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Arabidopsis VASCULAR-RELATED NAC-DOMAIN6 Directly Regulates the Genes That Govern Programmed Cell Death and Secondary Wall Formation during Xylem Differentiation [C][W]

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Xylem consists of three types of cells: tracheary elements (TEs), parenchyma cells, and fiber cells. TE differentiation includes two essential processes, programmed cell death (PCD) and secondary cell wall formation. These two processes are tightly coupled. However, little is known about the molecular mechanisms underlying these processes. Here, we show that VASCULAR-RELATED NAC-DOMAIN6 (VND6), a master regulator of TEs, regulates some of the downstream genes involved in these processes in a coordinated manner. We first identified genes that are expressed downstream of VND6 but not downstream of SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1 (SND1), a master regulator of xylem fiber cells, using transformed suspension culture cells in microarray experiments. We found that VND6 and SND1 governed distinct aspects of xylem formation, whereas they regulated a number of genes in common, specifically those related to secondary cell wall formation. Genes involved in TE-specific PCD were upregulated only by VND6. Moreover, we revealed that VND6 directly regulated genes that harbor a TE-specific *cis*-element, TERE, in their promoters. Thus, we found that VND6 is a direct regulator of genes related to PCD as well as to secondary wall formation.

INTRODUCTION

The xylem functions to transport water, nutrients, and signal molecules throughout the plant body. Xylem tissues consist of tracheary elements (TEs), xylem parenchyma cells, and xylem fiber cells. TEs function as conductive tubes to distribute water to all parts of the plant body. The rigid secondary cell walls of xylem fiber cells and TEs provide mechanical support for the plant body. To lose their cellular contents and act as a conductive tube, TEs undergo a rapid and unique type of programmed cell death (PCD) process, which is different from cell death in xylem fibers, in the final step of their differentiation (Obara et al., 2001; Courtois-Moreau et al., 2009).

PCD is a genetically regulated cell suicide process that occurs in various developmental processes in both plants and animals (Vaux and Korsmeyer, 1999; Kuriyama and Fukuda, 2002). During the differentiation of TEs, PCD is initiated by central vacuolar rupture (Obara et al., 2001). The cellular contents are degraded by hydrolytic enzymes, such as proteinases and nucleases, which are released by the vacuolar rupture (Funk et al., 2002; Ito and Fukuda, 2002). Cellular and biochemical analyses in *Zinnia* xylogenic culture have revealed a tight coupling of secondary cell wall formation and PCD in the TE-differentiating process (Fukuda, 2004). In addition, several transcriptome profiling analyses of TE differentiation have shown that genes related to PCD and secondary cell wall formation are expressed at similar times during TE differentiation (Demura et al., 2002; Milioni et al., 2002; Kubo et al., 2005), supporting a tight coupling of secondary cell wall formation and PCD in TE differentiation. Detailed analysis of the *ZINNIA CYSTEINE PROTEASE4* promoter revealed that tracheary element-regulating *cis*-element (TERE), a novel 11-bp *cis*-element, is necessary and sufficient for the immature TE-specific expression of genes (Pyo et al., 2007). TERE-like sequences are found in upstream regions of many genes related to both secondary cell wall formation and PCD in *Arabidopsis thaliana*. Therefore, TERE may be a key *cis*-element in the coordinated expression of genes related to PCD and secondary cell wall formation during TE differentiation. However, the transcription factor that binds TERE and participates in the coordinated gene expression remains elusive.

Recently, it was reported that transcription factors that belong to two similar subclasses of the NAC (NAM, ATAF1/2, and CUC2) domain proteins act as master switches of xylem cell differentiation (Kubo et al., 2005; Zhong et al., 2006; Mitsuda et al., 2005, 2007; Ko et al., 2007). *VASCULAR-RELATED NAC-DOMAIN6* (*VND6*) and *VND7*, which form a subclade with *VND1* to *VND5*, are expressed preferentially in potential metaxylem vessels and protoxylem vessels, respectively, and initiate their differentiation (Kubo et al., 2005). By contrast, NAC SECONDARY WALL THICKENING PROMOTING FACTOR (NST1) and SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1 (SND1), also known as *ARABIDOPSIS* NAC DOMAIN CONTAINING PROTEIN12 or NST3, which belong to the subclade closest to the VND family, are involved in xylem fiber cell differentiation (Zhong et al., 2006; Mitsuda et al., 2005, 2007; Ko et al., 2007). Indeed,

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nst1 snd1 double knockout plants show suppressed xylem fiber development (Mitsuda et al., 2007). Conversely, ectopic overexpression of *SND1*, *NST1*, or *NST2* induces ectopic deposition of secondary cell walls. However, apart from VND6 and VND7, these genes do not induce rapid PCD in cells.

Molecular studies of SND1 indicated that a network of transcription factors function downstream of SND1 and finally lead to secondary cell wall formation (Zhong et al., 2007, 2008; Zhou et al., 2009). SND1 directly regulates the expression of genes encoding the transcription factors MYB46, MYB83, MYB103, SND3, and KNAT7 (Zhong et al., 2007; McCarthy et al., 2009). *MYB46* and *MYB83* are specifically expressed in fiber and vessel cells, which form secondary cell walls. MYB46 and MYB83 upregulate genes involved in the biosynthesis of all three components of the secondary cell wall, namely, cellulose, xylan, and lignin. In addition to these direct targets of SND1, 10 other transcription factors have been reported to function downstream of SND1. Of them, MYB58 and MYB63, which are induced by MYB46, activate the expression of genes related to lignin biosynthesis (Zhong et al., 2008; Zhou et al., 2009). These facts suggest that SND1 functions as the master switch at the top of the hierarchy to upregulate MYB-type transcription factors, which, in turn, as the second and third regulators, upregulate the expression of genes encoding enzymes that catalyze secondary wall thickening during differentiation of xylem fiber cells. Similar regulation mechanisms for secondary wall formation are expected for VND6 and VND7 because VND6 and VND7 are able to regulate *MYB46* and *MYB83* expression (Zhong et al., 2008; McCarthy et al., 2009).

These findings lead us to the hypothesis that VND6 and VND7 directly or indirectly regulate genes that contain the TERE sequence to induce PCD and secondary wall formation in a coordinated manner. Therefore, in this study, we first sought to identify specific genes downstream of VND6 by comparing them with those downstream of SND1. To obtain precise results at high resolution, we established *Arabidopsis* suspension cell lines in which *VND6* or *SND1* was overexpressed after the addition of estrogen. Using these cell lines, we performed microarray experiments and identified many specific and common genes downstream of VND6 and SND1. Our results show that VND6 and SND1 regulate distinct aspects of xylem development, while they govern the expression of a number of genes in common, especially genes related to secondary wall formation. Furthermore, using leaf disc infiltration assays, electrophoretic mobility shift assays, and chromatin immunoprecipitation (ChIP)-PCR, we revealed that VND6 directly regulates genes harboring the TERE sequence in their promoters. Thus, we found that VND6 is a direct regulator of genes involved in PCD and secondary wall formation.

RESULTS

A Simple Gene Expression System Coupling PCD with Secondary Wall Formation

The NAC transcription factors VND6 and VND7 as well as SND1 are master regulators of the differentiation of metaxylem cells and fiber cells, respectively (Kubo et al., 2005; Zhong et al., 2006; Mitsuda et al., 2007). To understand the mechanisms by which these master regulators govern gene expression, we analyzed the gene expression profiles induced by these transcription factors. For this analysis, we used VND6, but not VND7, because VND6 preferentially functions as a homodimer, whereas VND7 functions as heterodimers with other VND proteins (Yamaguchi et al., 2008).

