Transcription switches for protoxylem and metaxylem vessel formation

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Land plants evolved xylem vessels to conduct water and nutrients, and to support the plant. Microarray analysis with a newly established *Arabidopsis* in vitro xylem vessel element formation system and promoter analysis revealed the possible involvement of some plant-specific NAC-domain transcription factors in xylem formation. *VASCULAR-RELATED NAC-DOMAIN6* (*VND6*) and *VND7* can induce transdifferentiation of various cells into metaxylem- and protoxylem-like vessel elements, respectively, in *Arabidopsis* and poplar. A dominant repression of *VND6* and *VND7* specifically inhibits metaxylem and protoxylem vessel formation in roots, respectively. These findings suggest that these genes are transcription switches for plant metaxylem and protoxylem vessel formation.

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Xylem vessels, a conductive component of the vascular tissues in plants, are found throughout the plant body. To colonize the land, plants have evolutionarily developed different types of xylem vessels that function in the long-distance transport of water, various nutrients, and signaling molecules throughout their life (Raven et al. 1999). Two types of vessels mature in characteristic positions within protoxylem and metaxylem of the primary xylem tissue that differentiates from the procambium during the early ontogeny of a plant. The protoxylem vessels, which commonly have annular and spiral thickenings, mature before the surrounding organs have elongated. These are frequently destroyed by the extension of the surrounding tissues. The metaxylem vessels, which usually have reticulate and pitted thickenings, mature after the surrounding organs complete their growth. In contrast to protoxylem vessels, they are not destroyed, and constitute the water-conducting tubes of the mature plant (Esau 1977). In *Arabidopsis* roots, two protoxylem vessels are typically formed at the outermost position of the vascular system, between which three to four metaxylem vessels develop (Supplementary Fig. S1).

Recent forward genetic and molecular biological approaches have revealed several aspects of xylem formation that are affected by several key genes (Ye 2002; Fukuda 2004). These genes are related to auxin transport and signaling, and include *PINFORMED1* (Gälweiler et al. 1998) and *MONOPTEROS* (Przemeck et al. 1996), the xylogen genes related to cell–cell interactions (Motose et al. 2004); genes related to brassinosteroid biosynthesis and signaling such as *CPD* (Mathur et al. 1998a), *DWF4* (Choe et al. 1998), and *BRLs* (Caño-Delgado et al. 2004; Zhou et al. 2004); genes related to pattern formation such as the *HD-ZIPIII* family genes (Ohashi-Ito and Fukuda 2003); the *KANADI* gene family (Emery et al. 2003); and *APL*, related to xylem–phloem switching (Bonke et al. 2003). However, the hierarchical genetic control of differentiation of individual xylem cells is still poorly understood. In this study, we identified VND6 and VND7, which belong to plant-specific transcription factors, NAC-domain proteins that can induce transdifferentiation of various types of cells into metaxylem- and protoxylem-like vessel elements, respectively. It is suggested that VND6 and VND7 are transcription switches for plant metaxylem and protoxylem vessel formation, respectively.

Results and Discussion

We have uncovered an expression profile of 9000 genes during xylem vessel element differentiation in an in vitro *Zinnia* cell culture (Demura et al. 2002). To gain an expression profile of xylem cell-differentiation-related genes in *Arabidopsis*, we established an in vitro xylem vessel element inducible system from *Arabidopsis* suspension cells. In this system, ~50% of subcultured cells of *Arabidopsis* ecotype Col-0 differentiate into xylem vessel elements within 7 d in the presence of 1 μM brassinolide and 10 mM boric acid (Fig. 1A,B). Microarray analysis with the *Arabidopsis* full-genome GeneChip array ATH1 (Affymetrix) indicated that 1705 genes showed more than an eightfold change in expression over the time course. These were clustered into 23 sets using the QT clustering method (Fig. 1C; Supplementary Table S1). Of these, genes in Set 3 (156 genes), Set 8 (58 genes), and Set 22 (10 genes) showed up-regulated expression just when the xylem vessel elements were actively forming (6 d after induction). They encoded a variety of proteins closely associated with particular morphogenetic events such as secondary cell-wall formation (cellulose synthases, xylanases, and laccases) and programmed cell death (nucleases and proteases). A promoter analysis of three selected genes—xylanase *AtXyn3* (At4g08160) (Sawa et al. 2005), cellulose synthase *IRX3* (At5g17420) (Taylor et al. 1999), and laccase (At2g38080) (Oh et al. 2003)—was conducted with the cyan or yellow fluorescence protein gene (*CFP*

