER chimeric protein is inactive since it is trapped in the cytoplasm through binding to a cytoplasmic complex. When estradiol is applied, the SND1 chimeric protein is released and thus can enter the nucleus to regulate the expression of its downstream target genes. In the presence of the protein synthesis inhibitor cycloheximide, estradiol activation of SND1 can still induce the expression of its direct target genes since this induction does not require new protein synthesis. The expression of the transcription factor genes was detected by real-time quantitative PCR analysis. Error bars represent the SE of three biological replicates.

(A) Diagram of the SND1 construct and the reporter construct used for direct target analysis. The SND1 construct (35S:SND1-HER) contains SND1 translationally fused with the regulatory region of HER and is driven by the CaMV 35S promoter. The reporter construct consists of the GUS reporter gene driven by the *MYB46* promoter linked with the CaMV 35S minimal promoter sequence.

(B) Transactivation analysis showing that the estradiol-activated SND1 induced MYB46 promoter–driven GUS reporter activity, and this induction was completely abolished by addition of the protein synthesis inhibitor cycloheximide (CHX). *Arabidopsis* leaf protoplasts transfected with the reporter (MYB46P:GUS), and effector (35S:SND1-HER) constructs were treated with estradiol (2 μM), cycloheximide (2 μM), or with estradiol together with various concentrations (2, 5, 10, and 20 μM) of cycloheximide. The GUS activity in the mock-treated (control) protoplasts was set to 1.

overexpression of SND3, MYB103, and KNAT7 was unable to induce ectopic secondary wall deposition in transgenic plants. Based on the observations that dominant repression of SND3, MYB103, and KNAT7 causes a reduction in secondary wall thickening (Figure 7) and that overexpression of SND3 and MYB103 results in an increase in secondary wall thickening (Figure 9), it is tempting to propose that SND3, MYB103, and KNAT7 are part of a transcriptional complex required for activation of secondary wall biosynthetic genes. Dominant repression or overexpression of one of the components in the complex may lead to a reduction or an increase, respectively, in the activity of the transcriptional complex, thus resulting in an alteration in the level of activation of secondary wall biosynthetic genes. Because activation of the secondary wall biosynthetic program may require all the components in the transcriptional complex, expression of one transcription factor in the absence of other components is thus insufficient to induce ectopic secondary wall deposition. The involvement of a transcriptional complex consisting of multiple transcription factors synergistically binding to the promoters of target genes to activate their expression has been considered to be a general characteristic of eukaryotic transcriptional regulation (Ogata et al., 2003). Although it is currently unclear how SND3, MYB103, and KNAT7 function in the transcriptional regulation of secondary wall biosynthesis, identification of *SND3*, *MYB103*, and *KNAT7* as direct targets of SND1 moves us a step closer to elucidating the cascade of transcription factors involved in regulation of secondary wall biosynthesis.

Transcriptional Network Regulating the Secondary Wall Biosynthetic Program

We have demonstrated that SND1 regulates the expression of a number of transcription factors that are important for secondary wall biosynthesis, and among them, SND3, MYB103, and KNAT7 together with MYB46 are direct targets of SND1 (Figures 1 and 11). These findings support the hypothesis that a transcription network comprising a hierarchy of transcription factors is involved in regulation of secondary wall biosynthesis (Figure 14). In this network, SND1 acts as a master switch at the top because it is able to activate the entire developmental program of secondary wall biosynthesis (Zhong et al., 2006). *MYB46*, a direct target of SND1, apparently also functions as a key regulator albeit at another level since it is also capable of activating the whole secondary wall biosynthetic program (Zhong et al., 2007a). However, the SND1-mediated transcriptional cascade is not solely through MYB46; SND1 also directly activates the expression of at least three other transcription factors, SND3, MYB103, and KNAT7. Therefore, SND1 exerts its functions through activating several direct target genes in the transcriptional cascade leading to secondary wall biosynthesis (Figure 14).