We recently established a novel in vitro transgenic system using *Arabidopsis* suspension cells in which *VND6-YFP* (for yellow fluorescent protein) was overexpressed after the addition of estrogen (Figure 1A; Oda et al., 2010). In this culture system, the cells harboring estrogen-inducible *VND6-YFP* synchronously deposited metaxylem-like pitted secondary cell walls 60 h after the addition of estrogen (Figure 1B). To compare the precise molecular functions of VND6 and SND1, we created a transgenic culture cell line in which *SND1-CFP* (for cyan fluorescent protein) was overexpressed after the addition of estrogen (Figure 1C). The culture cells overexpressing *SND1-CFP* deposited a secondary cell wall over the entire cell surface, without forming a pattern (Figure 1D). In plants overexpressing *VND6* or *SND1/NST3*, ectopic secondary cell wall deposits exhibited various patterns, depending on the organ evaluated. For example, overexpression of *VND6* induced secondary cell walls with a metaxylem-like pattern in roots and hypocotyls and an annular pattern in leaves (Kubo et al., 2005). Similarly, overexpression of *SND1* induced an annular pattern in leaves (Zhong et al., 2006; Mitsuda et al., 2007). These different patterns of ectopic secondary cell walls are thought to reflect cytoskeletal factors intrinsically expressed in each organ. The metaxylem-like pitted secondary cell walls seen in *VND6*-overexpressing cultured cells and the secondary cell walls lacking a pattern in *SND1*-overexpressing cultured cells resemble metaxylem vessel cells and fibers, respectively. We confirmed that *VND6*-overexpressing cells with pitted secondary cell walls lost their cellular contents in mature, as judged by 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) staining, indicating that they had differentiated into metaxylem vessel cells by means of PCD (Figure 1E). By contrast, *SND1*-overexpressing cells with secondary cell walls maintained vacuoles stained by BDECF, indicating that PCD was not induced in the *SND1*-overexpressing cells (Figure 1F). Both transgenic suspension cell lines were of high purity. About 50% of *VND6* transgenic cells showed a YFP signal, and 30% of *SND1* transgenic cells showed a CFP signal in their nuclei 24 h after the addition of estrogen (Figure 1G). In correspondence with the high frequency of transgenic cells, cells with secondary walls, as judged by wheat germ agglutinin (WGA) staining (Wojtaszek and Bolwell, 1995), comprised ~40% of the cultures 36 h after the addition of estrogen in both the *VND6*- and *SND1*-overexpressing cell cultures (Figure 1H). At 48 to 60 h, ~50 to 60% of cultured cells differentiated into xylem cells with secondary walls. These observations indicate that the in vitro culture systems reflect processes of xylem cell differentiation in planta and are appropriate for the analysis of xylem cell development. In particular, the high synchrony and rapid induction of differentiation in nondifferentiating cells allowed us to analyze early programs of gene expression induced by VND6 and SND1 in relation to xylem cell differentiation.

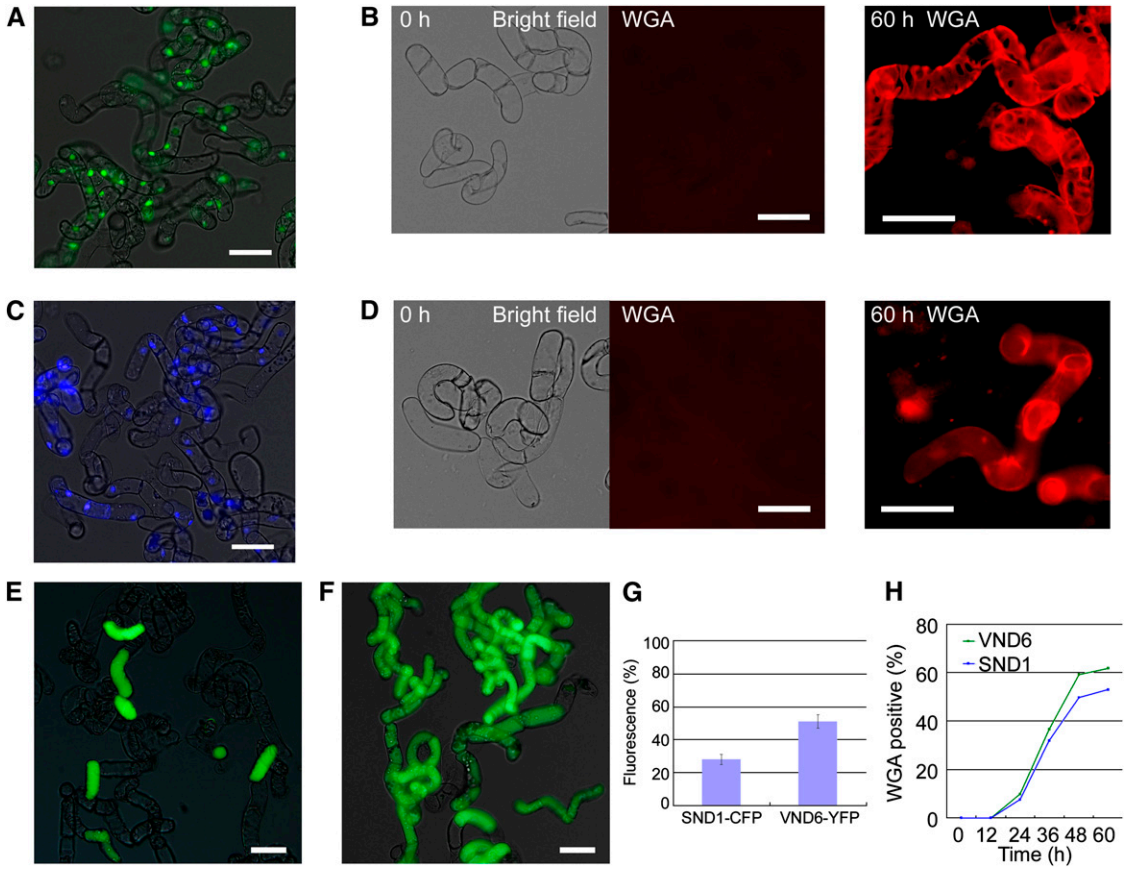

Figure 1. In Vitro Culture Systems Used in This Study.

(A) *VND6-YFP*–inducible transgenic cell line 24 h after the addition of estrogen. The image is an overlay of bright-field and fluorescence microscopy. Green fluorescence indicates expression of *VND6-YFP*.
(B) Secondary cell walls stained with WGA–AlexaFluor 596, 0 and 60 h after induction of *VND6-YFP*.
(C) *SND1-CFP*–inducible transgenic cell line 24 h after the addition of estrogen. The image is an overlay of bright-field and fluorescence microscopy. Cyan fluorescence indicates expression of *SND1-CFP*.
(D) Secondary cell walls stained with WGA–AlexaFluor 596, 0 and 60 h after induction of *SND1-CFP*.
(E) BCECF-stained vacuoles 108 h after *VND6-YFP* induction. The image is an overlay of bright-field and fluorescence microscopy.
(F) BCECF-stained vacuoles 108 h after *SND1-CFP* induction. The image is an overlay of bright-field and fluorescence microscopy.
(G) Percentage of transgenic cells containing fluorescence from SND1-CFP or VND6-YFP in their nuclei 24 h after induction. Error bars indicate SD; $n = 3$.
(H) Differentiation time course of xylem vessels and fibers.
Bars = 50 μm.

Identifying the Genes Downstream of VND6 and SND1 with Microarray Experiments

To investigate the target genes regulated by VND6 or SND1, we performed microarray experiments with the suspension-cultured cells we had established. Because the secondary cell wall deposition in *VND6* transgenic cells was first observed 18 h after the addition of estrogen, we decided to examine the initial molecular events induced by VND6 and SND1 12 h after the addition of estrogen, when genes involved in the morphological change are expected to be expressed. As a control, an *Arabidopsis* suspension cell culture (wild type), which was the origin of the *VND6*-harboring and *SND1*-harboring cell lines, was used (Gene Expression Omnibus accession number GSE20586). Expression levels for each gene at 0 and 12 h after the addition of estrogen were compared. Next, a one-way analysis of variance was performed to identify significant expression differences among the cultures. A total of 178 genes showed statistically significant changes ($P < 0.01$, $q < 0.10$), with over a fivefold change, in VND6-induced cells or SND1-induced cells. Of them, 98 genes were upregulated in both cell lines (see Supplemental Data Set 1 online; Figure 2A). However, 47 and 33 genes were identified as being upregulated preferentially by the induction of *VND6* (see Supplemental Data Set 2 online; Figure 2B) and of *SND1* (see Supplemental Data Set 3 online; Figure 2C), respectively. Our results indicated that, although VND6 and SND1 each

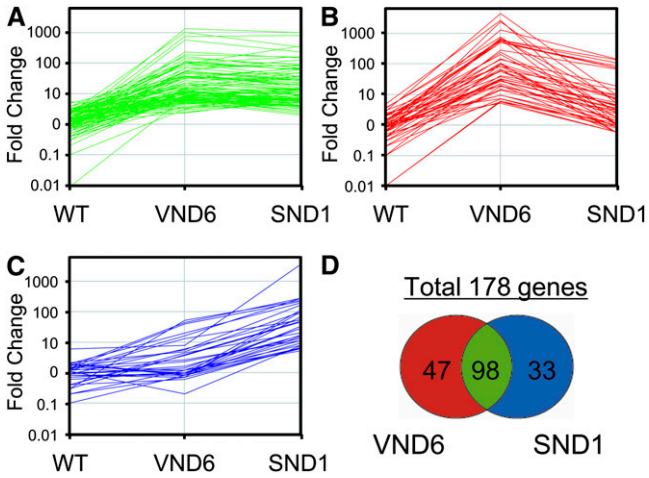

Figure 2. Summary of Microarray Results.

(A) Overview of fold changes of expression of genes identified as being downstream of VND6 and SND1. WT, wild type.

(B) Overview of fold changes of expression of genes downstream of VND6.

(C) Overview of fold changes of expression of genes downstream of SND1.

(D) Venn diagram of genes expressed downstream of VND6 and SND1.

had numerous distinctive targets, about half of the genes downstream of VND6 and SND1 overlapped (Figure 2D).

