Regulatory Mechanisms of Master Gene Expression in Xylem Vessel Formation

（道管分化マスター因子の発現制御機構の解明）

1181004

Hitoshi Endo

Nara Institute of Science and Technology

Graduate School of Biological Sciences

Laboratory of Plant Metabolic Regulation

(Professor Taku Demura)

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Table of content

Introduction

The xylem cells of the plant vascular system, vessel elements and fibers, conduct water and minerals throughout the plant body and provide mechanical strength to support the entire plant by forming thick secondary cell walls. The secondary cell walls mainly consist of polysaccharides, such as cellulose and hemicellulose, and a phenolic polymer, lignin. Since these components can be converted into bioenergy or biomaterials, xylem cells are regarded as a promising sustainable alternative to fossil fuels (Blanch et al. 2008, Yang et al. 2013).

Kubo et al. established an *in vitro* system for xylem vessel element transdifferentiation of Arabidopsis Col-0 suspension cultured cells (Kubo et al. 2005). Through transcriptome analysis, they isolated a number of genes that exhibit drastic changes in expression during transdifferentiation, including those encoding transcription factors and enzymes involved in secondary cell wall biosynthesis and programmed cell death (Kubo et al. 2005). Among these genes, *VASCULAR-RELATED NAC-DOMAIN7* (*VND7*), encoding a NAC-domain transcription factor, was found to be important for xylem vessel element differentiation. Overexpression of *VND7* induces ectopic differentiation of xylem vessel elements, while overexpression of dominant negative forms of *VND7* inhibits the normal differentiation of xylem vessel elements (Kubo et al. 2005, Yamaguchi et al. 2008, Yamaguchi et al. 2010a, Ohtani et al. 2011). These data strongly suggest that VND7 is a master regulator of xylem vessel differentiation, and have prompted further studies of this gene. VND7 regulates the expression of several transcription factors, such as those belonging to the MYB, NAC domain, and LATERAL ORGAN BOUNDARIES DOMAIN (LBD)/ASYMMETRIC LEAVES2 (ASL) families and KNOTTED1-LIKE HOMEODOMAIN PROTEIN7 (KNAT7) (Zhong et al. 2010, Yamaguchi et al. 2011). The expression of *MYB46* and its close homolog, *MYB83*, which regulate many genes related to secondary cell wall formation, was found to be up-regulated directly by VND7 (Zhong et al. 2010, Yamaguchi et al. 2011, Kim et al. 2012a, 2012b, 2013).

VND7 is post-translationally regulated; the stability of VND7 protein is controlled by proteasome-mediated proteolysis (Yamaguchi et al. 2008) and the transcriptional activation activity of VND7 is negatively regulated by VNI2, which encodes a NAC transcription factor (Yamaguchi et al. 2010b). In addition, *VND7*

transcription is strictly regulated in a spatiotemporal manner, suggesting the involvement of upstream transcription factors. Recent research revealed that xylem vessel formation is tightly regulated by a complex transcriptional network (Demura et al. 2007, Caño-Delgado et al. 2010, Ohashi-Ito and Fukuda 2010, Yamaguchi and Demura 2010, Brady et al. 2011, Miyashima et al. 2012). As several members of the Class III HD-ZIP (HD-ZIP III) transcription factor family, such as ATHB-8 and PHBULOSA (PHB), determine vascular patterning, these transcription factors may regulate *VND7* expression (Carlsbecker et al. 2010, Miyashima et al. 2011, Furuta et al. 2012). Soyano et al. (2008) reported that LOB-domain (LBD) transcription factors, LBD18/ASL20 and LBD30/ASL19, up-regulate *VND7* expression and act as components of a positive feedback loop that regulates *VND7* expression. However, because the expression of LBD18/ASL20 and LBD30/ASL19 itself is dependent on VND7 function, information about upstream transcription factors that regulate *VND7* expression is limited. Thus, the regulatory mechanism underlying *VND7* expression is still largely unknown.

In this study, I sought to decipher the transcriptional regulation mechanism underlying *VND7* expression. I screened the 73 transcription factors expressed during xylem vessel element differentiation for their ability to activate *VND7* expression. Dual luciferase assays using the *VND7* promoter showed that a number of transcription factors, including all members of the VND family (VND1 to VND7); ANAC075; two GATA transcription factors, GATA5 and GATA12; LBD18/ASL20; and LBD30/ASL19 have the potential to induce reporter gene expression. Promoter-reporter analysis revealed that A*NAC075*, *GATA5,* and *GATA12* are preferentially expressed in the vascular cylinder of the root tip region, where xylem vessels differentiate. Moreover, electrophoresis mobility shift assays (EMSAs) showed that VND1 to VND7 and GATA12 bind to the *VND7* promoter region. Overexpression of all seven *VND*s, *GATA12,* and *ANAC075* induced transdifferentiation into xylem vessel elements, without upregulating the expression of endogenous *VND7*. In addition, further studies using chemicals and mutants related to the epigenetic control of gene expression showed that *VND7* expression is likely to be repressed by the well-documented epigenetic marks, H3K27me3 and DNA methylation (Feng et al. 2010). Based on these results, I propose a mechanism that regulates *VND7* expression in a cell/tissue-specific manner.

Materials and methods

Plasmid construction

To generate the Gateway destination vector for the dual luciferase (LUC) transient transfection assay, the pA35SG effector plasmid (Yamaguchi et al. 2008) was digested with *Sma*I and ligated into *Eco*RV-digested GATEWAY Reading Frame Cassette (GWRFC) B (Invitrogen, http://www.invitrogen.com/). Likewise, the GAL4UAS:TATA:LUC reporter plasmid containing firefly *LUC* (Ohta et al. 2000) was digested with *Hind*III and *Sma*I, blunted using the BKL Kit (Takara Bio; http://www.takara-bio.com/), and ligated into *Eco*RV-digested GWRFC B. The resultant effector and reporter plasmids were designated as pA35G and pAGL, respectively. The amplified coding sequences (CDSs) of candidate transcription factors and the promoter regions of *VND1* to *VND7* were cloned into the pENTR/D/TOPO vector (Invitrogen; http://www.lifetechnologies.com), and then integrated respectively into the Gateway destination vectors, pA35G and pAGL, using LR Clonase (Invitrogen) (Table 1). For the effector control, the nucleotide sequence of the multi-cloning site (MCS) 5'-CACCTAGTGGATCCCCCGGGCTGCAGGAATTCGATATCAAGCTTATCGATA CCGTCGACCTCGTGATG-3', which includes a stop codon at the 5' end and a start codon at the 3' end, was used (Yamaguchi et al. 2008). The pBIG2113SF effector plasmids (binary vectors) were obtained from the RIKEN Arabidopsis full-length (RALF) cDNA library, which contains full-length cDNAs of the genes listed in Table 2. The pBIG2113SF effector vector was constructed as described previously (Ichikawa et al. 2006). The empty vector pBIG2113SF was used as the effector control (Ichikawa et al. 2006). A reference plasmid containing *Renilla reniformis* LUC was prepared as described in Ohta et al. 2000. For the promoter analysis, the promoter fragments of *GATA5*, *GATA12,* and *ANAC075* (for primer sequences see Table S26) were subcloned into the pENTR/D-TOPO vector, and then integrated into the GATEWAY destination vector, pBGYN (Kubo et al. 2005). To generate the overexpression plants, the CDSs of *VND1* to *VND7*, *GATA5*, *GATA12,* and *ANAC075* were subcloned into the PacI/AscI sites of the pER8 vector, which is part of an estrogen receptor-based chemical-inducible system for use in transgenic plants (Zuo et al. 2000). For the electrophoresis mobility shift assays (EMSAs), the NAC domain region of *VND1* to *VND7* (for the NAC region used in this study, see Table S27) and full-length *GATA12* were subcloned into the

pENTR/D-TOPO vector, and then integrated into the GATEWAY destination vector, pMAL-GWRFC (Yamaguchi et al. 2010b), using LR Clonase (Invitrogen).

Plant materials

Arabidopsis seeds were sterilized with 70% ethanol and Plant Preservative Mixture (PPM; Plant Cell Technology, http://www.plantcelltechnology.com/), and then placed on germination medium (GM) containing Murashige and Skoog (MS) medium, 1% sucrose, 0.05% MES-KOH (pH 5.8), B5 vitamins, and 0.3% phytagel (Sigma). The seeds were then placed at 4°C for 3 to 4 days, and then incubated in a growth chamber under continuous illumination at 22°C. To investigate the effects of 5-aza-2'-deoxycytidine (5-adC) or phytohormones, seedlings of Columbia-0 (Col-0), Landsberg erecta (Ler), mutant, and transgenic plants grown on GM were incubated in solution containing various combinations of phytohormones and 5-adC (Wako: http://www.wako-chem.co.jp) at 22°C for 5 or 6 days.

Transformation of plants

Arabidopsis thaliana (ecotype Col-0) was used as the wild type. Plants were germinated on sterile MS medium (0.6% agar) at 22℃ under continuous light conditions after cold treatment (at 4℃ in the dark for 2 to 3 days). Two to three weeks after germination, plants grown on plates were transferred to soil and further grown in a growth chamber at 22℃ with a photoperiod of 16 h of light and 8 h of darkness. The resultant plasmids were electroporated into *Agrobacterium* strain *GV3101::pMP90*, which in turn was used to transform plants by the floral dip method (Clough et al. 1998).

Dual luciferase transient transfection assay

The effector, reporter, and reference plasmids were delivered to the rosette leaves of 4- or 5-week-old Arabidopsis plants using the Biolistic PDS-1000/He Particle Delivery System (BIO-RAD, http://www.bio-rad.com) and LUC activity was assayed with the Dual-Luciferase Reporter Assay System (Promega, http://www.promega.com) using the Mithras LB940 or LB941 System (Berthold, http://www.bertholdtech.com).

Transient expression assay using *VND7*pro::*β-glucuronidase* (*GUS*) leaves

The effector plasmids were introduced into the rosette leaves of 18-day-old transgenic plants expressing the *GUS* reporter driven by the *VND7* promoter (*VND7pro*::*GUS*; Yamaguchi et al. 2008) by particle bombardment, as described above. After the bombardment, plants were incubated for 4 days at 22°C, and then fixed in 90% (v/v) acetone at -30°C. The leaves were washed with 100 mM sodium phosphate buffer (pH 7.0) three times, and incubated in reaction solution (1 mM 5-bromo-4-chloro-3-indolyl glucuronide, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 100 mM sodium phosphate, pH 7.0) at 37°C for 10 h. After washing with 30% ethanol, the samples were mounted in clearing solution (8 g chloral hydrate, 1 ml glycerol, and 2 ml water) and observed with a microscope equipped with Nomarski optics (BX51, Olympus; http://www.olympus-global.com/).