The other SND1-regulated transcription factors appear not to be direct targets of SND1 and are likely positioned further downstream in the transcriptional network (Figure 14). Overexpression of these transcription factors does not induce ectopic deposition of secondary walls except that MYB85 overexpression results in ectopic lignin deposition (Figure 9). It is likely that some of these transcription factors are involved in enhancing or fine-tuning the level of expression of secondary wall biosynthetic genes, whereas others may be part of a transcriptional complex directly involved in regulating the expression of genes in a particular biosynthetic pathway. This possibility is consistent with the finding that, whereas SND2 is able to induce the expression of cellulose biosynthetic genes, MYB85 can induce the expression of lignin biosynthetic genes. In addition, MYB52 and MYB54 appear to slightly induce the expression of genes in all three major secondary wall biosynthetic pathways. Although individually these SND1-regulated transcription factors are not sufficient to induce ectopic deposition of secondary walls, some of them are likely involved in the normal regulation of secondary wall biosynthesis because dominant repression of SND2, MYB85, MYB52, MYB54, and MYB69 causes a significant reduction in secondary wall thickening in fibers (Figures 7 and 8). Elucidation of the positions of these transcription factors in the SND1-mediated transcriptional cascade will undoubtedly contribute to our understanding of how the expression of secondary wall biosynthetic genes is coordinately regulated.

It is intriguing to find that in addition to SND1 and its homologs, many transcription factors are involved in the regulation of secondary wall biosynthesis. Although some of these transcription factors are shown to be required for secondary wall biosynthesis, we are still far from understanding how they function in the transcriptional network and why they are all needed. Considering that a few hundred genes are probably involved in the biosynthesis of secondary walls in wood formation (Mellerowicz et al.,

Figure 11. (continued).

(C) Direct activation of *MYB46* expression by SND1. The *MYB46* gene was drastically induced by estradiol treatment in protoplasts expressing SND1-HER (left panel; the expression level in the mock-treated [control] protoplasts was set to 1). Direct activation of *MYB46* by SND1 was demonstrated by inhibition of new protein synthesis with cyclohexamide during estradiol treatment (right panel; the expression level in the CHX-treated protoplasts was set to 1).

(D) The expression of secondary wall–associated transcription factors was highly induced by estradiol treatment in protoplasts expressing SND1-HER. The expression level in the mock-treated (control) protoplasts was set to 1.

(E) The expression of *SND3*, *MYB103*, and *KNAT7* is directly activated by SND1 in the absence of new protein synthesis in protoplasts expressing SND1-HER. The expression level in the CHX-treated protoplasts was set to 1.

(F) EMSA of SND1 binding to the promoter fragments of *SND3*, *MYB103*, and *KNAT7* genes. The NAC domain of SND1 fused with maltose binding protein (MBP) was incubated with biotin-labeled promoter fragments (located between −600 and −1 relative to the start codon) and subjected to EMSA by polyacrylamide gel electrophoresis. The biotin-labeled DNA fragments were detected with the chemiluminescence method. MBP was used as a control protein. For competition analysis, unlabeled corresponding promoter fragments (competitors) in 20-fold (+) molar excess relative to the labeled probes were included in the reactions.