Many genes related to secondary cell wall synthesis were induced by both VND6 and SND1. In particular, genes encoding cellulose synthases that function specifically in the synthesis of the secondary cell wall, including *CESA4*/*IRX5*, *CESA7*/*IRX3*, and *CESA8*/*IRX1* (Taylor et al., 2003), were upregulated by both VND6 and SND1 (see Supplemental Data Set 1 online). Secondary wall synthesis is regulated by a number of MYB transcription factors (Zhong et al., 2007, 2008; Zhou et al., 2009). In our experiments, *MYB46*, *MYB83*, and *MYB103* were upregulated by VND6 and SND1 (see Supplemental Data Set 1 online). Because these three MYB transcription factors are known to regulate secondary cell wall formation (Zhong et al., 2007, 2008; McCarthy et al., 2009), they may be involved in processes of secondary wall formation that are common to xylem vessels and fibers. In addition, seven genes associated with the cytoskeleton, such as genes encoding tubulin, kinesin, and myosin, were upregulated by both VND6 and SND1. Many genes involved in vesicle transport, such as Rab GTPases, were also upregulated by both proteins. These facts indicate that both xylem vessel and fiber differentiation require dynamic changes in both the cytoskeleton and vesicle transport.

The most remarkable difference was that VND6 induced many genes that encode degradative enzymes, such as *XCP1* (for *xylem cysteine protease1*), *XCP2*, *XSP1* (for *xylem serine protease1*), *RNS3* (for *ribonuclease3*), *ATMC9* (for *Arabidopsis thaliana metacaspase9*), lipase (*At4g18550*), *ATSBT1.1* (for *Arabidopsis thaliana subtilase 1.1*), and nucleoside phosphatase family protein (*At1g14240*) (see Supplemental Data Set 2 online). Of the proteins encoded by these genes, XCP1 and XCP2 were suggested to participate in autolysis of PCD during differentiation of xylem TEs (Zhao et al., 2000; Avci et al., 2008). Although it has not been confirmed that the other genes function in TE-specific PCD, they are thought to be involved in PCD because, like *XCP1* and *XCP2*, their expression levels were strikingly changed only in response to VND6 induction. In addition, multiple genes for a leucine-rich repeat receptor-like kinase (five genes), kinesin motor protein (two genes), LBD transcription factor (two genes), and peroxidase (two genes) were listed as being expressed preferentially following induction of VND6. Furthermore, some other genes, including genes encoding a polygalacturonase (PG; At1g70500), plastocyanin-like domain-containing protein (At5g26330), and an unknown protein (At3g52900) exhibited striking changes in expression.

By contrast, SND1 preferentially induced genes encoding enzymes related to monolignol biosynthesis, such as *PAL1* (for *phenylalanine ammonia-lyase1*), *CCoAOMT* (for *caffeoyl-CoA 3-O-methyltransferase*), and *4CL3* (for *4-coumarate 3*; see Supplemental Data Set 3 online). Lignins are synthesized from the oxidative coupling of monolignols. While the oxidative coupling-related genes, such as peroxidases and laccases, were upregulated in response to both VND6 and SND1, the genes related to the synthesis of hydroxycinnamyl alcohols were upregulated only in response to SND1. Induction of *SND1* also induced cell wall synthesis-related genes, namely, *IRX9*, *CSLB02*, and *FLA12*. IRX9 is required for xylan synthesis in the secondary cell wall (Brown et al., 2007). *CSLB02* is annotated as a cellulose synthase-like gene. *FLA12* encodes a fasciclin-like arabinogalactan protein, which is thought to have a role in the formation of the secondary cell wall (Ito et al., 2005). Changes in the levels of all of these genes in *SND1*-overexpressing cells were much larger than those in *VND6*-overexpressing cells, suggesting that these genes play a role in the formation of the secondary cell wall, especially in fiber cells. In addition, SND1 caused striking changes in the levels exhibited by some other genes, including those encoding *FLA7*, GDSL-motif lipase (*At1g54790*), and an unknown protein having the DUF579 motif (*At3g50220*).

MYB Genes Induced by VND6, SND1, or Both VND6 and SND1

In addition to *MYB46*, *MYB83*, and *MYB103*, many MYB genes were up- or downregulated by the overexpression of *VND6*, *SND1*, or both (see Supplemental Data Set 4 online). MYB58 and MYB63 regulate the lignin biosynthetic pathway (Zhou et al., 2009). MYB52, MYB54, MYB85, MYB42, MYB43, MYB69, and MYB20 are suggested to be involved in the regulation of secondary cell wall synthesis (Zhong et al., 2008). Of these, the expression of *MYB63*, *MYB52*, and *MYB54* was highly induced by *SND1* overexpression, whereas changes in the expression of the other genes were relatively small. These results suggest that *MYB63*, *MYB52*, and *MYB54* are relatively close downstream target genes of SND1. In addition, we identified five previously unidentified MYBs and MYB-related genes whose expression was promoted by VND6 and SND1. The expression of *MYB25* was upregulated preferentially by VND6. The expression of *MYB63*, *MYB52*, *MYB54*, and *At3g10590* was induced by *SND1* overexpression. Because *MYB63* positively regulates the lignin biosynthetic pathway (Zhou et al., 2009), SND1-induced *MYB63*

expression may contribute to the SND1-specific induction of *PAL1*, *CCoAOMT*, and *4CL3* expression.

Expression of Genes That Are Preferentially Induced by VND6

Genes that we have identified as being preferentially induced by VND6 but not SND1 are thought to be candidate players involved in vessel differentiation. Therefore, we generated chimeric genes consisting of the β-glucuronidase (GUS) reporter gene and the region 2.0 kb upstream of three of the candidate genes, which had not been analyzed in relation to vessel differentiation yet (Figure 3). We selected *ATMC9*, which encodes an Arg/Lys-specific Cys protease, from the degradative enzyme (putative PCD) category, polygalacturonase (At1g70500) from the cell wall category, and one unknown protein (At3g52900) that was strikingly induced by VND6. GUS staining in plants expressing *ATMC9* was observed only in the developing vasculature of whole plants (Figures 3A and 3B). In roots, *ATMC9* was expressed in developing protoxylem and metaxylem vessels but not in fully differentiated vessels. Expression of polygalacturonase was observed in differentiating vessels and stomata (Figures 3C and 3D), and expression of At3g52900 was observed in developing vessels in roots and leaves as well as stipules (Figures 3E and 3F). All three genes showed strong expression in developing vessels. These results suggest that our microarray analysis to identify genes downstream of VND6 could enrich for genes that function in vessel differentiation.

Quantitative RT-PCR Analysis

To examine whether the identified genes are direct downstream targets of VND6, SND1, or both VND6 and SND1, we performed quantitative RT-PCR analysis using cultured cells for several representative genes of PCD, monolignol synthesis, and secondary cell formation at 0, 3, and 6 h after the addition of estrogen. *VND6*- and *SND1*-overexpressing cells significantly induced the expression of *VND6* and *SND1*, respectively, at 3 h after the addition of estrogen (Figures 4A and 4B). At 6 h, the level of transcripts for *VND6* and *SND1* increased over 2000- and 400-fold, respectively. By contrast, VND6-induced transcription factor genes, including *LBD15*, *LBD30*, and *At5g07310*, were not expressed until 6 h after the addition of estrogen (see Supplemental Figure 1 online). Therefore, it is most likely that genes that are upregulated 3 h after the addition of estrogen may be direct targets of VND6. The RT-PCR analysis confirmed that expression of *XCP1* and *XCP2* was induced only in *VND6*-overexpressing cells, but not in wild-type cells or *SND1*-overexpressing cells (Figures 4C and 4D). Because the induction of *XCP1* and *XCP2* occurred at as early as 3 h, *XCP1* and *XCP2* may be direct targets of VND6. The expression of *MYB46*, *MYB83*, and *CESA4* transcripts increased 6 h after the induction of both *VND6*- and *SND1*-overexpressing cells (Figures 4G to 4I). In particular, increases in the *CESA4* and *MYB46* transcripts were striking. The increase in *MYB46* expression started at as early as 3 h after induction. These results suggest that distinctive genes related to secondary cell wall formation, including *CESA4* and *MYB46*, may be direct targets of both VND6 and SND1. Although the transcript levels of *PAL1* and *4CL3* increased 6 h after the addition of estrogen in *SND1*-overexpressing cells, these changes were much less than those of other genes examined (Figures 4E and 4F). Therefore, *PAL1* and *4CL3* might be indirect targets of SND1 and induced by an SND1-induced transcription factor.

Taken together, our findings suggest that although both VND6 and SND1 regulate secondary cell wall formation with some differences, VND6 and SND1 have unique functions: to upregulate gene expression related to rapid PCD and lignin monomer synthesis, respectively.