Electrophoretic mobility shift assay

Promoter fragments were labeled with biotin using the Biotin 3' End DNA Labeling Kit (Thermo, http://www.thermoscientific.com). Labeled probes were separated from unincorporated biotin-dUTP using a MERmaid SPIN Kit (MP-biomedicals, http://www.mpbio.com). The MBP-tagged N-terminal region of VND1 to VND7, containing whole NAC domains, and full-length GATA12 were expressed in *Escherichia coli* strain BL21*trxB* (DE3) and purified as previously described in Yamaguchi et al. (2010b). Approximately 20 fmol of biotinylated promoter fragments were incubated in reaction buffer (LightShift EMSA Optimization and Control Kit; Thermo) for 30 minutes at 4°C with 5 pmol of the purified recombinant protein for VND1-VND7 and 1, 2, 4, 6, and 8 pmol of the purified recombinant protein for GATA12 and/or an excess of unlabeled fragments as competitors. Protein bound to biotinylated DNA fragments was separated by polyacrylamide gel electrophoresis. The DNA was electroblotted onto positively charged nylon membrane (Hybond-N+; GE Health care, http://www.gelifesciences.com) and detected using the LightShift Chemiluminescent EMSA Kit, according to the manufacturer's instructions.

Real-Time PCR

Total RNA was prepared using the RNeasy Plant Mini Kit (Qiagen; http://www.qiagen.com) and RQ1 RNase-Free DNase (Promega; http://www.promega.com). cDNA synthesis was performed using oligo(dT)20 primer and Transcriptor Reverse Transcriptase (Roche; http://www.roche.com). Real-time PCR was performed using a Light Cycler 480 II and Light Cycler 480 SYBR Green I Master (Roche), as described in the manufacturer's protocols. The sequences of primers used for real-time PCR are presented in Table S26. *UBQ10* cDNA served as an internal control for all experiments.

Microscopy analysis

For all mounted plant observations, seedlings were fixed with 90% acetone for more than 1 week at -30℃. Samples were mounted on clearing solution (8 g of chloral hydrate, 1 mL of glycerol, and 2 mL of water) before observation. Nomarski images were captured using a polarizing microscope (BX51; Olympus) equipped with a digital camera (DP70; Olympus). To observe the roots of *VND7*pro*:YFP-NLS* (Kubo et al. 2005, Yamaguchi et al. 2008), *GATA5*pro*:YFP-NLS*, *GATA12*pro*:YFP-NLS*, and *ANAC075*pro*:YFP-NLS* plants, the samples were counterstained with propidium iodide and observed with a laser scanning confocal microscope (Zeiss AX10 observer. Z1) equipped with a digital camera (Zeiss LSM 710).

Analysis of DNA methylation levels using McrBC-PCR

DNA was extracted from two 7-day-old Col-0 seedlings treated with or without 1 μg/ml of 5-adC (Wako; http://www.wako-chem.co.jp) using a NucleoSpin Plant II Kit (MACHEREY-NAGEL: http://www.mn-net.com). Then, 60 ng of DNA was digested with 20 U of McrBC (New England Biolabs) and incubated for 12 h at 37℃, or incubated without enzyme in the same total volume and under the same conditions. Digested and undigested DNA samples were then subjected to PCR analysis using the primers listed in Table S26.

Chromatin immunoprecipitation and quantitative PCR

ChIP experiments were carried out as described by Helliwell et al. (2006) with modifications. Five-day-old Col-0 seedlings (0.25 g) were glaciated with liquid nitrogen and ground in a mortar. Chromatin was crosslinked by formaldehyde. Samples were then sonicated twice and centrifuged for 15 minutes at 20,000 g. The resulting supernatant containing mononucleosomes was incubated with or without Anti-trimethyl-Histone H3 (Lys27) Antibody (Millipore, http://www.millipore. com) for 2 h. Immunoprecipitation was carried out using the MAGnify ChIP System (Invitrogen, http://www.invitrogen.com). The supernatant containing chromatin was incubated with Dynabeads Protein G (Invitrogen) for 2 h at 4°C with rotation. The samples were then washed with lysis buffer (1 M HEPES (pH7.5), 2 M NaCl, 10% Triton X-100, 10% deoxycholate, 10% SDS), LNDET buffer (0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA) and twice with TE buffer for 5 min at 4°C. The protein-DNA crosslinks were reversed by heating at 65°C and the DNA was eluted with 300 µl of proteinase K in 200 mM NaCl, 40 mM Tris–HCl (8.0), and 10 mM EDTA at 55°C for 15 min. The supernatant was further incubated at 95°C for 15 min. DNA was isolated using a Qiagen PCR Clean-up Kit. Real-time quantitative PCR was carried out using SYBR Green and Platinum Taq DNA Polymerase (Invitrogen), according to the manufacturer's instructions. The reactions were carried out on a 7900HT Fast Real-Time PCR System (Applied Biosystems; http://www.appliedbiosystems.com). Primers used for qPCR analysis are listed in Table S26. The data were analyzed using the Absolute Quantification program (Applied Biosystems; http://www.appliedbiosystems.com) and Arabidopsis cDNA isolated from five-day-old Col-0 seedlings was used as the standard.

Results and Discussion

Part I

Identification of the transcription factors that regulate the expression of *VND7*

Results I

1-1. Selection of transcription factor genes that are expressed during vessel element differentiation

Previously Kubo et al. established an *in vitro* system for xylem vessel element transdifferentiation of *Arabidopsis* Col-0 suspension cultured cells, which was used to analyze the gene expression profile during the induction of xylem vessel element differentiation (Kubo et al. 2005). *VND7*, encoding a NAC domain transcription factor, was identified as being upregulated during xylem vessel element differentiation. Functional characterization revealed that VND7 directly or indirectly regulates a number of downstream target genes involved in xylem vessel differentiation in planta and established this transcription factor as a master regulator of xylem vessel differentiation (Kubo et al. 2005, Yamaguchi et al. 2008). However, the upstream regulatory mechanism underlying *VND7* expression remained largely unknown. To elucidate the mechanism, transcription factors that regulate *VND7* expression must be identified. Thus, I selected 73 transcription factor genes whose expression peaks at the same time or before *VND7* expression peaks in the *in vitro* differentiation system (Table 1, 2).

I obtained the CDSs of 26 of these transcription factors from the RIKEN full-length cDNA library and subcloned these fragments into a binary vector (pBIG2113SF) downstream of the *CaMV35S* promoter (Chikawa et al. 2006). I amplified the CDSs of the remaining 47 transcription factors from cDNAs derived from Arabidopsis seedlings and subcloned these fragments into a transient expression vector (pA35GS) downstream of the cauliflower mosaic virus 35S (*CaMV35S*) promoter. The resulting constructs were used as effector constructs in transient expression assays.

1-2. Identification of transcription factors that induce *VND7* promoter activity using a transient expression assay

To isolate the transcription factors that upregulate *VND7* expression, I performed a dual luciferase assay according to a previously published technique (Mitsuda and Ohme-Takagi 2009). I first confirmed that a 1000-bp promoter region of *VND7* flanked

by a 9-bp coding region was sufficient to induce xylem vessel-specific expression of a yellow fluorescent protein (YFP) reporter in the root (Fig. 1C). Then, I constructed a reporter construct that contained this region of the *VND7* promoter fused to firefly luciferase (LUC) (Fig. 1B). I delivered this reporter construct together with one of the effector constructs containing CDSs of the candidate transcription factors and a reference construct harboring *Renilla* LUC downstream of the *CaMV35S* promoter into *Arabidopsis* rossette leaves by particle bombardment. Fifteen of the candidate transcription factors induced firefly LUC activity (>3-fold compared with the control), and the increase was statistically significant for 11 of these (Welch's t-test, p-value < 0.05; Fig. 2; Table 1, 2; Table S1-25). Among these, GATA5, GATA12, and ANAC075 were identified as novel candidate transcription factors that regulate xylem vessel formation. I confirmed these results by transiently expressing the candidate transcription factors in the leaves of Arabidopsis plants stably expressing *VND7*pro::*GUS*, in which the 2-kb promoter region of *VND7* was fused to *GUS* (Yamaguchi et al. 2008), by particle bombardment. All of the candidate transcription factors induced GUS activity, indicating that they indeed activate *VND7* expression (Fig. 3, Table 3).

Moreover, I found that all *VND* genes strongly upregulated the *VND7* promoter activity (Table 2, 3, Fig. 3). Furthermore, the expression pattern of *VND7* overlapped with that of other *VND* genes (Kubo et al. 2005, Yamaguchi et al. 2008). These results suggest that *VND* family genes mutually regulate each other's expression during xylem vessel formation. To test this possibility, I performed a dual luciferase assay using all of the *VND* promoters as reporters and all of *VND* genes as effectors for all possible combinations. Although reporter gene expression driven by the *VND3*, *VND5,* and *VND6* promoters was preferentially elevated by a certain set of *VND* genes, the up-regulation of *VND7* promoter activity by VND genes was the most striking (Fig. 4).

1-3. *GATA5*, *GATA12,* and *ANAC075* are expressed in the vascular cylinder of roots

To investigate the spatial expression of the transcription factors identified as candidate genes that positively regulate *VND7* expression, I generated transgenic lines expressing YFP fused to the SV40 nuclear localization signal (*YFP-NLS*) under the control of the

GATA5, *GATA12, ANAC075,* or *VND7* promoter (Yamaguchi et al. 2008, 2010b). As previously reported (Yamaguchi et al. 2008, 2010b), *VND7pro::YFP-NLS* was specifically detected in the differentiating xylem vessels in the root tip region (Fig. 5A). By contrast, *GATA5pro::YFP* signal was detected in the root stele from the meristematic zone upward, while *GATA12pro::YFP-NLS* and *ANAC075pro::YFP-NLS* signals were detected in the root stele from the distal elongation zone upward (Fig. 5B-D). Microscopy observations indicated that all three transcription factors were expressed prior to the onset of *VND7* expression during vascular development in the root. To analyze the spatial expression pattern of these transcription factors in more detail, I observed the roots of the transgenic plants with confocal microscopy. As in the case of *VND7*, all three transcription factors were expressed in differentiating protoxylem vessel elements, which have a spiral secondary cell wall structure, and in putative precursors of metaxylem vessel elements located between two protoxylem vessels (Fig. 6A-D).

1-4. Overexpression of *VND1* to *VND7, GATA12,* and *ANAC075* causes ectopic xylem vessel element formation

To investigate the roles of ten of the identified transcription factors, I generated transgenic plants overexpressing *GATA5*, *GATA12*, *ANAC075,* and all seven *VND*s under the control of an estradiol-mediated induction system (Zuo et al. 2000). Ectopic xylem vessel elements with thickened secondary cell walls were induced by application of β-estradiol in the transgenic plants exogenously overexpressing all *VND* genes, *GATA12,* and *ANAC075* (Fig. 7), but not in the transgenic plants overexpressing *GATA5* (data not shown). Safranin-O staining revealed that these secondary cell walls were lignified (Fig. 8). Notably, ectopic xylem vessel elements were observed in the leaves, hypocotyls, and roots of inducible lines harboring the *VND* genes and *ANAC075* (Fig. 7B-H and J, 8B-H and J), but only in the roots in the *GATA12* inducible line (Fig. 7I, 8I), suggesting that GATA12 has a lower ability to induce transdifferentiation of xylem vessel elements than do the other tested transcription factors.