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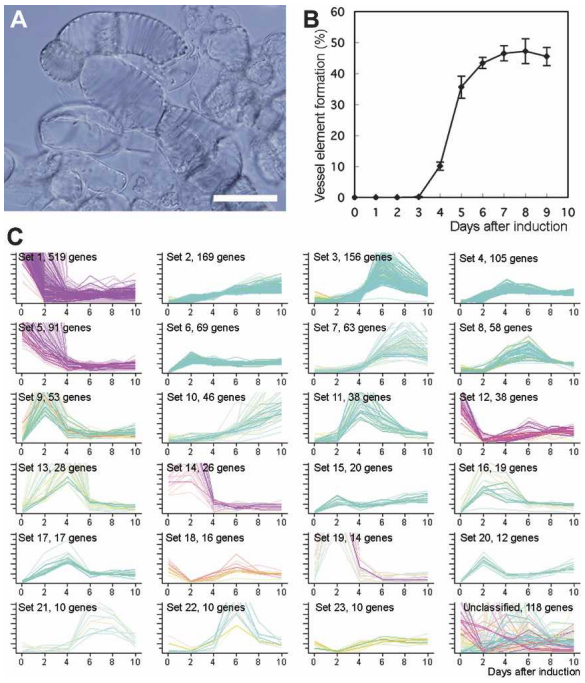

Figure 1. The *Arabidopsis* in vitro xylem vessel element formation system. (*A*) Xylem vessel elements induced in *Arabidopsis* suspension cells cultured for 7 d. Bar, 50 µm. (*B*) Frequency of xylem vessel element formation. Results are means ± SD ($n = 3$). (*C*) Hierarchical clustering of developmentally regulated genes during in vitro xylem vessel element formation. Colors indicate expression levels at 0 d after induction as follows: (magenta) high; (yellow) medium; (cyan) low.

or *YFP*, respectively) fused to the SV40 nuclear localizing signal (NLS). This revealed the immature xylem vessel-specific expression of these promoters in the roots (Supplementary Fig. S2A–C). Several putative transcription factors were also included in these gene sets. In particular, Set 3 included 16 genes that encoded transcription factors such as four NAC-domain proteins, five zinc finger proteins, and three Myb proteins (Supplementary Table S1). These might control the gene expression for xylem vessel formation. The four *Arabidopsis* NAC-domain proteins in Set 3 showed a significant similarity to the zinnia NAC-domain protein encoded by an expressed sequence tag, *Z567*, which showed up-regulated expression during the zinnia in vitro xylem vessel element formation (Demura et al. 2002). Several reports also indicated that NAC-domain proteins play pivotal roles, such as during morphogenesis and in hormone signaling in plants (Aida et al. 1997; Sablowski and Meyerowitz 1998; Xie et al. 2000; Duval et al. 2002; Tran et al. 2004). For these reasons, we focused on the NAC-domain-encoding genes for further analysis.

By searching the *Arabidopsis* genome for genes with similarity to the full-length sequence of *Z567*, we identified three additional NAC-domain genes that belong to the same subfamily. We designated these seven genes—At2g18060, At4g36160, At5g66300, At1g12260, At1g62700, At5g62380, and At1g71930—as *VASCULAR-RELATED NAC-DOMAIN PROTEIN 1* (*VND1*) to *VND7*, respectively (Fig. 2A; Supplementary Fig. S3). Microarray analysis revealed that *VND1*, *VND2*, and *VND6* as well as *VND3*, *VND4*, *VND5*, and *VND7* classified into Set 3, were up-regulated during in vitro xylem vessel element formation (Fig. 2B; Supplementary Table S1). This suggested the vascular formation-preferential expression of this gene family. The vascular cell-specific expression of all the *VND* genes was confirmed by the promoter analysis with the *YFP–NLS* and *GUS* as reporters (Fig. 2C; Supplementary Fig. S4). *VND1p* to *VND3p*::*YFP–NLS* were expressed preferentially in procambial cells adjacent to root meristem. Expression of *VND4p* to *VND7p*::*YFP–NLS* was observed predominantly in immature xylem vessels without obvious secondary wall thickenings. In particular, expression of *VND6p*::*YFP–NLS* was restricted to the central metaxylem vessels in a middle position of the root (Fig. 2C; Supplementary Fig. S4A–C), whereas that of *VND7p*::*YFP–NLS* was detected in the immature protoxylem vessels around the position just above the root meristem (Fig. 2C; Supplementary Fig. S4D). In shoots, no obvious expression of *VND1p*, *VND4p*, and *VND6p*::*GUS* was found (data not shown), but the expression of the others was observed preferentially in vascular cells (Supplementary Fig. S4E–H). An alignment of the VND proteins, the Z567 protein, and several other *Arabidopsis* NAC-domain proteins showed that the putative DNA-binding NAC-domain, which comprised five subdomains, I to V, was highly conserved among NAC-domain proteins (Supplementary Fig. S3). The VND and Z567 proteins specifically contained two novel conserved domains in the C-terminal region, which might be associated with transcription activation (Supplementary Fig. S3; Duval et al. 2002).