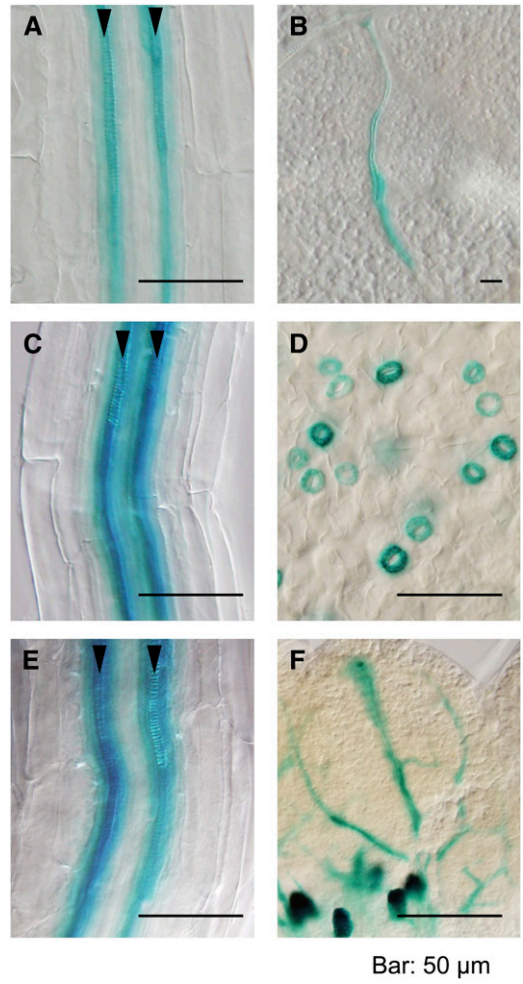

Figure 3. Expression Patterns of VND6-Induced Genes, as Determined by GUS Reporter Assays.

Arabidopsis seedlings harboring a chimeric gene consisting of the region 2.0 kb upstream of the VND6-induced gene fused to a GUS reporter gene were grown for 7 d. Arrowheads indicate vessel cells. Bars = 50 μm.
(A) and **(B)** Expression patterns of *ATMC9* in roots **(A)** and in the first true leaf **(B)**.
(C) and **(D)** Expression patterns of a *PG* gene (At1g70500) in roots **(C)** and in a cotyledon **(D)**.
(E) and **(F)** Expression patterns of a gene encoding an unknown protein (At3g52900) in a root **(E)** and in true leaves and stipules **(F)**.

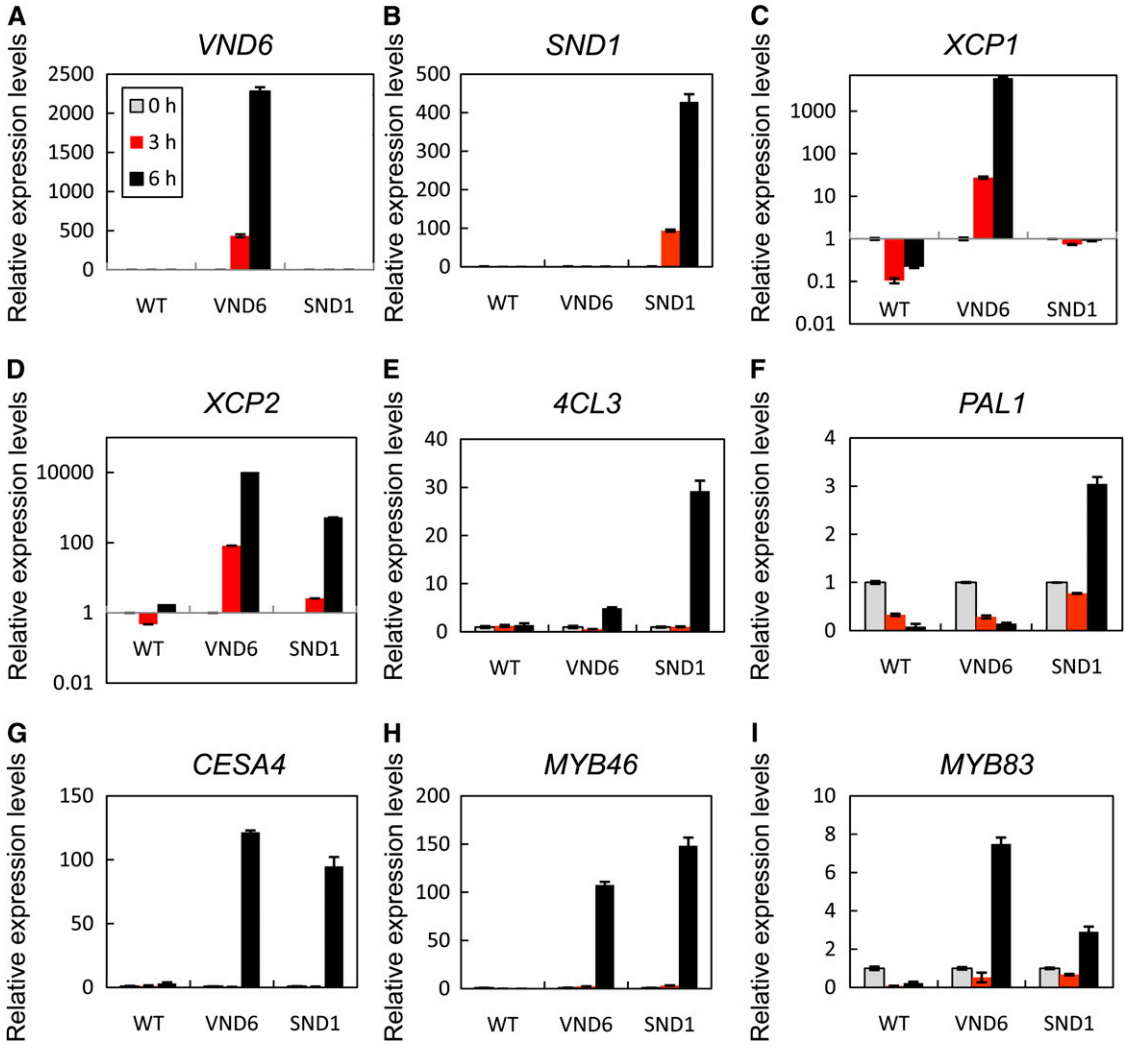

Figure 4. Quantitative RT-PCR Analysis of Genes Downstream of VND6, SND1, or Both VND6 and SND1.

Bars show relative expression levels for each gene in three genotypes: wild-type (WT) cells, VND6-induced cells, and SND1-induced cells at 0 h (gray), 3 h (red), and 6 h (black) after the addition of estrogen. *VND6* **(A)**, *SND1* **(B)**, *XCP1* **(C)**, *XCP2* **(D)**, *4CL3* **(E)**, *PAL1* **(F)**, *CESA4* **(G)**, *MYB46* **(H)**, and *MYB83* **(I)**. Note that *y* axes in **(C)** and **(D)** indicate log scale because changes in their gene expression were very wide. Error bars indicate SD; $n = 3$.

TERE Is a Direct Target of VND6

The results from the microarray and quantitative RT-PCR analyses suggest that *XCP1*, *XCP2*, *CESA4*, and *MYB46* may be direct targets of VND6. We identified a *cis*-element (TERE) that is responsible for TE-specific expression (Pyo et al., 2007). The TERE core sequence is CTTNAAAGCNA. We searched for the TERE sequence in the region 1000 bp upstream of the start of the 5′ untranslated region of genes that were upregulated by VND6, SND1, or both and found that genes induced by VND6, such as *XCP1*, *XSP1*, and *PG*, had a TERE sequence in their upstream regions (see Supplemental Data Set 2 online). The TERE sequence was found in the upstream regions of several VND6-regulated genes (8/47, 17.0%), was absent upstream of genes that were regulated by SND1 but not by VND6 (0/33, 0%), and occurred in the upstream regions of a few genes that were regulated by both SND1 and VND6 (3/98, 3.1%). Interestingly, half of the genes that were classified as encoding degradative enzymes (with a putative role in PCD) had the TERE sequence in their upstream regions (4/8). In addition, we determined the frequency of the TERE sequence in the 1000-bp upstream region of all *Arabidopsis* genes and found that 1.39% (463/33282) of genes have a TERE sequence in their 1000-bp upstream regions. These results indicate that genes upregulated by VND6 have a TERE sequence in their upstream regions at a significantly higher percentage than do genes in the entire *Arabidopsis* genome. This suggests that VND6 binds to TERE sequences and directly upregulates genes containing this sequence.