To further investigate whether overexpression of the transcription factors that induce ectopic xylem vessel element formation upregulates the mRNA levels of endogenous *VND7*, I analyzed the expression of *VND7* and its downstream target genes,

LBD30/ASL19, *MYB46*, *XYLEM CYSTEINE PROTASE1* (*XCP1*), and *CELLULOSE SYNTHASE A7* (*CESA7*) (Zhong et al. 2010, Yamaguchi et al. 2011), using quantitative RT-PCR analysis. RNA was extracted from 5-day-old seedlings treated with or without β-estradiol for 24 h (Fig. 9). Surprisingly, the mRNA levels of endogenous *VND7* were not markedly changed by overexpression of any of the analyzed transcription factors (Fig. 9A-H). By contrast, the mRNA levels of the downstream genes were upregulated 24 h after induction of the *VND* genes, whereas only *MYB46* was upregulated by *ANAC075* induction and none of the target genes was upregulated by *GATA12* (Fig. 9), presumably reflecting differences in the frequency of ectopic xylem vessel element formation in each overexpression lines (Fig. 7).

1-5. VND1 to VND7 bind to the *VND7* promoter region *in vitro*

VND6 and VND7 were reported to regulate downstream gene expression through *cis*-elements termed secondary wall NAC-binding elements (SNBEs) or tracheary element-regulating *cis*-elements (TEREs) (Pyo et al. 2007, Ohashi-Ito et al. 2010, Zhong et al. 2010, Yamaguchi et al. 2011). Expression analysis suggested that overexpression of *VND1* to *VND5* upregulated the direct target genes of VND6 and VND7 in the absence of an increase in endogenous *VND7* expression (Fig. 9A-G). These data suggest that VND1 to VND5 recognize and bind to the SNBE or TERE motifs. Interestingly, the *VND7* promoter region, used in the dual LUC assay, contains a SNBE and TERE-like motif (SNBE/TERE-like motif; -425 to -408) (Fig. 10, 11I; Pyo et al. 2007, Zhong et al. 2010). To test whether VND1 to VND7 directly bind to the *VND7* promoter, I conducted electrophoresis mobility shift assays (EMSAs) in which I combined a biotinylated 603-bp *VND7* promoter fragment (-1 to -603 bp relative to the start codon) with maltose binding protein-tagged (MBP-tagged) N-terminal regions of VND proteins that include the whole NAC domain (Table S27). When the probe was incubated with any of the VND proteins, one or more shifted bands were detected (Fig. 11A-G). These shifted bands were significantly reduced but not completely eliminated by the application of an excess amount (x 200) of the 33-bp DNA fragment of the *VND7* promoter, containing the SNBE/TERE-like motif (-428 to -396) (Fig. 11A-G).

To obtain direct evidence that VND proteins bind to the 33-bp fragment of the *VND7* promoter, the 33-bp fragment was biotinylated and used as a probe in EMSAs. In

the presence of MBP-VND3 (as a representative of VND1 to VND6) and MBP-VND7 (as a possible positive control known to bind to the TERE motif; Yamaguchi et al. 2011), a shifted band was observed and the application of an excess amount (x200) of unlabeled fragment strongly decreased the signal (Fig. 12). Moreover, when a mutated 33-bp fragment harboring three nucleotide substitutions in the SNBE/TERE-like motif was used as a competitor, the shifted bands were clearly detected (Fig. 12). These results suggest that VND proteins bind to the SNBE/TERE motif located in the promoter region of *VND7*.

Furthermore, I found that the *VND7* promoter also contains a putative GATA-binding motif (AG-motif; AGATCCAA) previously described in *Nicotiana tabacum* GATA16 (AGP1; Sugimoto et al. 2003) in the region spanning -411 to -404 bp (Fig. 11I). The amino acid sequence of the GATA DNA-binding domain of the reported AGP1 and Arabidopsis GATA12 exhibit high levels of similarity (Fig. 13). Interestingly, the AG-motif was shown to overlap with the SNBE/TERE-like motif (-425 to -408 bp) (Fig. 11I). Based on these findings, I performed an EMSA using N-terminal MBP-tagged full-length GATA12 (MBP-GATA12). When the biotinylated 603-bp *VND7* promoter fragment was incubated with GATA12, free probe disappeared, but no clearly shifted bands were detected (Fig. 11H). The application of an excess amount (x 1000) of 33-bp DNA fragment containing the AG-motif did not restore the appearance of free probe (Fig. 11H, I). These data suggest that GATA12 binds to the *VND7* promoter region, but that its target site is not within the 33-bp region harboring the AG-motif. To further examine whether GATA12 binds to the *VND7* promoter, I performed an EMSA using a series of different concentrations of GATA12 protein (Fig. 14). I found that the level of biotinylated 603-bp *VND7* free probe declined as the concentration of GATA12 proteins increased, and shifted bands appeared (Fig. 14).

Discussion I

In this study, I aimed to identify the transcription factors that regulate the expression of *VND7* using a transient expression assay. Interestingly, all of the *VND* family genes strongly induced VND7 promoter activity (Table 2, Fig. 4). However, the transactivation activity of VND7 for other *VND* genes was not strong (Fig. 4). Moreover, the transient expression of VNDs in *VND7*pro::*GUS* transgenic plants showed that all VNDs induced ectopic GUS activity in the leaf epidermal cells (Fig. 3, Table 3), suggesting that VNDs induce the activity of the *VND7* promoter, which was exogenously integrated into the genome *in vivo*. All of these results suggest that, among the *VND* gene family, *VND7* is the main target of VND transcription factors.

I identified *LBD15/ASL11*, *LBD30/ASL19,* and *LBD18/ASL20* in the dual luciferase assay and showed that they have the ability to induce *VND7* promoter activity *in vivo* (Table 2, 3). Whereas all of these genes were reported to be downstream targets of VND7 (Zhong et al. 2010, Yamaguchi et al. 2011), *LBD30/ASL19* and *LBD18/ASL20* were shown to maintain *VND7* expression during xylem vessel element differentiation via a feedback pathway (Soyanoa et al.2008) and *LBD15/ASL11* and *LBD30/ASL19* were shown to be direct targets of VND7 (Ohashi-Ito et al. 2010, Zhong et al. 2010, Yamaguchi et al. 2011). In addition, *SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN 2* (*SND2*), which is known to be upregulated by NST3/SND1, a master regulator of fiber cell differentiation (Zhong et al. 2008), also induced *VND7* promoter activity (Table 2, 3). Since *SND2* is expressed not only in fiber cells but also in xylem vessels, it is plausible that *SND2* expression is positively regulated not only by NST3/SND1 but also by VND7. Thus, I propose that these four transcription factors are involved in a positive feedback loop that regulates *VND7* expression during xylem vessel differentiation.

In the dual luciferase assay, I also identified REV as a candidate regulator of *VND7* expression (Table 2, 3). This protein belongs to the Class III HD-Zip transcription factor family and is known to function with several bHLHs, such as LHW and TMO5, in the initiation of vasculature and differentiation of xylem vessels (Baima et al. 2001, Carlsbecker et al. 2010, Miyashima et al. 2011, Furuta et al. 2012, De Rybel et al. 2013, Ohashi-Ito et al. 2013a, 2013b). However, other HD-ZipIII genes did not exhibit any significant induction of *VND7* expression (Table 2) and the promoter region

of *VND7* was not detected in a genome-wide binding-site analysis of *REV* (Brandt et al. 2012), suggesting that members of the HD-ZipIII gene family do not directly regulate *VND7* expression, but rather enhance its expression by promoting vascular development. Class III HD-Zip genes are known to be regulated by microRNA165 and 166 (miR165/166) (Carlsbecker et al. 2010, Miyashima et al. 2011). To overcome the post-transcriptional inhibition of *REV* expression, I used a microRNA resistant version of *REVOLUTA* (*REV*avb) (Zhong and Ye 2004) as an effector in my assay. I found that REVavb did not induce *VND7* promoter activity (Table 2).

Furthermore, I identified three previously undescribed transcription factors that induce *VND7* promoter activity, namely a NAC domain-containing transcription factor, ANAC075, and two GATA domain-containing transcription factors, GATA5 and GATA12 (Table 2, 3). *ANAC075* was proposed as a candidate gene that controls cell wall development based on the findings of an *in silico* analysis (Shen et al. 2009). Although ANAC075 is one of the closest homologs of SND2, *ANAC075* has not been reported as a downstream target of *VND* or *NST* (Ohashi-Ito et al. 2010, Zhong et al. 2010, Yamaguchi et al. 2011), suggesting that ANAC075 might not be included in the positive feedback loop that regulates *VND7* expression. Twenty-nine GATA domain-containing transcription factors have been identified in Arabidopsis. Although this gene family has been implicated in various biological functions, such as embryogenesis, morphogenesis, circadian clock regulation, light responses, seed dormancy, and hormone crosstalk (Liu et al. 2005, Manfield et al. 2007, Luo et al. 2010, Nawy et al. 2010, Kanei et al. 2012, Zhang et al. 2013), it has not been shown to function in vascular development. According to a previous report, *GATA5* and *GATA12* both belong to sub-family I of the GATA domain-containing transcription factors, and clustering analysis based on expression patterns showed that they are grouped into the same expression clade (Manfield et al. 2007). In addition to *GATA5* and *GATA12*, *GATA10* is also included in this sub-family and expression clade (Manfield et al. 2007). I showed that *GATA5* and *GATA12* expression peaks before *VND7* during *in vitro* xylem vessel differentiation, while *GATA10* expression is not significantly changed (Fig. 15). These data suggest that GATA5 and GATA12 are the only members of the sub-family I to contribute to xylem vessel formation.

I also showed that the four identified regulators of *VND7* are expressed in differentiating protoxylem vessel elements and the metaxylem pole (Fig. 6B-D). It has

been reported that *VND1* to *VND6*, *LBD18*/*ASL20,* and *LBD30*/*ASL19* are expressed in the procambial zone, protoxylem, and/or metaxylem vessels (Kubo et al. 2005, Yamaguchi et al. 2008, Soyanoa et al 2008). These data and previous reports suggest that the transcription factors isolated in my screen may coordinately regulate *VND7* expression during xylem vessel formation.