To define the function of VND proteins in vascular development, we expressed the *VND* genes ectopically under the control of the cauliflower mosaic virus 35S promoter (*35S*). None of the *35S*::*VND1* to *VND5* transgenic seedlings showed obvious morphological changes (data not shown). In contrast, we found transdifferentiation of various types of cells into xylem vessel elements, without changing cell shapes, in the hypocotyls and roots of the *35S*::*VND6* and *VND7* transgenic seedlings (Fig. 3A–C). The transdifferentiation into xylem vessel elements occurred mainly in the epidermis of the hypocotyls (Fig. 3A) and in the xylem parenchyma cells, some pericycle, endodermal, and cortex cells of the developed roots (Fig. 3B). Surprisingly, in the roots, the morphology of the transdifferentiated xylem vessel elements in *35S*::*VND6* and *35S*::*VND7* plants was clearly different. Whereas *VND6* induced xylem vessel elements with reticulate and pitted thickenings of the secondary wall that were similar to metaxylem vessels, *VND7* induced xylem vessel elements with annular and spiral thickenings that were similar to protoxylem vessels (Fig. 3B; Supplementary Fig. S1B). The transdifferentiation into xylem vessel elements also occurred in several cell types of adult *35S*::*VND6* and *VND7* plants. These included the epidermal cells such as guard cells of rosette leaves (Fig. 3C).

A promoter analysis of the three immature xylem vessel-specific genes, *AtXyn3*, *IRX3*, and the laccase gene (At2g38080), indicated their ectopic expression in *35S*::*VND7* plants (Supplementary Fig. S2D–F). This suggested that at least a part of immature xylem vessel-specific genes were regulated by *VND7*. To examine the generality of the *VND6* and *VND7* function in plants, *VND6* and *VND7* were overexpressed in poplar plants under the control of *35S*. This resulted in the transdifferentiation of mesophyll or epidermal cells in the leaves