To test this hypothesis, we examined the binding activity of VND6 to the *XCP1* promoter, which contained the complete

TERE sequence at –122 to –112 bp upstream of the start codon. The interaction between VND6 and the *XCP1* promoter with (ProXCP1-130) and without (ProXCP1-100) the TERE sequence was examined using the *Nicotiana benthamiana* infiltration assay (Figure 5A). Leaf discs were infiltrated with *VND6* and either of the two *XCP1* promoter:intron GUS constructs as an effector and a reporter, respectively. When the expression of *VND6* was induced by estrogen, ectopic transdifferentiation of epidermal cells into TEs occurred in both the ProXCP1–130 and the ProXCP1–100 leaf discs, confirming active function of the introduced VND6 in both of the leaf discs (Figures 5B to 5E). However, VND6 induced GUS activity only in the ProXCP1-130 leaf discs, but not in the ProXCP1-100 leaf discs (Figures 5B to 5E). To test whether the 130-bp region upstream of *XCP1* was sufficient to trigger the *XCP1* promoter activity responsible for TE-specific expression, we generated transgenic plants harboring the 130-bp region upstream of *XCP1* fused to the sequence encoding the YFP marker and a nuclear localization signal (Pro*XCP1*-130:*YFP-NSL)*. The YFP signal was observed specifically in developing vessel cells in roots (Figure 5F). These results suggest that the TERE sequence may be a *cis*-element that is recognized by VND6 and thereby triggers the TE-specific expression of genes. To investigate the specific interaction between VND6 and the TERE sequence, we quantified GUS activity driven by ProXCP1-130 in transgenic leaf discs overexpressing VND6 or SND1 (Figure 5G). While VND6 induced significant GUS activity, SND1 did not (Figure 5H, left). To determine if the TERE sequence is recognized by VND6, we produced four types of mutated ProXCP1-130 constructs, ProXCP1-130(CTT), ProXCP1-130 (AA), (ProXCP1-130(GC), and ProXCP1-130(CA), containing

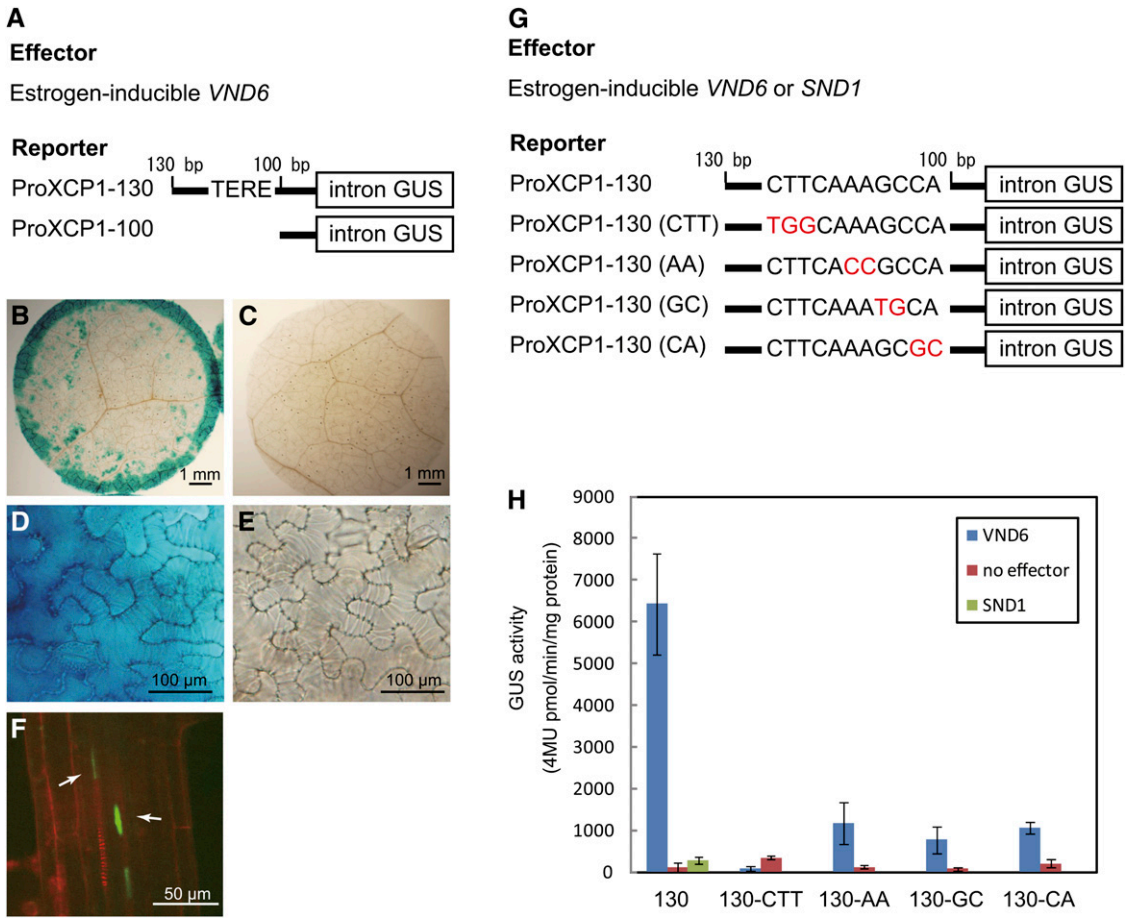

Figure 5. GUS Assay of the Interaction between TERE and VND6.

(A) Design of the infiltration assay in *N. benthamiana*. Estrogen-inducible *VND6* was used as an effector. ProXCP1-130, which contained the TERE sequence, and ProXCP1-100, which lacked the TERE sequence, were used as reporters.

(B) and **(C)** GUS-stained leaf discs infiltrated with the effector and the ProXCP1-130 reporter **(B)** or ProXCP1-100 reporter **(C)**.

(D) and **(E)** Magnified image of **(B)** and **(C)**, respectively.

(F) Expression pattern of ProXCP1-130:YFP-NLS in a root. Arrows indicate the YFP-NLS signal in vessels.

(G) Diagram of GUS activity assays. ProXP1-130 (CTT), ProXCP1-130 (AA), ProXCP1-130 (GC), and ProXCP1-130 (CA) contained two or three nucleotide substitutions in the TERE sequence.

(H) GUS activity assays performed on leaf discs infiltrated with combinations of an effector and a reporter as shown in **(G)**. Error bars indicate SD; $n = 3$.

two or three nucleotide substitutions in TERE (Figure 5G). These constructs were introduced into the *N. benthamiana* leaf discs together with the *VND6* construct (Figure 5H). ProXCP1-130 (CTT), which has substitutions in the first three nucleotides of the TERE sequence, did not exhibit GUS activity. Leaf discs harboring other mutated ProXCP1-130 constructs (AA), (GC), or (CA) exhibited 10 to 20% of the GUS activity of the wild-type 130-bp promoter (Figure 5H). These results suggest that TERE is a *cis*-element required for VND6 recognition and that the first three nucleotides are most important for the recognition.

We used ChIP-PCR to confirm that genes containing the TERE sequence are directly regulated by VND6 (Figure 6). In vitro–cultured cells in which *VND6-YFP* had been overexpressed for 24 h were fixed with formaldehyde, and chromatin was prepared from the cells. Chromatin DNA was fragmented, and VND6 binding fragments were enriched by immunoprecipitation with an anti-GFP antibody, which had a high affinity for GFP protein. We examined whether the promoter sequence of TE-related genes that have a TERE sequence, including *XCP1*, *ATMC9*, *ATSBT1.1*, *PG*, and *CESA4*, were enriched in the immunoprecipitated fraction. We also included *XCP2*, which encodes a PCD-related enzyme, and *MYB46*, which encodes a key transcription factor downstream of VND6, in our assay. The sequences upstream of *XCP2* and *MYB46* do not contain a perfect TERE sequence but do contain similar sequences, CTCTAAAGCAA and ATTGTAAGCAA, respectively. Fragments of the *XCP1*, *XCP2*, *ATMC9*, *ATSBT1.1*, *MYB46*, *CESA4*, and *PG* promoter were significantly enriched in an extract from cells in which *VND6-YFP* was overexpressed relative to those from cells in which *VND6-YFP* expression had not been induced (Figure 6). The 450-bp sequence upstream of TERE in the *XCP1* promoter showed lower enrichment than did the *XCP1* promoter that included TERE. By contrast, the *ATHB-15/CNA* promoter, which directs procambium-specific expression of genes (Ohashi-Ito and Fukuda, 2003) and does not contain TERE, was not enriched in an extract from *VND6-YFP*–overexpressing cells (Figure 6). These findings suggest that VND6 binds to TERE and directly upregulates TE-specific genes related to not only transcription factors but also enzymes for both PCD and secondary wall formation.

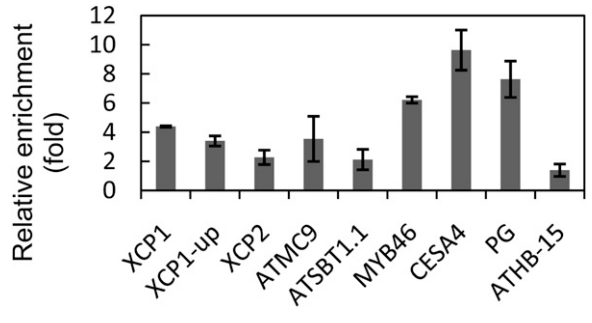

Figure 6. Real-Time ChIP-PCR Analysis to Evaluate the Interaction between the TERE Sequence and VND6.