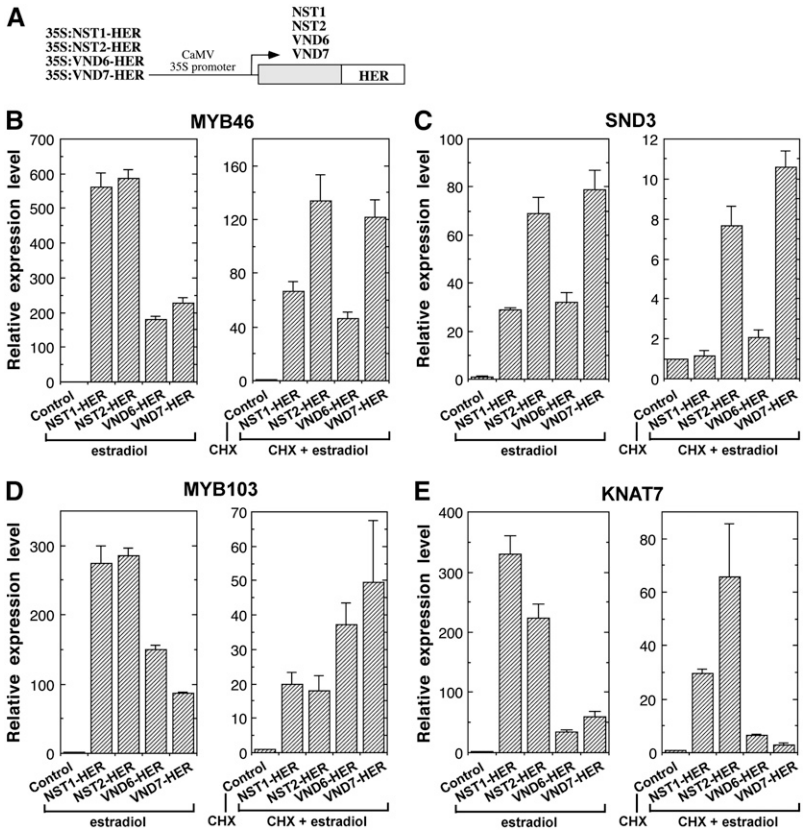

Figure 12. Direct Activation of the *MYB46*, *SND3*, *MYB103*, and *KNAT7* Genes by SND1 Homologs.

SND1 homologs, NST1, NST2, VND6, and VND7, were fused with the regulatory region of the human estrogen receptor, and the fusion proteins were expressed in *Arabidopsis* leaf protoplasts. The protoplasts were treated with CHX alone (control) or CHX plus estradiol. The expression of the transcription factor genes was detected by real-time quantitative PCR analysis. The expression level of each gene in the mock-treated (control) or CHX-treated protoplasts was set to 1. Error bars represent the SE of three biological replicates.

(A) Diagram of the NST1, NST2, VND6, and VND7 constructs used for direct target analysis. The constructs (35S:NST1-HER, 35S:NST2-HER, 35S:VND6-HER, and 35S:VND7-HER) consist of NST1, NST2, VND6, and VND7 translationally fused with the regulatory region of HER and driven by the CaMV 35S promoter.

(B) to **(E)** The expression of *MYB46* **(B)**, *SND3* **(C)**, *MYB103* **(D)**, and *KNAT7* **(E)** was induced by estradiol treatment of protoplasts expressing NST1-HER, NST2-HER, VND6-HER, and VND7-HER in the absence (left panels) or presence (right panels) of cycloheximide.

2001), it is reasonable to predict that many transcription factors are required to coordinately regulate the expression of these secondary wall biosynthetic genes. It is possible that the regulation of individual biosynthetic pathways of secondary wall components, such cellulose, xylan, and lignin, may require common and unique transcription factors. It has been suggested that some *MYB* (Patzlaff et al., 2003a, 2003b; Goicoechea et al., 2005) and *LIM* (Kawaoka et al., 2000) genes are specifically involved in regulating the expression of lignin biosynthetic genes. Among the SND1-regulated transcription factors, MYB85 overexpression leads to specific induction of lignin biosynthetic genes and ectopic lignin deposition (Figures 9 and 10), suggesting that it is involved in regulation of lignin biosynthesis. This finding is consistent with a previous report showing that pine *MYB1*, a homolog of *MYB85*, is expressed in secondary xylem of wood and its recombinant protein is able to bind to the AC element commonly present in the promoters of monolignol biosynthetic genes (Patzlaff et al., 2003b). Other SND1-regulated transcription factors, such as SND2, SND3, and MYB103, may play a role in regulation of cellulose biosynthesis, since they are able to induce the expression of cellulose biosynthetic genes when overexpressed (Figure 10). It is anticipated that further studies of secondary wall–associated transcription factors will reveal how the biosynthetic pathways of secondary wall components are coordinately regulated. It should be noted that although SND2, SND3, MYB103, and MYB85 differentially regulate the expression of secondary wall biosynthetic genes, dominant repression of these transcription factors all leads to a reduction in the amount of cellulose and xylan (Table 2). This reduction could be due to a defect in the biosynthesis of one wall component, which may result in impediment of the overall secondary wall biosynthesis and assembly. It has been shown that a reduction in any one of the three major secondary wall components (i.e., cellulose, xylan, and lignin) can cause a