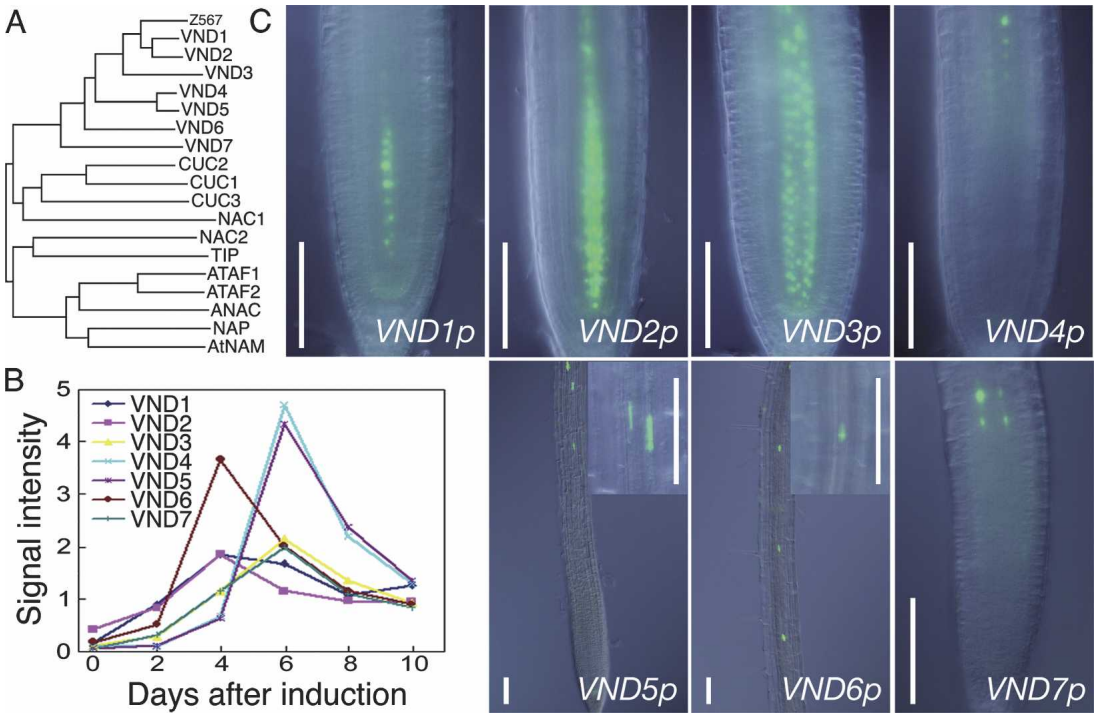

Figure 2. Characterization of the *VND* genes. (*A*) Neighbor-joining tree of selected NAC-domain proteins of zinnia and *Arabidopsis*. Amino acid sequences were aligned by using the MAFFT program. (*B*) Expression patterns of *VND1* to *VND7* during in vitro xylem vessel element formation revealed by microarray analysis. (*C*) Expression of *VNDp*::*YFP–NLS* in *Arabidopsis* roots. Images of differential interference contrast (DIC) and YFP fluorescence were merged. Bars, 100 µm.

of *35S*::*VND6* and *VND7* poplar plants into metaxylem- and protoxylem-like vessel elements, respectively (Fig. 3D).

To examine the subcellular location of the VND6 and VND7 proteins, VND6 and VND7 fused to YFP were overexpressed by the *35S* promoter in *Arabidopsis*. VND6–YFP and VND7–YFP fluorescence was located in the nucleus of transgenic plants, in which transdifferentiation to xylem vessel elements occurred similarly to that in *35S*::*VND6* and *VND7* plants (Supplementary Fig. S5). This result suggested that the VND6 and VND7 function as nuclear transcription regulators.

To investigate the intrinsic *VND6* and *VND7* function further, we analyzed loss-of-function phenotypes of *VND6* and *VND7*. Transfer-DNA (T-DNA) insertion mutants of *VND6* (GABI_567F08) and *VND7* (SALK_063329, SALK_112924 and SALK_115812), and transgenic plants with the expression of antisense RNA or RNAi for *VND6* and *VND7* did not show any detectable defects in morphology (data not shown). This lack of loss-of-function phenotype of *VND6* and *VND7* may be presumably due to gene redundancy. However, transgenic seedlings with overexpressed VND6 and VND7 proteins fused to the SRDX strong repression domain driven by *35S* (*VND6–SRDX* and *VND7–SRDX*) displayed defects in growth and vessel formation in the roots (Fig. 4; Hiratsu et al. 2003). Although root growth in *VND6–SRDX* seedlings was inhibited slightly, that in *VND7–SRDX* seedlings was inhibited strongly (Fig. 4B,C; Supplementary Fig. S6). Overexpression of VND6–SRDX repressed central metaxylem vessel formation, while protoxylem vessel formation was normal (Fig. 4E; Supplementary Fig. S6). In contrast, overexpression of VND7–SRDX repressed protoxylem vessel formation without affecting metaxylem vessel formation (Fig. 4F; Supplementary Fig. S6). A control experiment revealed that such defects were not observed in transgenic seedlings with overexpression of SRDX fused with another *Arabidopsis* NAC-domain protein, NAC1 (Xie et al. 2000; data not shown).

Auxin, cytokinin, and brassinosteroids play pivotal roles in xylem vessel formation (Fukuda 2004). Therefore, we investigated the effects of phytohormones on expression levels of *VND6* and *VND7* by culturing hypocotyls of wild-type and transgenic *VND6p* and *VND7p*::*GUS Arabidopsis* seedlings in the presence of these phytohormones for 5 d (Fig. 5; Supplementary Fig. S7). The presence of both auxin and cytokinin was required for significant expression of both *VND6* and *VND7* (Fig. 5). In the combination, *VND6p*::*GUS* was expressed strongly in newly proliferating cells derived presumably from pericycle, while *VND7p*::*GUS* was expressed only in some cells just beside xylem vessels (Supplementary Fig. S7). The highest expression of *VND6* and *VND7* was detected in the presence of all three hormones (Fig. 5). Under this condition, new cell proliferation was prevented in the cultured hypocotyls, in which expression of *VND7* extended along by the vessel strands and *VND6* was expressed in limited vascular cells at the edge of the hypocotyls (Supplementary Fig. S7). These results indicated that *VND6* and *VND7* function downstream of the signal transduction pathway of these phytohormones.