Fragments of promoters of *XCP1*, XCP2, *ATMC9*, *ATSBT1.1*, *MYB46*, *CESA4*, *PG*, *ATHB-15*, and *XCP1-up* (a fragment of the *XCP1* promoter from which the TERE-like sequence was removed; the section 450 bp upstream of the TERE segment) were subjected to ChIP assays. Error bars indicate SD; $n = 3$.

To further examine the direct binding of VND6 to TERE, we performed electrophoretic mobility shift assays (EMSAs) using the recombinant glutathione *S*-transferase (GST)-VND6 protein and a 24-bp promoter fragment of *XCP1* that included TERE (Figure 7A). Upon binding of VND6, the bands corresponding to the *XCP1* fragments shifted. The amount of shifted band increased in proportion to the amount of VND6 added. However, VND6 did not bind to a mutant fragment of *XCP1* that included three nucleotide substitutions at the first three positions of TERE (mt-XCP1; Figure 7B). We also tested six different TERE-like sequences from *MYB46*, *CESA4*, *PG*, *XCP2*, *ATMC9*, and *ATSBT1.1* using EMSA (Figure 7B). VND6 was able to bind to fragments of *CESA4*, *PG*, *ATMC9*, and *ATSBT1.1* but not to those of *MYB46* and *XCP2* (Figure 7C). The first three nucleotides of the TERE-like sequence were not conserved in *MYB46* and *XCP2* (Figure 7B). These results indicate that VND6 directly binds to the upstream region of *XCP1*, *CESA4*, *PG*, *ATMC9*, and *ATSBT1.1* through TEREs and that the first three nucleotides of TERE are critical for the binding. *MYB46* and *XCP2* are likely regulated by VND6 directly but not through TERE.

DISCUSSION

In Vitro Suspension Cell Culture Systems Harboring an Estrogen-Inducible Master Gene Are Suitable for Studying a Hierarchical Gene Expression Network

In this work, we identified the genes that act downstream of VND6 and SND1 and their targets. To do this, we employed *Arabidopsis* suspension-cultured cells harboring master genes whose expression is controlled by an estrogen-inducible system. The combination of this gene-inducible system and *Arabidopsis* suspension culture was efficient for elucidating the genes downstream of VND6 and SND1. These systems were thought to be excellent resources for the analysis of not only hierarchical gene expression networks but also of many aspects of molecular biological processes for the following reasons. (1) Suspension culture provides sufficient amounts of materials needed for various analyses. (2) Gene expression in the system is synchronously induced in the majority of the cell population, which has homogeneous characteristics. Therefore, we can follow stage-specific events, including hierarchical gene expression profiles, in high resolution. (3) This conditional gene-inducible system allows us to analyze genes that cause cell death or severe defects in plants. Indeed, although PCD occurred in *VND6*-expressing cells treated with estrogen, the cells not exposed to estrogen grew healthily and normally. Thus, transformed cells can be maintained as a cultured line for a long time.

Regulation of Hierarchical Gene Expression by VND6 and SND1

Here, we revealed the differences and commonalities of downstream gene regulation by VND6 for vessel differentiation and by SND1 for fiber differentiation using VND6- and SND1-induced

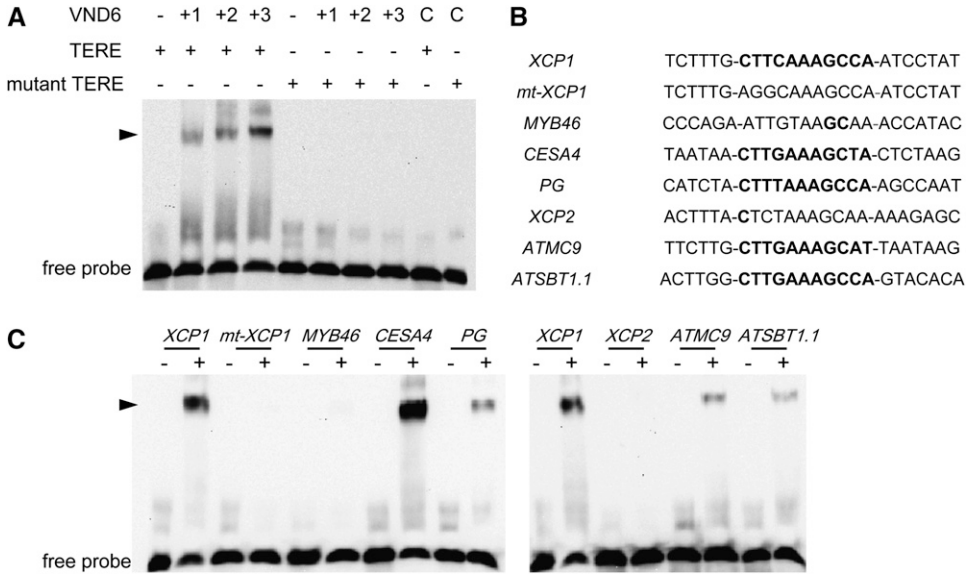

Figure 7. EMSAs of VND6 Binding to the TERE Region of *XCP1* Promoter Fragments.

(A) EMSAs using the GST-VND6 NAC domain protein and the TERE of *XCP1* or a mutated version of the TERE of *XCP1*. +1, +2, and +3 indicate the amount of protein (+2 means twofold of +1; +3 means threefold of +1). C indicates a negative control using GST-CLV1 instead of GST-VND6. An arrowhead indicates shifted bands.

(B) Sequences used in the EMSAs. TERE sequences recognized by VND6 are indicated as bold letters. Mt *XCP1*, a mutant version of *XCP1* in which the first three nucleotides of TERE were substituted with AGG.

(C) EMSAs using various TERE sequences and the VND6 protein (+). Arrowhead indicates shifted bands. −, Absence of VND6.

cultured cell lines. VND6 and SND1 regulated a number of genes in common, especially those related to secondary cell wall formation. Specific genes downstream of VND6 encode enzymes involved in PCD, such as enzymes for autolysis. Genes encoding enzymes that catalyze lignin monomer biosynthesis were preferentially upregulated by SND1.

Some plant master transcription factors induce a hierarchical gene expression network (Ito et al., 2004; Zhong et al., 2008). Microarray experiments revealed that VND6 and SND1 also induce a hierarchical gene expression network. These master transcription factors induce the expression of genes that encode other transcription factors, such as *MYB46*, *MYB83*, and *MYB103*, as well as genes for various enzymes related to secondary wall formation, including lignification and PCD. We demonstrated that genes encoding transcription factors such as *MYB46*, which are ranked higher in the gene expression hierarchy, and genes for enzymes such as *XCP1* and*CESA4*, which are ranked lowest, are both direct targets of VND6. Similarly, it has been reported that genes both for transcription factors such as *MYB46*, *MYB83*, *MYB103*, *SND3*, and *KNAT7* and for enzymes such as *4CL1* are direct targets of SND1 (Zhong et al., 2008; McCarthy et al., 2009). MYB46 and MYB83 are known to regulate redundant biosynthetic pathways for all three major secondary wall components: cellulose, lignin, and xylan (Zhong et al., 2007; McCarthy et al., 2009). Therefore, the two NAC transcription factors are thought to regulate genes that, on the one hand, encode enzymes that are directly related to xylem differentiation and, on the other, function indirectly to induce other transcription factors, such as MYBs.

VND6 Regulates a Part of PCD Specifically and Directly

The differentiation of vessel cells is the result of an orchestrated construction of secondary wall structure, which involves cellulosic thickening and lignification, and PCD, which involves cellular autolysis. Systematic analysis of gene expression has revealed that many genes involved in both secondary wall formation and modification and PCD are simultaneously expressed just before morphological changes of TEs take place (Demura et al., 2002; Milioni et al., 2002; Kubo et al., 2005; Pesquet et al., 2005). In this study, using a microarray analysis of transcripts from VND6-induced *Arabidopsis* cultured cells, we also found that VND6 upregulated many genes that encode enzymes related to PCD and secondary wall formation, such as proteases, nucleases, cellulose synthases, and peroxidases. These findings suggest the existence of a common transcriptional regulation system by which genes related to both secondary wall formation and PCD are upregulated in vessel cells. We found that the TERE *cis*-sequence confers vessel cell-specific expression to genes related to both secondary wall formation or modification and PCD (Pyo et al., 2007). In this article, we showed that VND6 binds the TERE sequence and activates the TERE-containing promoter in planta but not a mutated promoter that has substitutions in the TERE sequence. These results demonstrate that TERE is one of the target sequences of VND6.