I demonstrated ectopic xylem vessel element formation in transgenic plants overexpressing each of the *VND* genes (Fig. 7B-H, 8B-H). However, endogenous *VND7* expression was not markedly upregulated in these transgenic plants, suggesting that *VND* overexpression upregulates downstream target genes independently of upregulating endogenous *VND7* expression, still inducing transdifferentiation. Several research groups have reported that secondary cell wall-related NAC transcription factors bind to *cis*-elements termed secondary wall NAC-binding elements (SNBEs) or tracheary element-regulating *cis*-elements (TEREs) (Pyo et al. 2007, Zhong et al. 2010). These elements exist in the promoter region of the direct target genes of VND7, including *LBD30*, *MYB46,* and *XCP1* (Zhong et al. 2010, Yamaguchi et al. 2011). It is likely that VND1 to VND5 also bind to these *cis*-elements and cooperatively regulate xylem vessel differentiation together with VND7. In the transgenic plants overexpressing *GATA12*, neither *VND7* nor its downstream targets were upregulated, whereas ectopic xylem vessel elements were observed (Fig. 7I, 8I). Further analyses, including the isolation of target genes of GATA12 during xylem vessel formation, are needed to establish the molecular function of GATA12. Since LBD30 was shown to participate in a positive feedback loop regulating *VND7* expression (Soyanoa et al 2008), it could be expected that the upregulated expression of *LBD30* caused by the overexpression of *VND* genes positively regulates *VND7* expression. However, endogenous *VND7* expression was not markedly upregulated in lines overexpressing *VND* genes (Fig. 9A, B, C, D, F). This might be simply because the expression level of *LBD30* was not enough to drive the positive feedback loop. Further analysis is needed clarify the feedback mechanisms governing *VND7* expression.

I also examined whether VND1 to VND7 could directly bind to the *VND7* promoter using EMSA. These results showed that VND1 to VND7 do indeed bind to the *VND7* promoter (Fig. 11A-G). Moreover, most of the samples showed multiple bands (Fig. 11B, D, E, F, G). Since VND7 forms homodimers and/or heterodimers with other VND proteins (Yamaguchi et al. 2008), it is possible that the other VND proteins

also form homo- or heterodimers, and that the multiple bands are due to the formation of dimers. Furthermore, when a mutated fragment of the *VND7* promoter with three nucleotide substitutions in the SNBE/TERE-like motif was used as a competitor, shifted bands were still observed for both VND3 and VND7 (Fig. 12A, B). These results suggest that VND proteins bind to the SNBE/TERE motif and directly regulate the expression of genes involved in xylem vessel differentiation through the SNBE or TERE motif. In addition, I showed that GATA12 binds to the *VND7* promoter region, since the level of *VND7* free probe decreased with increasing concentrations of GATA12 (Fig. 14). Therefore, GATA12 also directly regulates the expression of *VND7,* and probably in a cell type-specific manner, since endogenous *VND7* mRNA was not upregulated in the seedlings of the overexpressors (Fig. 9H).

In this part of my study, I succeeded in isolating 15 transcription factors that could potentially regulate the expression of *VND7*, a master regulator of vessel formation. Three of the 15 transcription factors had not previously been reported to be involved in vascular development. Furthermore, LBD18/ASL20, LBD30/ASL19, LBD15/ASL11, and SND2 seem to form a positive feedback loop that regulates *VND7* expression. Moreover, all seven VND proteins directly regulate *VND7* expression. In addition, my results suggest that VND1 to VND5 promote xylem vessel formation independently of the upregulation of endogenous *VND7* expression (Fig. 7B-H, 8B-H, 9A-F). Signaling molecules often regulate vascular development and vascular specification by controlling the expression of specific transcription factors (Demura and Fukuda 2007, Ohashi-Ito and Fukuda 2010, Miyashima et al. 2012). Recent advances in our understanding of vascular development show that multiple types of signaling molecules regulate vascular development. For instance, Okushima et al. (2007) and Soyano et al. (2008) reported that *LBD18/ASL20* expression is regulated by auxin. The expression of transcription factors newly identified in this study might also be regulated by some molecular signal. To decipher how xylem vessel elements are specified, it is important to characterize the identified transcription factors in detail. The results obtained in this study also suggest that certain additional unknown factors would strongly repress *VND7* expression in non-xylem cells (Fig. 15), since overexpression of *VND1* to *VND6* and *GATA12* could not induce the expression of endogenous *VND7,* even though they had the ability to bind to the *VND7* promoter and induce its activity (Table 2, 3, Fig. 3, 11, 12, 14). Thus, additional undefined factors are required for the

precise regulation of *VND7* expression *in planta*.

Table 1. List of transcription factors used in the dual luciferase assay (pBIG2113SF vector)

Category	AGI no.	Description	Fold change[a]	S.D.[b]	p-value[c]
AP2	*At5g05410*	DREB2	**1.00**	0.50	0.9527
	At5g25190	AP2/ERF	**1.01**	0.49	0.9549
	At5g61600	AP2/ERF	**1.02**	0.24	0.9426
bHLH	*At4g36540*	BEE2	**0.96**	0.44	0.8836
	At3g26744	ICE1/SREAM/SCRM	**0.92**	0.50	0.9034
bZIP	*At5g15830*	ATBZIP3	**0.74**	0.35	0.3106
Homeobox	*At5g41410*	BEL1/BELL 1/ MYC6.12	**0.49**	0.11	0.0478
MADS	*At3g02310*	SEPALLATA2	**0.93**	0.36	0.8619
MYB	*At1g22640*	MYB3	**0.67**	0.08	0.3961
	At5g16600	MYB43	**1.08**	0.17	0.8869
NAC	*At1g28470*	SND3	**0.45**	0.07	0.0408
	At1g34190	ANAC017	**0.39**	0.04	0.0399
	At1g77450	ANAC032	**0.64**	0.07	0.1010
	At3g04420	ANAC048	**0.63**	0.09	0.0839
	At4g28530	ANAC074	**0.93**	0.23	0.6943
	At5g39610	ANAC092/ATNAC2/ATNAC6	**0.73**	0.39	0.3824
	At5g13180	VNI2	**0.83**	0.25	0.6366
TCP	*At2g31070*	TCP10	**1.29**	0.81	0.6210
WRKY	*At2g30590*	WRKY21	**1.00**	0.41	0.9663
	At3g04670	ATWRKY39/WRKY39	**0.49**	0.27	0.1282
	At2g46400	ATWRKY46/WRKY46	**0.57**	0.17	0.1639
zf-C2H2	*At1g66140*	ZFP4	**0.60**	0.21	0.2716
zf-C3HC4	*At1g26800*	zinc finger C3HC4	**0.52**	0.29	0.1449
	At3g23060	zinc finger C3HC4	**0.57**	0.39	0.2310
	At5g08750	zinc finger C3HC4	**0.36**	0.09	0.0810
zf-Dof	*At1g64620*	Dof-type zinc finge	**0.83**	0.08	0.6224

[a] Fold change = relative luciferase activity

[b] S.D. = standard deviation of three replicates

[c] p-value = Welch's t-test; numbers in blue $P < 0.05$

Table 2. List of transcription factors used in the dual luciferase assay (pA35G vector)

Category	AGI no.	Description	Fold change[a]	S.D.[b]	p-value[c]
ARF	*At1g19850*	MP/ARF5	**0.87**	0.07	0.6725
bHLH	*At1g63650*	ATMYC-2/ EGL1/ EGL3	**1.50**	0.50	0.3793
	At1g35460	bHLH	**0.82**	0.23	0.4692
	At1g68810	TMO5-like1	**2.11**	1.11	0.2203
	At1g51140	bHLH	**0.84**	0.14	0.5339
	At1g29950	bHLH	**1.49**	0.27	0.1796
	At3g56980	ORG3	**0.95**	0.24	0.3812
	At5g64340	SAC51	**0.74**	0.22	0.4092
	At5g09460	SAC51like	**0.83**	0.09	0.5466
bZIP	*At5g49450*	ATBZIP1	**2.64**	0.36	0.0082
CCAT	*At4g14540*	NF-YB3	**2.01**	1.60	0.3889
GATA	*At5g66320*	GATA5	**10.32**	1.39	0.0056
	At5g25830	GATA12	**7.93**	2.72	0.0454
Homeobox	*At1g62990*	KNAT7	**0.92**	0.14	0.7769
	At5g06710	HAT14	**1.43**	0.05	0.0587
	At4G32880	AtHB8	**2.00**	0.70	0.1578
	At1g52150	AtHB15	**2.30**	0.90	0.1141
	At2g34710	PHB	**1.55**	0.46	0.1631
	At1g30490	PHV	**1.82**	0.27	0.0176
	At5g60690	REV	**3.80**	0.90	0.0150
	At5g60690	REVavb[d]	**0.96**	0.19	0.9364
LBD	*At2g40470*	LBD15	**12.27**	4.34	0.0459
	At2g45420	LBD18	**5.92**	3.24	0.1182
	At4g00220	LBD30	**7.10**	2.00	0.0224
	At1g31320	LBD4	**1.24**	0.26	0.4532
MYB	*At3g10760*	MYB	**0.98**	0.23	0.9253
	At2g38090	MYBlike	**0.47**	0.27	0.0881
	At1g69580	MYBlike	**1.11**	0.54	0.7851
NAC	*At4g28500*	SND2	**3.39**	0.59	0.0088
	At4g29230	ANAC075	**6.70**	1.71	0.0277
	At5g64530	XND1	**2.09**	0.46	0.0419
	At2g18060	VND1	**61.33**	10.37	0.0095
	At4g36160	VND2	**30.42**	4.44	0.0067
	At5g66300	VND3	**41.87**	21.19	0.0789
	At1g12260	VND4	**14.73**	6.87	0.0730
	At1g62700	VND5	**62.70**	30.50	0.0348
	At5g62380	VND6	**47.71**	18.89	0.0502
	At1g71930	VND7	**32.85**	0.94	0.0000
NF-YC2	*At1g56170*	HAP5B	**0.60**	0.19	0.1361
TUB	*At1g76900*	ATTLP1	**0.73**	0.44	0.4386
	At1g43640	ATTLP5	**0.80**	0.53	0.6048
zf-C2H2	*At1g67030*	ZFP6	**2.02**	0.46	0.0457
	At5g03510	Zinc finger C2H2	**0.47**	0.16	0.1288
zf-C3HC4	*At5g55970*	MDA7.1	**0.66**	0.22	0.2819
	At5g17600	RING-H2 zinc finger	**0.82**	0.18	0.3283
	At1g72220	RING-H2 zinc finger-ATL3	**1.82**	0.91	0.2445
zf-Dof	*At5g60200*	TMO6/Dof5.3	**1.57**	0.54	0.2352
	At5g66940	Dof5.8	**0.59**	0.34	0.1901

[a] Fold change = relative luciferase activity; numbers in red Fold > 3

[b] S.D. = standard deviation of three replicates

[c] p-value = Welch's t-test; numbers in blue $P < 0.05$

[d] REVavb = REVOLUTA microRNA RESISTANT VERSION (Zhong and Ye 2004)