It is well known that cell fate in vertebrates is sometimes regulated by master regulator genes such as the MyoD family, which has a remarkable capacity to direct the skeletal muscle cell-specific transcriptional program

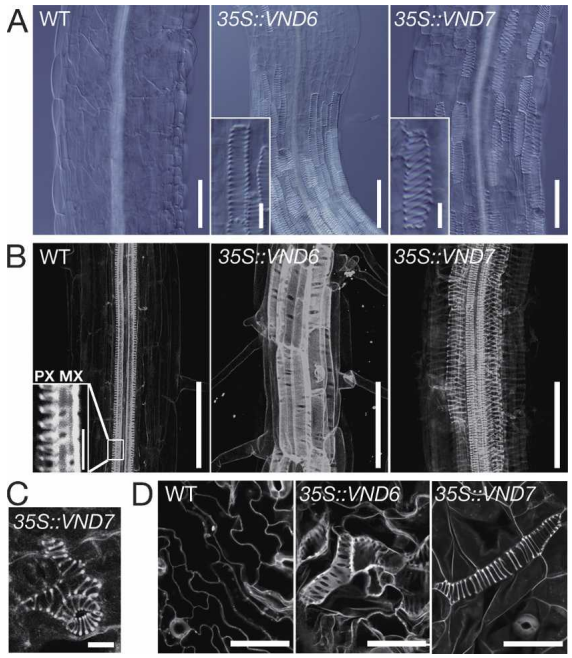

Figure 3. Overexpression phenotypes of *VND6* and *VND7*. (*A*) Longitudinal DIC images of *Arabidopsis* hypocotyls. (*B*) Longitudinal confocal laser scanning microscope (CLSM) images of *Arabidopsis* roots. (*C*) CLSM image of epidermis of *Arabidopsis* rosette leaf. (*D*) CLSM images of epidermis in the poplar leaves. (PX) Protoxylem vessel; (MX) metaxylem vessel. Bars: *A*,*B*, 100 µm; *insets* in *A*, 20 µm; *C*, *insets* in *B*, 10 µm; *D*, 50 µm.

(Pownall et al. 2002). In contrast, only little is known about the master genes directing cell fate in plants, except for *GL2* and *PLT* for differentiation of plant hair cells and the quiescent center as a stem cell niche in root, respectively (Schiefelbein 2003; Aida et al. 2004). Data presented here indicate that *VND6* and *VND7* independently have the ability to regulate cell fate of metaxylem and protoxylem vessel elements, respectively, probably through controlling the expression of different target genes. Identification of target genes is one of the most important next steps toward the further understanding of the role of VND6 and VND7. The transdifferentiation into vessel elements by overexpression of *VND6* and *VND7* occurred not only in vascular cells such as those in the stele of the roots, but also in nonvascular cells such as epidermal cells including guard cells without intervening cell division and changing cell shape in *Arabidopsis*, which strengthened the idea that *VND6* and *VND7* are master regulators for vessel cell fate.

The *VND* genes form a subfamily that belongs to a large family of the NAC-domain transcription factor genes. Other members of the NAC-domain-containing gene family, *CUP-SHAPED COTYLEDON 1* (*CUC1*), *CUC2*, and *CUC3*, are partly functionally redundant in shoot apical meristem formation (Vroemen et al. 2003), and both the dominant repression of CUC1 or CUC2 with the SRDX repression domain and the *cuc1/cuc2* double mutations cause the loss of the shoot apical meristem (Hiratsu et al. 2003). Expression of the *VND* genes partially overlaps in some vascular tissues, suggesting partially redundant functions. Indeed, similarly to *CUC* genes, loss-of-function mutations, T-DNA insertions, and antisense or RNAi expression of the *VND6* and *VND7* genes did not show any detectable defects in morphology, while the dominant repression of *VND6* and *VND7* repressed metaxylem and protoxylem formation, respectively. Further analysis of the redundant functions of VND members is necessary to understand the hierarchical gene regulation responsible for the development of various types of vascular cells, as well as for xylem vessel element formation.