Two genes, *XCP1* and *XCP2*, have been shown to be involved in PCD during xylem vessel differentiation in *Arabidopsis* (Zhao et al., 2000; Avci et al., 2008). The two genes are upregulated by VND6 induction. In addition, the list of VND6-induced genes

includes several genes, *XSP1*, *ATMC9*, *RNS3*, lipase family protein (*At4g18550*), nucleoside phosphatase family protein (*At1g14240*), and *ATSBT1.1*, that may be related to PCD and specifically to autolysis. For example, *ATMC9* encodes a caspase-like protein, which does not function as a caspase but as an Arg/Lys-specific Cys protease (Vercammen et al., 2004). We examined the expression pattern of *ATMC9* and found that *ATMC9* was specifically expressed in differentiating vessels but not in fully differentiated vessels. This result suggests the involvement of ATMC9 in the PCD process. Four of eight genes classified into the degradative enzyme (putative PCD) category contained the TERE sequence in their upstream regions. The EMSA analysis showed that three of them (*XCP1*, *ATMC9*, and *ATSBT1.1*) were bound directly by VND6 through TERE. The transcript for *XCP1* was strikingly upregulated as early as within 3 h after induction of *VND6*. Furthermore, VND6 can activate the expression of *XCP1* promoter-driven intron GUS in planta but not that of a mutated *XCP1* promoter that has substitutions in the TERE sequence. These findings suggest that VND6 initiates at least a part of PCD directly by activating PCD-related genes through binding the TERE sequence in their promoters.

By contrast, *SND1*, which is the master regulator gene for differentiation of xylem fibers, did not induce this PCD-related gene expression. This result is consistent with the fact that xylem fibers do not involve rapid PCD in their developmental process. SND1 preferentially induced two genes for proteases, which might be involved in fiber-specific slow PCD. Because these genes do not contain TERE sequences in their promoter regions, the mechanisms that regulate PCD during TE differentiation and fiber differentiation are thought to be different. SND1 binds to two sites on the *MYB46* promoter (Zhong et al., 2007). The target sequence described for SND1 at one site differs from the TERE sequence. The other site, which is 114 bp long, contains a TERE-like binding sequence. The TERE-like sequence includes a nonconserved nucleotide at the first position. Because our EMSA analysis indicated the importance of the first three nucleotides of TERE sequence for VND6 binding, the TERE-like sequence in the SND1 binding site may not function as a TERE. On the other hand, *MYB46* is also upregulated by VND6 and considered as a direct target of VND6, as judged from the ChIP-PCR experiment. However, EMSA analysis indicated that VND6 does not bind to the TERE-like sequence of *MYB46*. These results suggest that VND6 may regulate *MYB46* directly through binding to sites other than TERE. Because *MYB46* is expressed both by SND1 and VND6, *MYB46* expression might be regulated through a common *cis*-element(s) both by SND1 and VND6.

VND6 and SND1 Upregulate Genes Involved in Secondary Cell Wall Formation

Both VND6 and SND1 induced many genes related to secondary cell wall synthesis, including genes encoding cellulose synthases that function specifically in the synthesis of the secondary cell wall, *CESA4*/*IRX5*, *CESA7*/*IRX3*, and *CESA8*/*IRX1* (Taylor et al., 2003). Both VND6 and SND1 also upregulated the expression of *MYB46*, *MYB83*, and *MYB103*. It is known that MYB46 and MYB83 act redundantly as master switches for secondary cell wall formation (McCarthy et al., 2009) and regulate biosynthetic pathways for all three major secondary wall components: cellulose, lignin, and xylan (Zhong et al., 2007; McCarthy et al., 2009). Therefore, many genes identified as being downstream of both VND6 and SND1 might be regulated through MYB46, MYB83, or both. Although both vessels and fibers form secondary cell walls, their characteristics are different. For example, vessel cells have patterned secondary cell walls, while fiber cells have cell walls without a pattern. We recently identified a novel gene, *MIDD1*, which encodes a novel microtubule-associated protein. MIDD1 accumulates in the pits of the secondary cell wall of metaxylem vessels and determines the secondary cell wall patterns (Oda et al., 2010). This gene is upregulated downstream of VND6 and SND1. Indeed, our gene lists contain many uncharacterized genes, which may play roles in undiscovered processes during xylem development. For example, VND6-specific downstream genes include two genes related to the cytoskeleton. These genes might confer unique features on vessel morphogenesis, such as patterned secondary cell wall formation.

SND1, but not VND6, preferentially upregulated genes involved in lignin monomer synthesis, such as *PAL1*, *4CL3*, and *CCoAOMT*. Because these genes are expressed a little later than

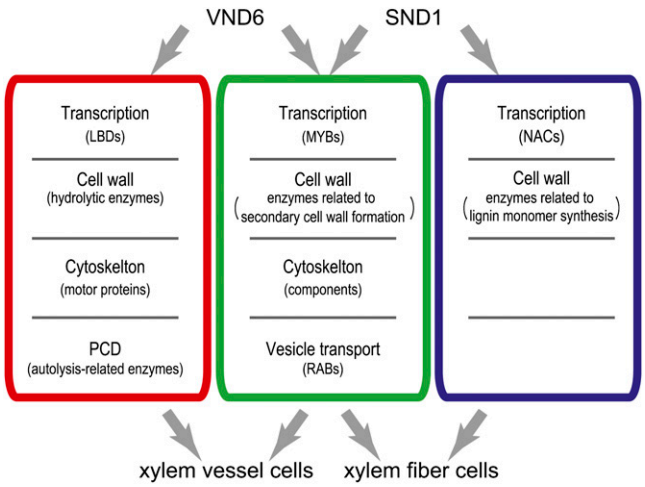

Figure 8. Diagrammatic Representation of VND6 and SND1 Gene Regulation.

Major events induced by VND6 (left), SND1 (right), and both (middle). Representative proteins that participate in the events are indicated in parentheses. Whereas MYBs are regulated by both VND6 and SND1, LBDs and NACs are specifically regulated by VND6 and SND1, respectively. In the cell wall, VND6 specifically regulates the expression of genes that encode hydrolytic enzymes, while SND1 regulates genes involved in lignin monomer synthesis. VND6 and SND1 commonly regulate genes that function in secondary cell wall formation, including genes involved in cellulose synthesis and lignin polymerization. Genes related to TE-specific PCD are regulated only by VND6. VND6 induces the expression of genes that encode specific motor proteins, and both VND6 and SND1 induce the expression of genes that encode components of the cytoskeleton, such as tubulin. VND6 and SND1 both upregulate the expression of genes related to vesicle transport, such as RABs.

[See online article for color version of this figure.]

the direct targets of SND1, they may be indirect targets of SND1 that are regulated by unknown SND1-induced transcription factors. By contrast, genes that encode enzymes that catalyze the polymerization of lignin monomers, such as peroxidases and laccases, were upregulated by both VND6 and SND1. Tokunaga et al. (2005) reported that some amounts of lignin precursors are provided from other xylem cells during the process of lignification in TEs. Our findings are consistent with this. In addition to these genes, we identified various genes that were induced in a VND6- and SND1-specific manner. Functions of most of these genes are still unknown. Further analysis of these genes may elucidate differences in function and cell structure between vessel cells and xylem fibers.

In conclusion, we revealed the gene regulation frameworks needed to perform vessel and fiber differentiation by identifying genes that are expressed downstream of VND6 and SND1. VND6 and SND1 are master regulators that dictate the final differentiation of secondary wall formation of vessel cells and fiber cells, respectively. At the next rank in the hierarchy, MYB46 and MYB83 function as master regulators for secondary cell wall formation under both VND6 and SND1. On the other hand, only VND6 initiates vessel-specific PCD by directly inducing the expression of some PCD-related genes (Figure 8).

METHODS

Cell Culture and Transformation

Arabidopsis thaliana suspension cells, strain Columbia-0, were cultured in 27 mL of modified Murashige and Skoog (MS) medium, pH 5.8, containing 4.33 g·L^{-1} of MS inorganic salts, 4.1 μM 2,4-D, 3.0% (w/v) sucrose, and vitamins, including 8 mg·L^{-1} nicotinic acid, 8 mg·L^{-1} pyridoxine-HCl, 80 mg·L^{-1} thiamine-HCl, and 800 mg·L^{-1} myoinositol. The cells were agitated on a rotary shaker at 124 rpm at 23°C in the dark. At weekly intervals, 12-mL aliquots of the culture were transferred to 15 mL of fresh medium in a 100-mL culture flask.

To establish a transformant strain, 3-d-old cells were cocultured with *Agrobacterium tumefaciens* strain GV3101 (MP90) harboring estrogen-inducible *SND1-CFP* in MS medium supplemented with 50 mg·L^{-1} acetosyringone for 2 d. Then, 0.5 g·L^{-1} claforan was added to the cell suspension and the cells were cultured for a further 5 d. Thereafter, the cell suspension was transferred into 15 mL fresh medium and maintained as described above in medium supplemented with 50 mg·L^{-1} hygromycin B over 4 weeks, and the surviving cells were used for experiments.

To induce differentiation, a 1-mL aliquot of 7-d-old transformant cell culture was suspended in 9 mL of 2,4-D–free MS medium. After 3 min of settling, 5 mL supernatant was removed to adjust cell density. The remaining cell suspension was supplied with 2 μM estradiol and cultured on a rotary shaker at 124 rpm at 23°C in the dark. To stain secondary cell walls, the cells were incubated with 1 mg·L^{-1} WGA–AlexaFluor 594 (Invitrogen) for 15 min.