Table 3. Summary of transient expression assay using *VND7pro*::*GUS* plants

Effector		Cell type	Leaf No.																	Frequency (No. positive leaves / total)
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
Control	MCS	SCW (A + C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	0 / 15
		GUS (B + C)	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	-	-	1 / 15
		SCW/GUS (C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	0 / 15
VND1	At2g18060	SCW (A + C)	0	0	0	0	0	0	2	0	0	1	4	2	3	2	0	-	-	6 / 15
		GUS (B + C)	0	0	0	0	0	0	1	0	0	1	1	1	2	0	0	-	-	5 / 15
		SCW/GUS (C)	0	0	0	0	0	0	0	0	0	0	1	1	2	0	0	-	-	3 / 15
VND2	At4g36160	SCW (A + C)	0	0	0	0	5	3	2	2	0	0	0	0	0	0	0	-	-	4 / 15
		GUS (B + C)	0	0	0	0	2	1	2	2	0	0	0	1	1	0	0	-	-	6 / 15
		SCW/GUS (C)	0	0	0	0	2	1	2	2	0	0	0	0	0	0	0	-	-	4 / 15
VND3	At5g66300	SCW (A + C)	2	3	2	0	0	1	0	0	5	3	0	1	3	3	2	-	-	10 / 15
		GUS (B + C)	2	2	3	0	0	1	0	1	2	2	0	3	4	2	3	-	-	11 / 15
		SCW/GUS (C)	2	2	1	0	0	1	0	0	1	2	0	1	3	2	2	-	-	10 / 15
VND4	At1g12260	SCW (A + C)	1	1	0	0	0	0	0	0	3	3	0	0	0	4	2	-	-	6 / 15
		GUS (B + C)	1	1	0	0	0	0	1	0	3	2	1	0	3	1	1	-	-	9 / 15
		SCW/GUS (C)	1	1	0	0	0	0	0	0	2	2	0	0	0	0	0	-	-	4 / 15
VND5	At1g62700	SCW (A + C)	0	3	0	0	0	1	0	2	0	2	2	8	5	4	1	8	-	10 / 16
		GUS (B + C)	0	2	0	0	0	1	0	4	0	2	1	7	4	3	1	6	-	10 / 16
		SCW/GUS (C)	0	2	0	0	0	1	0	2	0	2	1	7	4	3	1	6	-	10 / 16
VND6	At5g62380	SCW (A + C)	2	0	8	0	0	5	9	2	1	9	3	0	3	5	0	0	-	10 / 16
		GUS (B + C)	1	0	1	0	0	3	2	1	0	5	2	0	5	1	1	1	-	11 / 16
		SCW/GUS (C)	1	0	1	0	0	3	2	1	0	4	1	0	2	1	0	0	-	9 / 16
VND7	At1g71930	SCW (A + C)	3	0	0	2	2	2	4	2	3	14	0	4	0	0	8	4	-	11 / 16
		GUS (B + C)	0	0	0	0	1	0	0	0	3	6	2	1	0	0	1	1	-	7 / 16
		SCW/GUS (C)	0	0	0	0	1	0	0	0	1	3	0	1	0	0	1	1	-	6 / 16
GATA5	At5g66320	SCW (A + C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	0 / 15
		GUS (B + C)	0	1	2	1	0	0	1	2	0	3	0	1	0	0	0	-	-	7 / 15
		SCW/GUS (C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	0 / 15
GATA12	At5g25830	SCW (A + C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0 / 17
		GUS (B + C)	0	0	0	1	1	2	0	1	0	0	0	0	0	0	1	1	4	7 / 17
		SCW/GUS (C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0 / 17
LBD15	At2g40470	SCW (A + C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	0 / 15
		GUS (B + C)	0	0	1	0	1	0	0	2	1	0	0	2	0	1	3	-	-	7 / 15
		SCW/GUS (C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	0 / 15
LBD30	At4g00220	SCW (A + C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	0 / 16
		GUS (B + C)	0	0	0	1	0	0	1	0	0	0	1	2	1	1	0	4	-	7 / 16
		SCW/GUS (C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	0 / 16
ANAC075	At4g29230	SCW (A + C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	0 / 16
		GUS (B + C)	7	0	1	0	1	3	3	2	4	0	3	1	5	6	1	1	-	13 / 16
		SCW/GUS (C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	0 / 16
REV	At5g60690	SCW (A + C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	0 / 15
		GUS (B + C)	0	0	0	0	2	0	0	0	0	1	0	0	0	0	2	-	-	3 / 15
		SCW/GUS (C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	0 / 15
SND2	At4g28500	SCW (A + C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	0 / 15
		GUS (B + C)	0	0	0	1	0	1	0	0	0	0	0	0	1	4	1	-	-	5 / 15
		SCW/GUS (C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	0 / 15

The number of cells with patterned secondary cell wall formation (SCW) and / or VND7pro::GUS activity (GUS) in each leaf. A, B, C in "Cell type" colum indicate (A) cell with patterned SCW, (B) cell with GUS activity and (C) cell with both SCW and GUS activity. A detailed description of cell types provided in Fig. 3. Blue, yellwow and green color shadeing indicates leaf that has cells with SCW, GUS or both SCW and GUS, respectively.

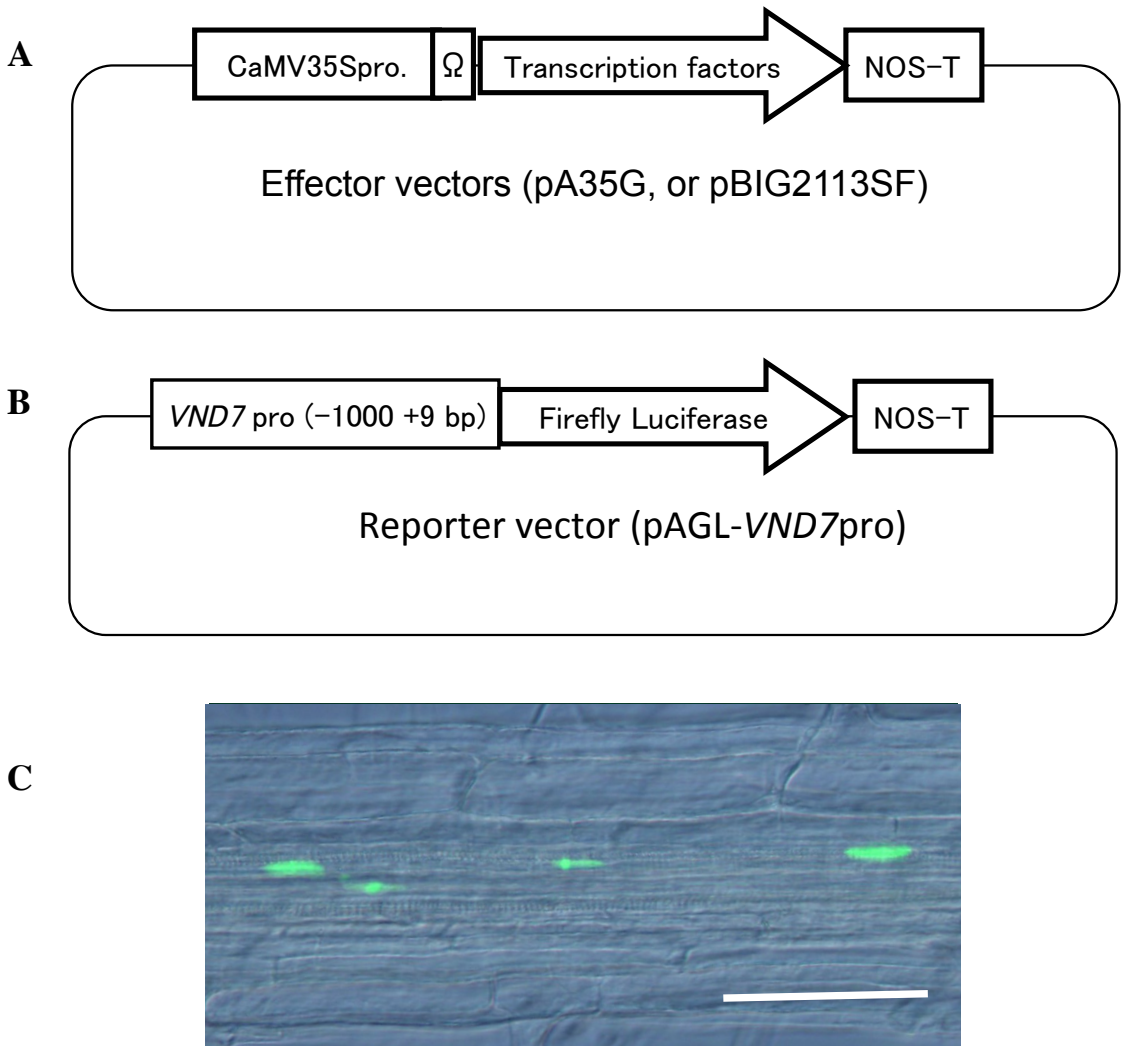


Fig. 1. Schematic diagrams of vectors used in the dual luciferase reporter assay and confirmation that a 1000-bp promoter region of *VND7* induces xylem vessel-specific expression. (A) The effector vector and (B) reporter vector. (C) Expression pattern of *VND7pro::YFP-NLS*. Images of differential interference contrast (DIC) and YFP fluorescence were merged. Bar = 100 μm.

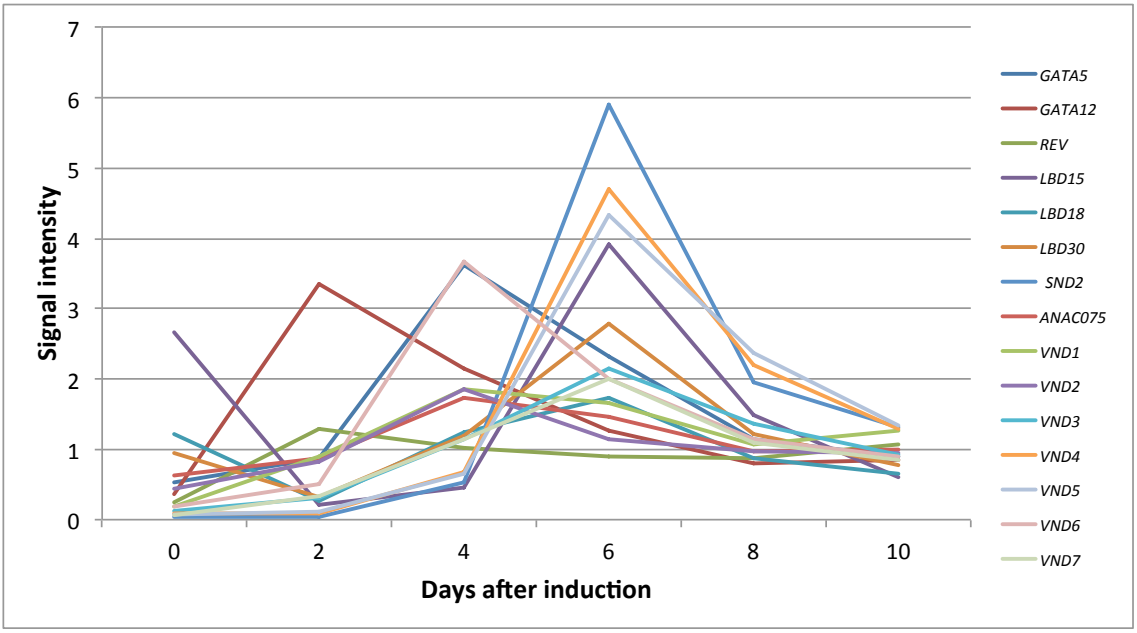


Fig. 2. Expression patterns of the transcription factors that induce *VND7* promoter activity during *in vitro* xylem vessel element differentiation, as revealed by microarray analysis (Kubo et al. 2005).