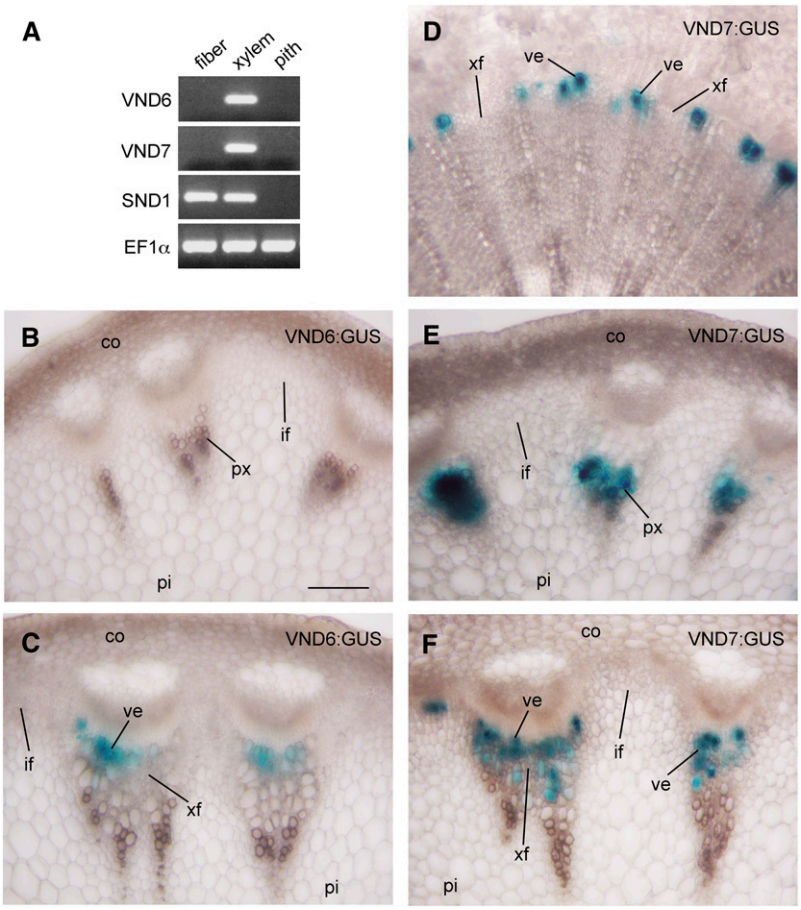

Figure 13. Expression Patterns of the *VND6* and *VND7* Genes in *Arabidopsis* Inflorescence Stems.

The expression patterns of the *VND6* and *VND7* genes were examined using RT-PCR and GUS reporter gene analyses. The *VND6* and *VND7* genes, including a 3-kb 5′ upstream sequence, the entire coding region, and a 2-kb 3′downstream sequence, were fused with the GUS reporter gene, and the expression constructs (VND6:GUS and VND7:GUS) were transformed into *Arabidopsis* plants for expression analysis. co, cortex; if, interfascicular fiber; pi, pith; px, protoxylem; ve, vessel; xf, xylary fiber. Bar in **(B)** = 56 m for **(B)** to **(F)**.

(A) RT-PCR analysis of the expression of *VND6* and *VND7* in laser-dissected cells showing their specific expression in xylem but not in interfascicular fibers or pith cells compared with *SND1* expression in both xylem and interfascicular fibers. The expression of the *EF1*α gene is shown as an internal control. Shown are the representative data from three biological replicates.

(B) Cross section of an elongating internode of VND6:GUS plants showing the absence of GUS staining.

(C) Cross section of a nonelongating internode of VND6:GUS plants showing GUS staining in developing vessels but not in xylary fibers of the metaxylem or interfascicular fibers.

(D) Cross section of a root of VND7:GUS plants showing GUS staining in developing vessels but not in xylary fibers of the secondary xylem.

(E) Cross section of an elongating internode of VND7:GUS plants showing GUS staining in vessels of the protoxylem.

(F) Cross section of a nonelongating internode of VND7:GUS plants showing GUS staining in developing vessels but not in xylary fibers of the metaxylem or interfascicular fibers.

decrease in the overall secondary wall biosynthesis (Zhong et al., 1998, 2005; Taylor et al., 2004), suggesting that the biosynthesis of secondary wall components is a highly coordinated process.

SND1 and Its Close Homologs, NST1, NST2, VND6, and VND7, Activate the Same Set of Direct Targets

We have demonstrated that NST1, NST2, VND6, and VND7, close homologs of SND1, are able to activate the same set of direct target genes as SND1, including MYB46, SND3, MYB103, and KNAT7 (Figure 12). This finding suggests that these NACs execute their functions in the same way as SND1. Because these secondary wall NACs are expressed in different types of secondary wall–containing cells and all of them are able to turn on the entire secondary wall biosynthetic program (Kubo et al., 2005; Mitsuda et al., 2005, 2007; Zhong et al., 2006, 2007b), we propose that they are functional homologs acting as master switches that regulate secondary wall biosynthesis in