To determine the extent of cell death, the vacuolar lumen of the cells was stained with 1 μM BCECF (Invitrogen) 12 h after the onset of differentiation. Dead cells lose the fluorescence conferred by BCECF after rupture of their vacuoles, whereas viable cells preserve the vacuolar fluorescence of BCECF.

Microarray Analysis

ATH1 genome arrays (Affymetrix) were used for gene expression detection. Total RNA was extracted with TRIzol reagent (Invitrogen) and purified with an RNeasy Micro kit (Qiagen) following the manufacturer's instructions. Probe synthesis was performed with the GeneChip One-Cycle Target Labeling and Control Reagents kit or the GeneChip 3′ IVT Express kit (Affymetrix) following the manufacturer's protocol. Hybridization and washes were performed as described in the GeneChip Expression Analysis Technical Manual (Affymatrix). Signal detection and global normalization were performed using Affymetrix GeneChip Operating Software (version 1.4) with standard parameters. Comparison analysis was performed using Affymetrix GeneChip Operating Software (version 1.4). The expression of each gene was compared between 0 h as the baseline sample and 12 h as the experimental sample with standard parameters in the same genotype samples. Three biological replicate data points were used for statistical analysis. A one-way analysis of variance among wild-type cells, VND6-induced cells, and SND1-induced cells was performed using log2-transformed data. Statistical significance was set at $P < 0.01$. Q-values (Storey and Tibshirani, 2003) were calculated to estimate the false discovery rate and genes having $q > 0.1$ were excluded, except for VND6 itself. Genes showing significant expression differences with over a fivefold change in VND6-induced cells or SND1-induced cells were identified. Genes having a greater expression level in wild-type cells than in VND6-induced or SND1-induced cells were excluded. To classify the identified genes, values of fold change ratio (fold change in VND6-induced cells versus fold change in SND1-induced cells) were calculated. Genes that had a fold change ratio of >4 were classified into the VND6-specific list, while genes that had a fold change ratio of <0.25 were classified into the SND1-specific list. Genes that had values between 0.25 and 4 were listed as common downstream genes. To search for TEREs in the upstream regions of the gene lists, we used Patmatch (http://Arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl) and examined both forward and reverse sequences.

Quantitative RT-PCR

Total RNA was prepared using an RNeasy plant mini kit (Qiagen) with RNase-free DNase I (Qiagen). cDNA was synthesized using SuperScript III RT (Invitrogen) with oligo(dT)12-18 primers. The quantitative RT-PCR was performed using a Light Cycler 1.2 apparatus (Roche) by monitoring the amplification with appropriate universal probes (VND6; 2, SND1; 5, XCP1; 69, XCP2; 163, 4CL3; 16, PAL1; 63, CESA4; 140, MYB46; 35, MYB83; 101, Roche). The primers used for RT-PCR were designed using the website for the Universal ProbeLibrary Assay Design Center (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=uplct_030000). Quantitative PCR analysis was conducted using the first-strand cDNA as a template with the LightCycler TaqMan Master (Roche). The relative mRNA levels were normalized using the concentration of an ATMBF1A reference gene. Because the expression of genes encoding tubulin and actin was highly variable in VND6-overexpressing cells, these genes were not suitable reference genes. The data used were the average of three replicates.

DNA Manipulation

Vectors based on Gateway cloning technology (Invitrogen) were used for most manipulations. The estrogen-inducible CFP fusion construct was made from pER8 (Zuo et al., 2000). CFP was inserted into *Asc*I and *Spe*I sites of pER8, and then Gateway cassette A (Invitrogen) was inserted into the *Xho*I site. pMDC7 was used to generate the estrogen-inducible vector used in the infiltration analysis (Curtis and Grossniklaus, 2003). The *XCP1* promoter region was amplified from plant genomic DNA with the primers listed in Supplemental Table 1 online. *XCP1* promoters with substitutions were amplified by PCR with primers containing substitutions. Promoter intron-GUS was made in the pGWB434-intron GUS vector, which was a gift from T. Nakagawa (Shimane University; Nakagawa et al., 2007). Promoter YFP-NLS was constructed in a pBGYN vector (Kubo et al., 2005).

Growth Conditions for *Arabidopsis* and *N. benthamiana*

Arabidopsis ecotype Columbia was used as the wild-type plant. Seedlings were germinated on half-strength MS agar plates in a Percival incubator with 24 h light for 7 to 10 d at 22°C.

Nicotiana benthamiana seedlings were grown in vermiculite with 24 h light at 24°C.

N. benthamiana Infiltration Assays

A. tumefaciens strain GV3101 (MP90) carrying expression constructs was grown in Luria-Bertani media with appropriate antibiotics and suspended in infiltration buffer (10 mM MES, pH 5.7, containing 10 mM $MgCl_2$ and 150 μM acetosyringone). The cultures were adjusted to an OD_{600} of 1.0 and incubated at room temperature for at least 3 h prior to infiltration. Equal volumes of cultures of different constructs were mixed for coinfiltration and then mixed with agrobacterial cultures (OD_{600} of 1.0) carrying a p19 silencing suppressor at a ratio of 1:1 (Voinnet et al., 2003). The resulting cultures were infiltrated into 3- to 4-week-old *N. benthamiana* leaves. The leaf samples were harvested 3 d after infiltration and incubated in water containing 5 μM estrogen for 24 h (histochemical GUS staining) or for 8 h (detection of GUS activity) at 24°C.

For detection of histochemical GUS staining, samples were first placed in 90% acetone on ice for 1 h and then into reaction buffer solution [100 mM sodium phosphate, pH 7.0, containing 500 mg/mL 5-bromo-4-chloro-3-indolyl-β-glucuronide cyclohexylammonium salt, 1 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6$, 10 mM EDTA, and 0.1% Triton X-100] at 37°C. Samples were mounted in water or chloral hydrate:glycerol:water (8:3:1, w/v/v) and viewed with an Olympus BX51 microscope equipped with Nomarski optics or a Leica MZ 16F microscope.

For detection of GUS activity, two leaf discs (7 mm in diameter) were collected and ground in 200 μL GUS extraction buffer (50 mM NaH_2PO_4-Na_2HPO_4, pH 7.0, containing 10 mM 2-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine, and 0.1% Triton X-100). After centrifugation at 12,000*g* for 10 min at 4°C, 100 μL of the supernatant was mixed with 400 μL of GUS assay solution (2 mM 4-methylumbelliferyl-D-glucuronide in extraction buffer). A 250-μL aliquot was removed immediately and added to 500 μL of 0.2 M sodium carbonate. The remainder of the reaction mixture was incubated at 37°C for 90 min and then added to 500 μL of 0.2 M sodium carbonate. The production of 4-methylumbelliferone during this incubation was determined fluorometrically (excitation at 365 nm; emission at 455 nm). The concentration of protein was determined with Bradford's reagent (Bio-Rad). GUS activity is expressed as the rate of 4-methylumbelliferone production per mg protein.

EMSA

The NAC domain of VND6 was cloned into a pENTR-D-TOPO vector and then transferred into pDEST15 (Invitrogen). The recombinant GST-VND6 protein was purified using Glutathione Sepharose 4B (GE). As a negative control, we used GST-CLV1 protein purified in the same way. Fragments of promoters were labeled with a Biotin 3′ End DNA labeling kit (Thermo Scientific). Biotin-labeled probes and the GST-fused proteins were incubated in 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 0.05 μg/μL poly dI-dC) for 30 min at room temperature. The protein-probe complexes were separated by PAGE. The biotin-labeled probes were detected using a LightShift Chemiluminescent EMSA kit (Thermo Scientific) and with a ChemiDocXRS system (Bio-Rad).

ChIP-PCR

ChIP experiments were performed according to the method of Morohashi et al. (2007). Cultured cells harboring estrogen-inducible VND6 treated with estrogen or DMSO for 24 h were fixed. Immunoprecipitation was performed using 1 μL of anti-GFP antibody (ab290; Abcam). PCR was performed using a LightCycler (version 3.5; Roche) with a SYBR green method (Thunderbird SYBR qPCR mix; Toyobo).

Accession Numbers

The data discussed in this article have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus and are accessible through GEO Series accession number GSE20586. Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: VND6, At5g62380; SND1, At1g32770; XCP1, At4g35350; XCP2, At1g20850; ATMC9, At5g04200; ATSBT1.1, At1g01900; CESA4, At5g44030; MYB46, At5g12870; polygalacturonase, At1g70500; ATHB-15, At1g52150; MIDD1, At3g53350; and ATMBF1A, At2g42680.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Quantitative RT-PCR for Transcription Factors Downstream of VND6.

Supplemental Table 1. List of Primers Used in This Study.

Supplemental Data Set 1. List of Both VND6- and SND1-Induced Genes.

Supplemental Data Set 2. List of VND6 Preferentially Induced Genes.

Supplemental Data Set 3. List of SND1 Preferentially Induced Genes.

Supplemental Data Set 4. List of Myb Genes That Are Up- or Downregulated in Response to VND6 Overexpression.

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