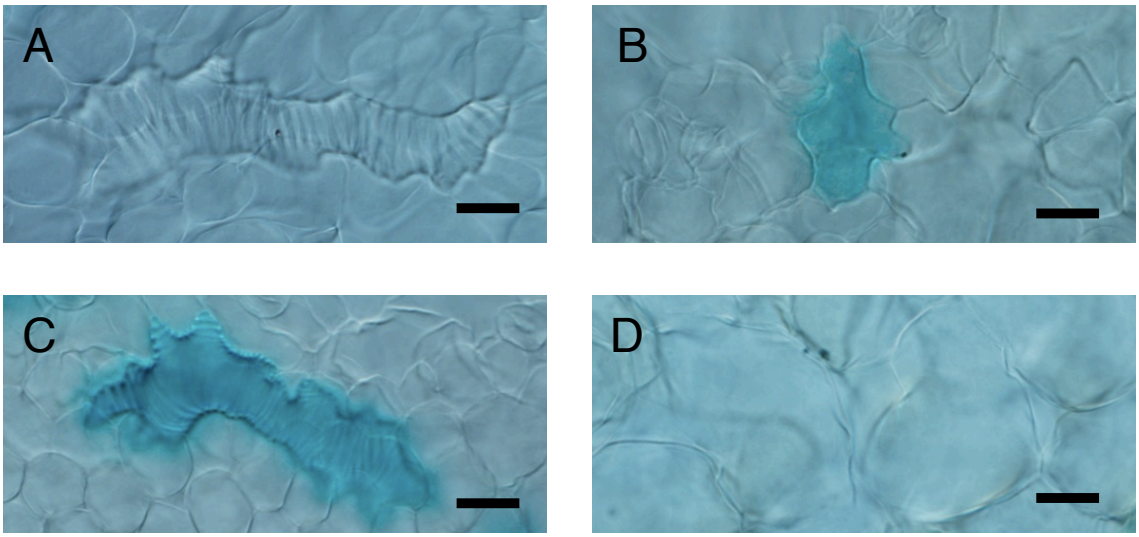


Fig. 3. Patterned secondary cell wall formation (SCW) and ectopic *VND7* promoter::*GUS* activity (GUS activity) induced by the overexpression of the candidate transcription factors. The leaves of *VND7* promoter::*GUS* plants that a *VND7* overexpression vector was introduced by particle bombardment. The plants were incubated at 22°C in a growth chamber for 4 days after bombardment. Four kinds of epidermal cells were present: (A) cells with patterned SCW, (B) cells with GUS activity, (C) cells with both SCW and GUS activity, and (D) cells lacking SCW and GUS activity. Bar = 30 μm. The frequency of each cell type is shown in Table 3.

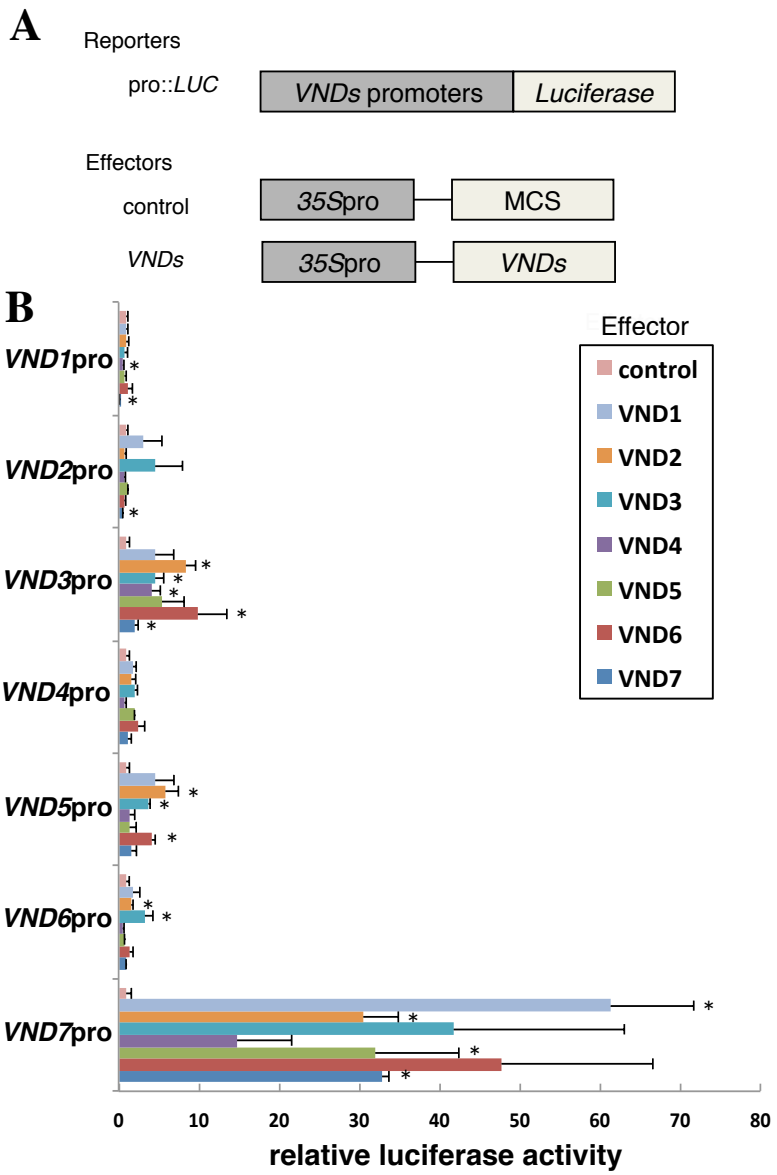


Fig. 4. *VND* family genes upregulate *VND7* expression *in vitro*. (A) Schematic diagrams of the effector and reporter plasmids used in luciferase transactivation assays. The reporter plasmids contain the promoters of *VND1* to *VND7* upstream of the firefly luciferase reporter gene. The effector plasmids contain *VND1* to *VND7* or a multi-cloning site (MCS) downstream of the CaMV35S promoter. (B) Results of transient expression assays. The reporter, effector and reference constructs were introduced into *Arabidopsis* leaves. The reporter gene activity was normalized by the activity of *Renilla* luciferase. Error bars indicate SD (n = 3). Asterisks indicate statistically significant differences (Welch's t-test; *P < 0.05) compared to the values for the control effector.

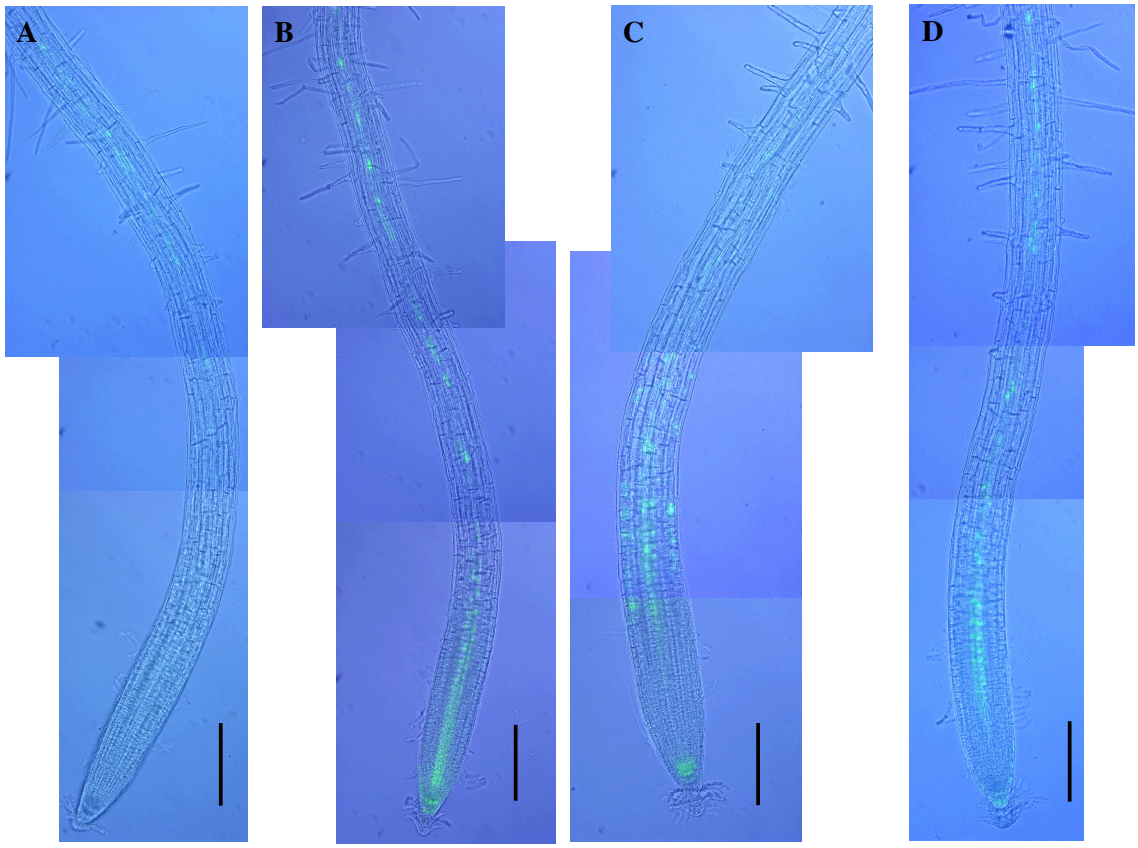


Fig. 5. The expression profiles of candidate transcription factors regulating *VND7* expression in the root. Expression patterns of (A) *VND7*pro::*YFP-NLS*, (B) *GATA5*pro::*YFP-NLS*, (C) *GATA12*pro::*YFP–NLS*, and (D) *ANAC075*pro::*YFP–NLS* in roots. DIC and YFP fluorescence images were merged. Bar = 200 μm.