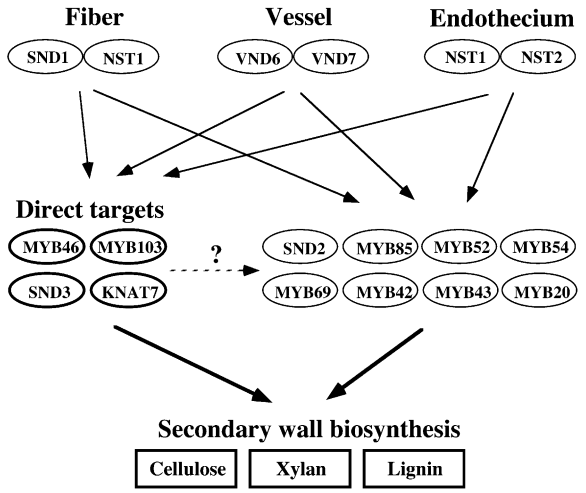

Figure 14. Diagram of the Transcriptional Network Regulating Secondary Wall Biosynthesis in Different Cell Types.

SND1, NST1, NST2, VND6, and VND7 are cell type–specific, functionally redundant master switches activating the entire secondary wall biosynthetic program. They induce the expression of the same set of downstream transcription factors. Among them, MYB46, SND3, MYB103, and KNAT7 are the direct targets of these master switches. MYB46 is also able to activate the entire secondary wall biosynthetic program. It is proposed that these master switches activate a cascade of transcription factors, which in turn induce the expression of the biosynthetic genes for secondary wall components, including cellulose, xylan, and lignin.

different cell types (Figure 14). Whereas SND1 and NST1 activate the secondary wall biosynthetic program in fibers, VND6 and VND7 specifically regulate secondary wall biosynthesis in vessels. NST1 and NST2 act together in regulating secondary wall biosynthesis in endothecium of anthers. It is apparent that plants evolved to have cell type–specific, functionally redundant NAC genes to regulate secondary wall biosynthesis in different types of secondary wall–containing cells.

Close homologs of SND1 and SND1-regulated transcription factors are found in tree species, such as poplar (*Populus trichocarpa*), pine, eucalyptus, and spruce (*Picea glauca*), and some of them were shown to be expressed in developing wood (Patzlaff et al., 2003a, 2003b; Karpinska et al., 2004; Goicoechea et al., 2005; Bedon et al., 2007), suggesting that they may also regulate secondary wall biosynthesis during wood formation. Therefore, it is likely that the transcriptional network regulating secondary wall biosynthesis is conserved between herbaceous *Arabidopsis* and woody tree species. Further studies on the transcriptional regulation of secondary wall biosynthesis in *Arabidopsis* will undoubtedly provide a foundation for elucidating the molecular mechanisms underlying wood formation in tree species. Knowledge gained from such studies will potentially allow us to manipulate the biosynthetic pathways of cellulose, xylan, and lignin by altering the expression of certain transcription factor genes.

METHODS

Gene Expression Analysis

For analysis of gene expression in different cell types, interfascicular fiber cells, xylem cells, and pith cells were isolated from inflorescence stems of 6-week-old *Arabidopsis thaliana* plants using the PALM microlaser system (PALM Microlaser Technologies), and the isolated cells were used for RNA isolation and subsequent gene expression analysis as described (Zhong et al., 2006).

For analysis of gene expression in different organs, total RNA was isolated from *Arabidopsis* seedlings, leaves, stems, flowers, and roots using a Qiagen RNA isolation kit. The seedlings used were 2 weeks old. Mature leaves were from 6-week-old plants. Mature roots were from 8-week-old plants. Stems from 6-week-old plants were divided into top, middle, and bottom parts, which represent the rapidly elongating internodes, internodes near cessation of elongation, and nonelongating internodes, respectively.

For quantitative PCR analysis, total RNA was treated with DNase I and used for first-strand cDNA synthesis. The first-strand cDNA was then used as template for real-time quantitative PCR analysis with the QuantiTect SYBR Green PCR Kit (Clontech). The relative mRNA levels were determined by normalizing the PCR threshold cycle number of each gene with that of the EF1α reference gene. The expression level of each gene in the wild-type control or in the sample with the lowest expression level was set to 1, and the data reported were the average of three biological replicates.