Overexpression of these genes in poplar leaves can also induce transdifferentiation of mesophyll and epidermal cells into metaxylem- and protoxylem-like vessel elements with different secondary walls. This result suggests that the hierarchical genetic control of vessel formation might be conserved evolutionarily. Therefore, functional analyses of *VND* genes may provide basic knowledge for improvements in wood biomass production.

Materials and methods

Plant materials

Fifteen milliliters of *Arabidopsis* Col-0 suspension cells (Mathur et al. 1998b) were transferred to 35 mL of a freshly modified Murashige and Skoog (MS) medium supplemented with 1 µg/mL 2,4-dichlorphenoxyace-

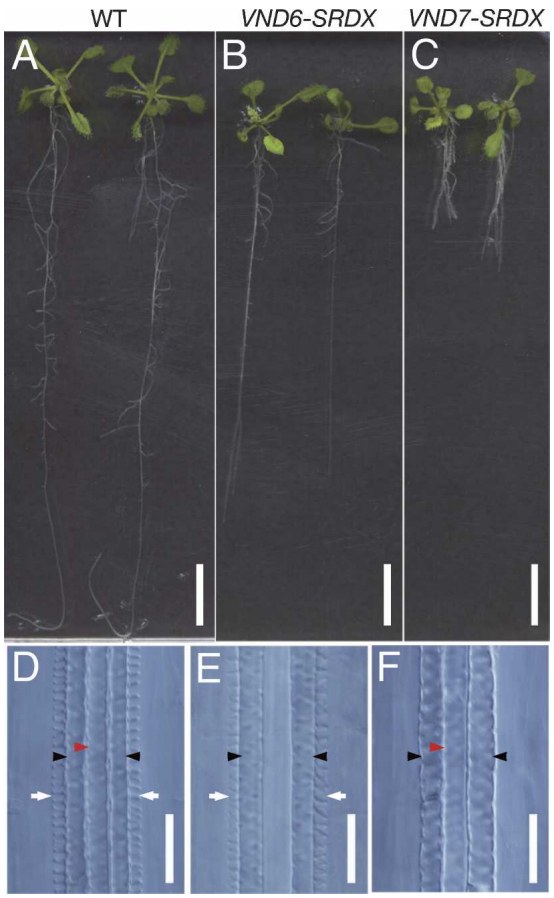

Figure 4. Dominant repression phenotypes of *VND6* and *VND7* in *Arabidopsis*. (*A–C*) Fourteen-day-old seedlings. (*D–F*) Longitudinal DIC images of vascular bundles in a middle region of the 9-d-old roots. White arrows indicate protoxylem vessels. Black and red arrowheads indicate primary and central metaxylem vessels, respectively. (*A*,*D*) Wild type. (*B*,*E*) *VND6–SRDX*. (*C*,*F*) *VND7–SRDX*. Bars: *A–C*, 10 mm; *D–F*, 20 µm.

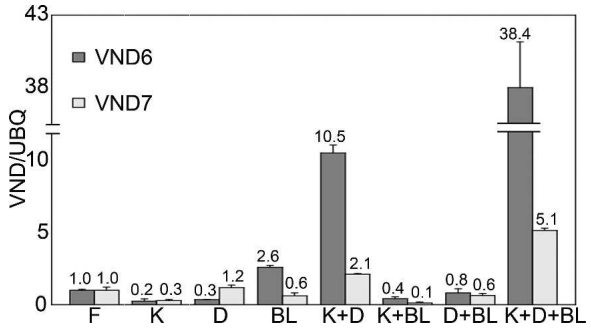

Figure 5. Effects of phytohormones on expression levels of *VND6* and *VND7* in cultured hypocotyls. (F) Hormone free; (K) 50 ng/mL kinetin; (D) 500 ng/mL 2,4-dichlorphenoxyacetic acid; (BL) 1 µM brassinolide. Relative expression levels between *VND6* and *UBQ*, and *VND7* and *UBQ* are shown as compared with the hormone-free condition. Results are means ± SD ($n = 3$).