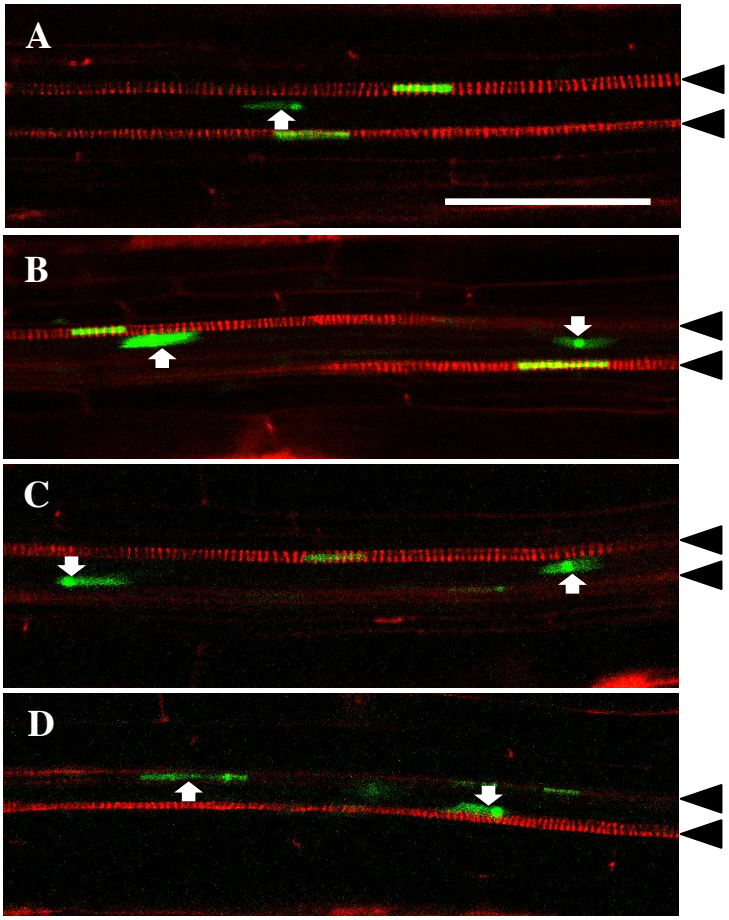


Fig. 6. Confocal microscopy analysis of the expression patterns of the candidate transcription factors regulating *VND7* expression in the root. Confocal microscopy images of the roots of transgenic plants expressing (A) *VND7*pro::*YFP-NLS*, (B) *GATA5*pro::*YFP-NLS*, (C) *GATA12*pro::*YFP–NLS,* and (D) *ANAC075*pro::*YFP–NLS*. A and D, 9-day-old plants; B and C, 10-day-old plants. The roots were counterstained with propidium iodide to visualize the outlines of cells. Black arrowheads indicate protoxylem vessel strands, while white arrows indicate fluorescent signals in metaxylem precursor cells. Bar = 100 μm.

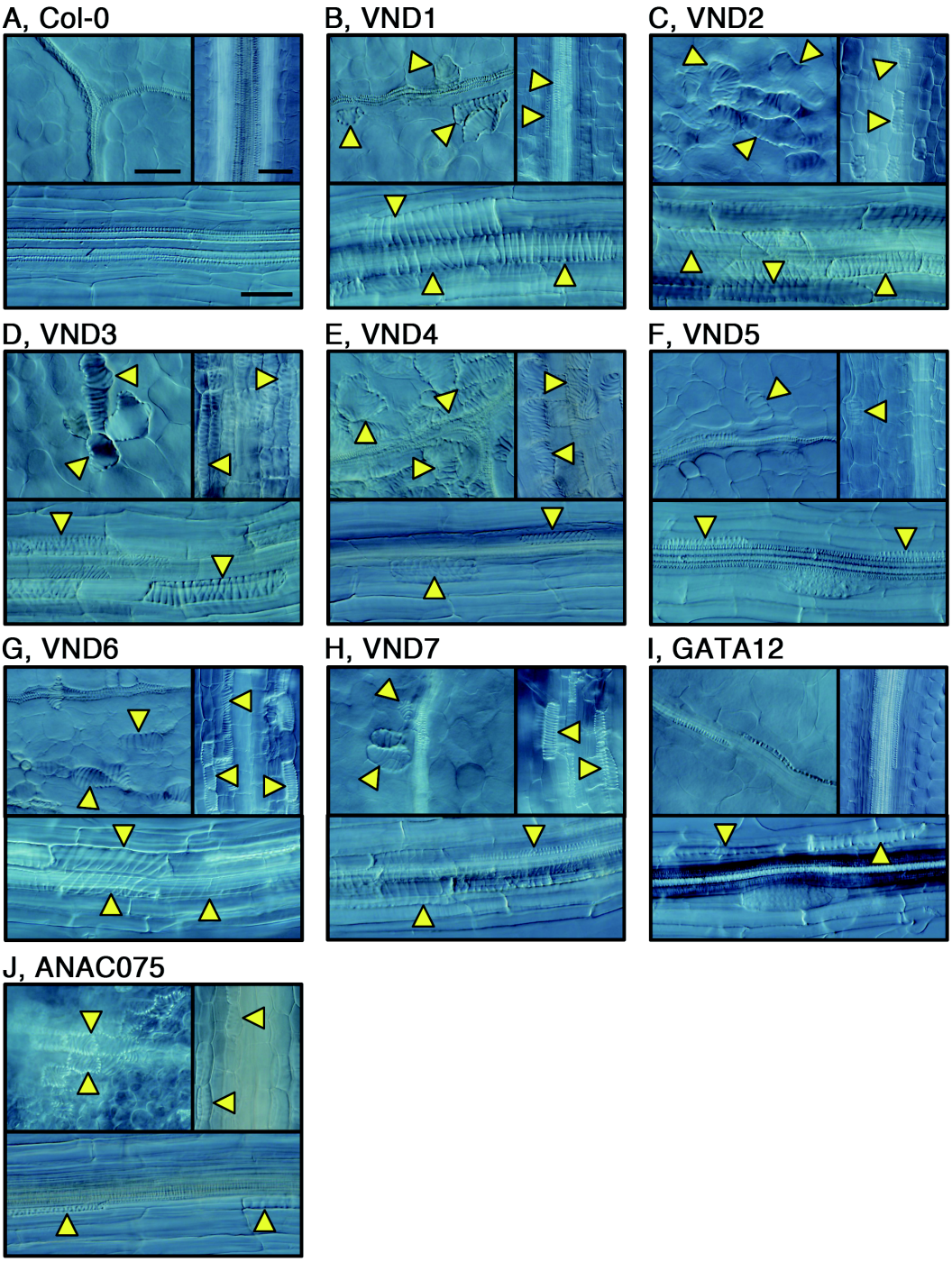


Fig. 7. Morphology of transgenic plant organs overexpressing transcription factors shown to upregulate *VND7* expression. DIC images of leaves (top left), hypocotyls (top right), and roots (bottom) of 7-day-old (A) Col-0 and transgenic plants expressing (B–H) *VND1–VND7*, (I) *GATA12*, and (J) *ANAC075* in an estrogen-inducible system. Plants were grown on media containing 10 μM β-estradiol for 7 days before analysis. Yellow arrowheads indicate the induced ectopic xylem vessel-like elements. Bar = 50 μm.

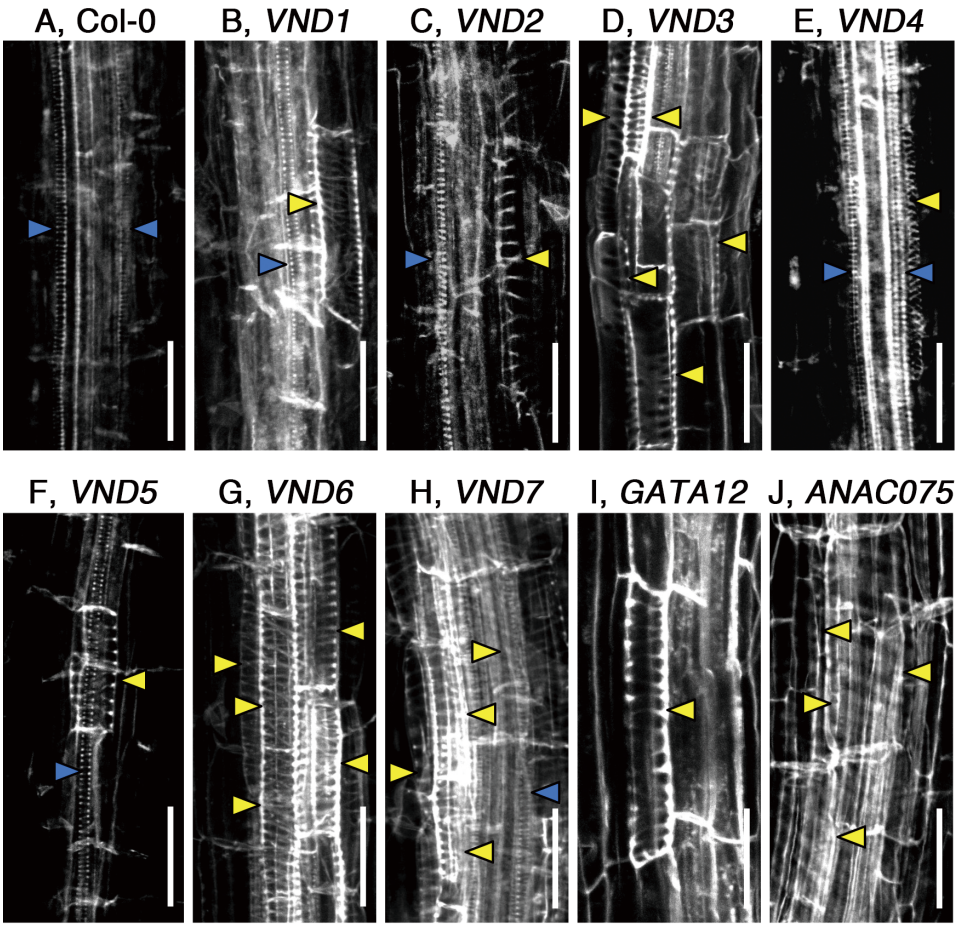


Fig. 8. Lignification patterns in the ectopic xylem vessel-like elements formed upon overexpression of the identified transcription factors. (A–J) Confocal microscopy images of the roots of 7-day-old seedlings. (A) Col-0 and (B–J) transgenic plants harboring estrogen-inducible *VND1* to *VND7*, *GATA12*, and *ANAC075*. Cells were treated with 10 μM of β-estradiol for 5 days before imaging. Plants were stained with Safranin-O, which stains lignin. Bar = 50 μm. Blue and yellow arrowheads indicate the endogenous and ectopic xylem vessel elements, respectively.