GUS Reporter Gene Analysis

The developmental expression patterns of the transcription factors were studied using the GUS reporter gene. Genomic DNA fragments containing a 3-kb 5′ upstream sequence, the entire coding region, and a 2-kb 3′downstream sequence were used to ensure the sequences used contained all the *cis*-elements required for the endogenous gene expression. The GUS reporter gene was inserted in frame right before the stop codon of the transcription factor genes and then cloned into the binary vector pBI101 (Clontech) to create the GUS reporter constructs. The constructs were transformed into wild-type *Arabidopsis* plants by means of *Agrobacterium tumefaciens*–mediated transformation. The first generation of transgenic plants was selected on kanamycin and used for expression analysis of the GUS reporter gene as described previously (Zhong et al., 2005).

In Situ mRNA Localization

Wild-type *Arabidopsis* inflorescence stems were fixed in 2.5% formaldehyde and 0.5% glutaraldehyde and embedded in paraffin. Sections (10 μm thick) were cut, mounted onto slides, and hybridized with digoxigenin-labeled gene-specific antisense RNA probes synthesized using the DIG RNA labeling mix (Roche). The hybridization signals were detected with alkaline phosphatase-conjugated antibodies against digoxigenin and subsequent color development with alkaline phosphatase substrates. As a negative control, sense RNA probes were hybridized with the stem sections, and no signals were observed under the same hybridization and detection conditions.

Subcellular Localization of Transcription Factors

The full-length cDNAs of transcription factors were fused in frame at the C terminus with the YFP cDNA and ligated between the CaMV 35S promoter and the nopaline synthase terminator in pBI221 (Clontech). The constructs were introduced into *Arabidopsis* leaf protoplasts by polyethylene glycol–mediated transfection (Sheen, 2001). After a 20-h incubation

period, the transfected protoplasts were examined for yellow fluorescence signals using a Leica TCs SP2 spectral confocal microscope (Leica Microsystems). Images were saved and processed with Adobe Photoshop Version 7.0 (Adobe Systems).

Transcriptional Activation Analysis in Yeast

The full-length cDNAs of transcription factors were fused in frame with the GAL4 DNA binding domain in the pAS2-1 vector (Clontech). The constructs were transformed into the yeast strain CG-1945 containing the *His3* and *LacZ* reporter genes. The transformed yeast cells were grown on SD (synthetic defined) plates with or without histidine and subjected to β-galactosidase activity assay.

Dominant Repression and Overexpression of Transcription Factors

The transcription factor dominant repression constructs were created by fusing the full-length cDNAs in frame with the dominant EAR repression sequence (Hiratsu et al., 2004), which was ligated downstream of the CaMV 35S promoter in pBI121 (Clontech). The transcription factor overexpression constructs were produced by ligating the full-length cDNAs downstream of the CaMV 35S promoter in pBI121. The constructs were introduced into wild-type *Arabidopsis* plants by *Agrobacterium*-mediated transformation (Bechtold and Bouchez, 1994). Transgenic plants were selected on kanamycin (50 mg/L), and the first generation was used for examination of phenotypes.

Microscopy

The lower part of inflorescence stems was fixed in 2% formaldehyde and embedded in Low Viscosity (Spurr's) resin (Electron Microscopy Sciences) as described (Burk et al., 2006). One-micrometer-thick sections were cut with a microtome and stained with toluidine blue for light microscopy. For transmission electron microscopy, 85-nm-thick sections were cut, post-stained with uranyl acetate and lead citrate, and observed using a Zeiss EM 902A transmission electron microscope (Carl Zeiss). Wall thickness was measured in metaxylem vessels and in the interfascicular fibers next to the endodermis. For lignin staining, 50-μm-thick sections of stems were stained with phloroglucinol-HCl, which was shown as bright red color. For each repression and overexpression construct, at least 10 transgenic plants with the most severe phenotypes were examined.

Cell Wall Composition Analysis

Inflorescence stems were ground into a fine powder in liquid nitrogen and extracted in 70% ethanol at 70°C. The resulting cell wall residues were dried in a vacuum oven at 60°C and used for analysis of monosaccharide composition following the procedure described by Hoebler et al. (1989). Briefly, cell walls were incubated with 70% sulfuric acid at 37°C for 60 min, followed by addition of inositol as the internal standard and dilution with water to 2 N sulfuric acid. After heating for 120 min at 100°C, the solution was cooled and treated with 25% ammonium solution. After reduction with sodium borohydride in dimethyl sulfoxide, the solution was heated for 90 min at 40°C, followed by sequential treatment with glacial acetic acid, acetic anhydride, 1-methylimidazole, dichloromethane, and water. The organic layer containing the alditol acetates of the hydrolyzed cell wall sugars was washed three times with water, and sugars were analyzed on an Agilent 6890N gas-liquid chromatography equipped with a 30 m × 0.25 mm (i.d.) silica capillary column DB 225 (Alltech Associates).