tic acid and 3% sucrose every 7 d, and subcultured on a rotary shaker at 120 rpm in the dark at 22°C. For xylem vessel element induction, a 7.5-mL aliquot of 7-d-old subcultured cells was transferred into 42.5 mL of fresh medium that included 1 µM brassinolide and 10 mM H_3BO_3, and cultured as described above. The frequency of xylem vessel element formation was calculated as the proportion of xylem vessel elements to the number of living cells and the vessel elements. *Arabidopsis* seeds were sterilized by 70% ethanol and PPM (Plant Biotechnology), and then placed on germination medium (GM) containing MS medium, 1% sucrose, 0.05% MES-KOH (pH 5.8), B5 vitamins, and 0.3% phytagel (Sigma). They were treated for 4 d at 4°C, and then incubated in growth chamber under continuous illumination at 22°C. To investigate phytohormone effects on the expression levels of *VND6* and *VND7*, hypocotyls of 5-d-old *Arabidopsis* Col-0 seedlings grown on GM in dimly lit conditions were excised and incubated on modified MS media supplemented with 2% glucose and various combinations of phytohormones for 5 d in the dark at 22°C. Transformation and regeneration of the poplar hybrid aspen (*Populus tremula* L. × *Populus tremuloides* Michx.) were carried out as described previously (Nilsson et al. 1992).

RNA extraction

Two independent samples were pooled for microarray and RT–PCR. Total RNA was prepared as described previously (Nishitani et al. 2001), and further purified by using the RNeasy Mini Kit (QIAGEN).

Microarray analysis

Microarray experiment was performed by using ATH1 GeneChips (Affymetrix) according to the manufacturer's instructions as described in Supplemental Materials. The complete data set is available on the TAIR Web site (http://arabidopsis.org).

Plasmid construction and plant transformation

To obtain a full-length cDNA of zinnia *Z567* (GenBank accession no. AB217775), a rapid amplification of cDNA ends (RACE)–PCR was performed with the SMART RACE cDNA amplification kit (Clontech). For the promoter analysis, we generated pBGGUS, pBGYN, and pBGCN binary vectors in which the GATEWAY cassette (Invitrogen) was fused to the 5′-ends of *GUS*, the *YFP–NLS*, and *CFP–NLS* (Clontech) fragments on pSMAB704, respectively. For overexpression and antisense RNA expression, we generated binary vectors containing 35S with the GATEWAY cassette in the sense (pH35GS) and antisense (pH35GA) orientation on pSMAH621, respectively. For YFP-fusion protein expression and SRDX-fusion protein expression, we generated binary vectors containing 35S with the sense GATEWAY cassette followed by YFP (pH35GY) and the SRDX strong repression domain (pH35GEAR) on pSMAH621, respectively. Promoter fragments and cDNAs of *Arabidopsis* genes were amplified from genomic DNA and cDNA pools of the Col-0 ecotype by PCR with gene-specific primer sets, respectively (Supplementary Table S2). They were then subcloned into a pDONR or pENTR/D/TOPO vector (Invitrogen), and then integrated into the GATEWAY binary vectors using LR clonase (Invitrogen). The resulting plasmids were electroporated into *Agrobacterium* strain GV3101∷pMP90, which was used to transform *Arabidopsis* ecotype Col-0 with the floral dip method. Multiple transgenic lines were obtained by selection on GM supplemented with 10 µg/mL bialaphos or 20 µg/mL hygromycin.

Histology

For observation of the *promoter∷reporter* lines, 4-, 5-, or 7-d-old seedlings were used. A GUS staining method was previously described (Pyo et al. 2004). The 7- and 9-d-old seedlings of the overexpression lines and the dominant repression lines, respectively, were fixed in 90% (v/v) acetone for 2 h to 2 d, rinsed with 100 mM phosphate buffer (pH 7.2) at least three times, and then cleared in a clearing solution (8 g of chloral hydrate, 1 mL of glycerol, and 2 mL of water). Fluorescent and DIC images were observed under a microscope (BX51; Olympus) and photographed with a digital DP70 camera (Olympus). CLSM images of *Arabidopsis* and poplar stained by safranin-O (Kitin et al. 2000) were detected under a confocal laser scanning microscope (FV-1000; Olympus). Images were processed with Adobe Photoshop CS.

RT–PCR

From total RNA after DNase treatment, cDNAs were synthesized by SuperScriptII reverse transcriptase (Invitrogen) with oligo(dT) primers. Semiquantitative RT–PCR was undertaken by using QuantiTect SYBR green PCR kit (QIAGEN) with a LightCycler (Roche) according to the manufacturers' instructions. Gene-specific primer sets for *VND6*, *VND7*, and ubiquitin (At5g57860) are described in Supplementary Table S2.

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