Transactivation Analysis in *Arabidopsis* Leaf Protoplasts

For testing the ability of transcription factors to induce the expression of secondary wall biosynthetic genes, the full-length cDNAs of transcription factors were ligated between the CaMV 35S promoter and the nopaline synthase terminator in the pBI221 vector (Clontech) to create the effector constructs. The reporter constructs were created by placing 1-kb promoter fragments (located between −1 to −1000 bp relative to the start codon) of *CesA8*, *IRX9*, and *4CL1* genes in front of the GUS reporter gene in pBI221 (Clontech). *Arabidopsis* leaf protoplasts were cotransfected with the reporter and effector constructs together with a construct containing the firefly luciferase gene driven by the CaMV 35S promoter for determination of the transfection efficiency. After a 20-h incubation, transfected protoplasts were lysed and the soluble extracts were used for analysis of GUS and luciferase activities as described (Gampala et al., 2001) or for real-time quantitative PCR analysis of the effector mRNA level. The GUS activity was normalized against the luciferase activity in each transfection, and the data were the average of three biological replicates.

Estrogen-Inducible System for Identification of Direct Targets of SND1

The full-length cDNA of *SND1* was fused to the C terminus of the regulatory region of HER (Zuo et al., 2000) and ligated between the CaMV 35S promoter and the nopaline synthase terminator in the pBI221 vector (Clontech). The SND1-HER expression construct was introduced into *Arabidopsis* leaf protoplasts alone or together with the MYB46P:GUS construct (Sheen, 2001). To activate SND1, the protoplasts were treated with 2 μM estradiol (Sigma-Aldrich) for 6 h. The control protoplasts were mock-treated with the same concentration (0.1%) of ethanol used to dissolve estradiol. To inhibit new protein synthesis, the protein synthesis inhibitor cycloheximide (2 μM) was added 30 min before addition of estradiol. Under this condition, new protein synthesis was completely inhibited, as tested by the GUS reporter activity analysis. After treatments, the protoplasts were harvested and subjected to total RNA isolation and subsequent quantitative RT-PCR analysis or used for GUS activity analysis. The expression level of each gene in the control protoplasts without addition of estradiol was set to 1, and the data were the average of three biological replicates.

EMSA

The NAC domain of SND1 fused with MBP was used in an EMSA with the promoter fragments (−600 to −1 from the start codon) of *SND3*, *MYB103*, and *KNAT7* (Zhong et al., 2007a). The promoter fragments were PCR-amplified and biotin-labeled at the 3′ end using the Biotin 3′ end DNA labeling kit (Pierce). The biotin-labeled DNA fragments were incubated for 30 min with 100 ng of SND1-MBP in the binding buffer [10 mM Tris, pH 7.5, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM $MgCl_2$, 0.05% Nonidet P-40, and 100 ng/μL poly(dI-dC)]. The SND1-bound DNA fragments were separated from the unbound ones by polyacrylamide gel electrophoresis. The DNA was electroblotted onto nitrocellulose membrane and detected by the chemiluminescent method.

Statistical Analysis

The data in the experiments of quantitative PCR, transcriptional activation of the GUS reporter gene, and measurement of cell wall thickness were subjected to statistical analysis using the Student's *t* test program (http://www.graphpad.com/quickcalcs/ttest1.cfm). The quantitative differences between two groups of data for comparison in all of these experiments were shown to be statistically significant ($P < 0.001$).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: MYB46

(At5g12870), SND1 (At1g32770), SND2 (At4g28500), SND3 (At1g28470), MYB20 (At1g66230), MYB85 (At4g22680), MYB103 (At1g63910), MYB42 (At4g12350), MYB43 (At5g16600), MYB20 (At1g66230), MYB52 (At1g17950), MYB54 (At1g73410), MYB69 (At4g33450), KNAT7 (At1g62990), NST1 (At2g46770), NST2 (At3g61910), VND6 (At5g62380), VND7 (At1g71930), ATHB8 (4g32880), and IFL1 (At5g60690).

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