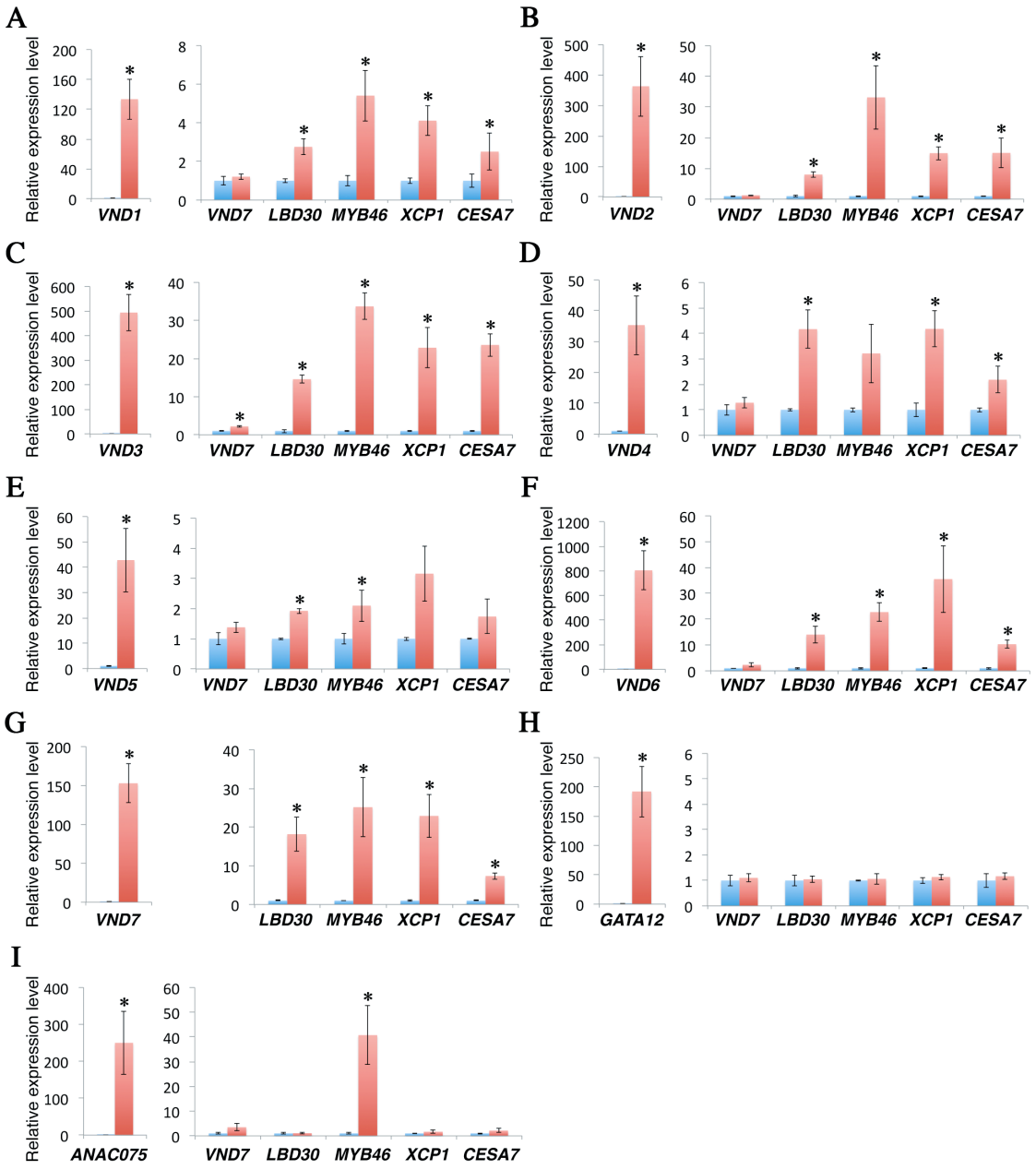


Fig. 9. Expression analysis of *VND7* and genes known to be involved in xylem vessel formation. Quantitative RT-PCR was used to analyze the expression of the indicated genes. Five-day-old transgenic seedlings harboring (A-G) *VND1* to *VND7* and (H) *GATA12* were treated with (EST) or without (mock) 10 μM β-estradiol for 24 h. mRNA levels for each of the indicated genes were normalized to *UBQ10* mRNA. Error bars indicate SD (n = 3). Asterisks indicate statistically significant differences (Welch's t-test; *P < 0.05) compared with the values of the mock treatment.

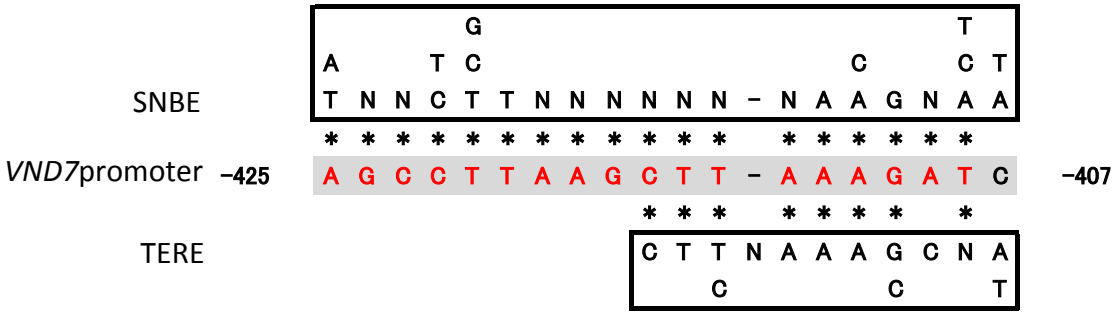


Fig. 10. Comparison of the *VND7* promoter (-425 to -407 bp) and SNBE or TERE consensus sequences. Asterisks indicate sequence matches between SNBE or TERE (Pyo et al. 2007, Zhong et al. 2010) and the *VND7* promoter sequence, and red font shows these matching nucleotides in the *VND7* promoter sequence.

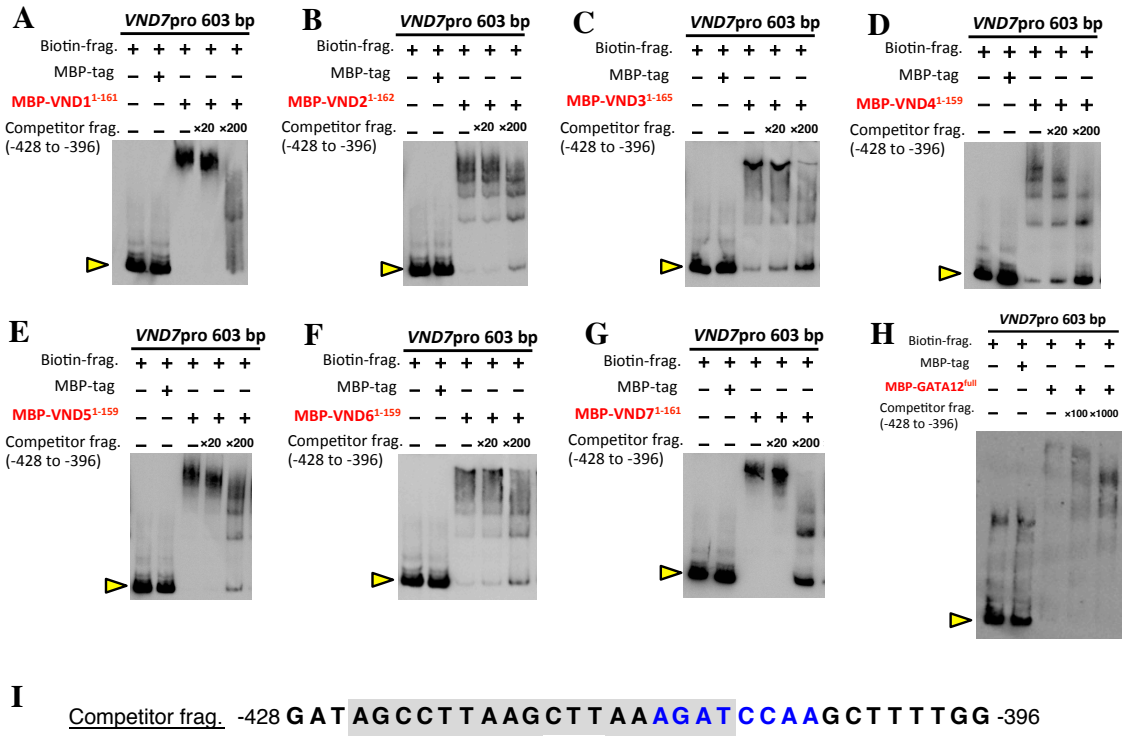


Fig. 11. VND proteins and GATA12 bind to the *VND7* promoter. EMSA was performed using MBP-tagged (A-H) VND1 to VND7 and GATA12 proteins. Biotin-labeled *VND7* promoter fragment (-1 to -603 bp) was incubated with (+) or without (-) MBP or the MBP-tagged VND and GATA12 proteins. The 33-bp *VND7* promoter fragment (-428 to -396 bp) was used for competition analysis. Yellow arrowheads indicate free probe. (I) Nucleotide sequences of the 33-bp *VND7* promoter fragments used for EMSA. The gray shading and blue letters indicate the SNBE/TERE-like motif and AG-motif, respectively.

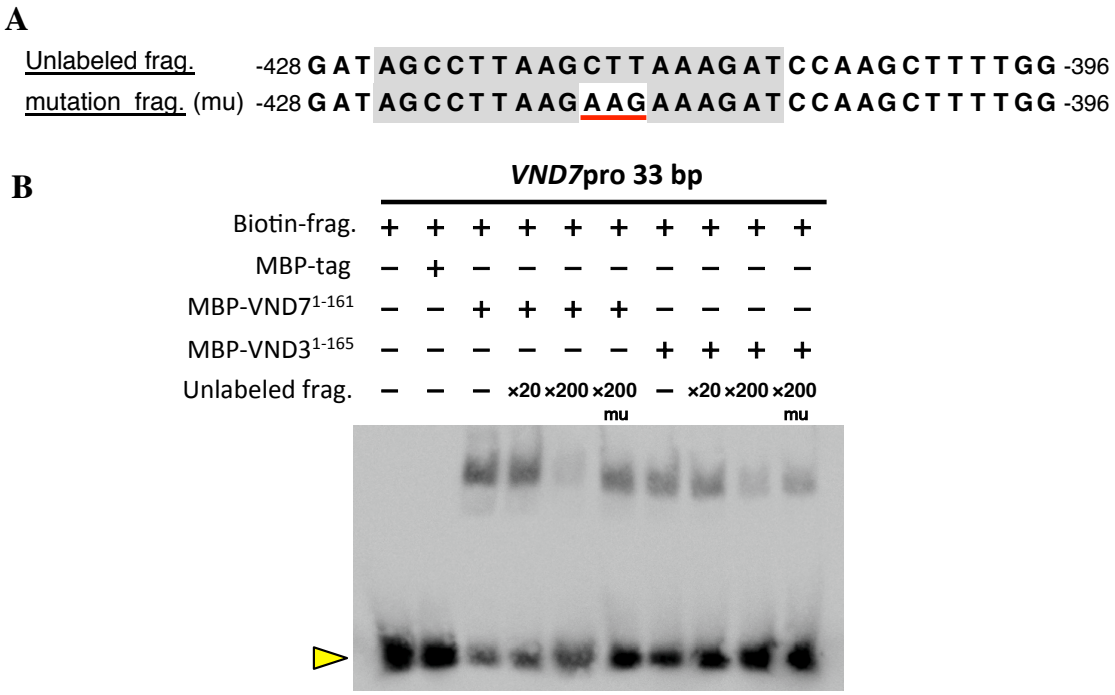


Fig. 12. VND proteins bind to the 33-bp *VND7* promoter region containing the SNBE/TERE motif. (A) Nucleotide sequences of the 33-bp *VND7* promoter fragment used for EMSAs. Gray shading and red underlining represent the SNBE/TERE-like motif and mutated nucleotide sequence, respectively. (B) The 33-bp *VND7* promoter fragment labeled with biotin was incubated with (+) or without (-) MBP, MBP-VND3, or MBP-VND7 proteins. The unlabeled *VND7* promoter fragment or mutated fragment (mu) was used for competition analysis. Yellow arrowheads indicate free probe.

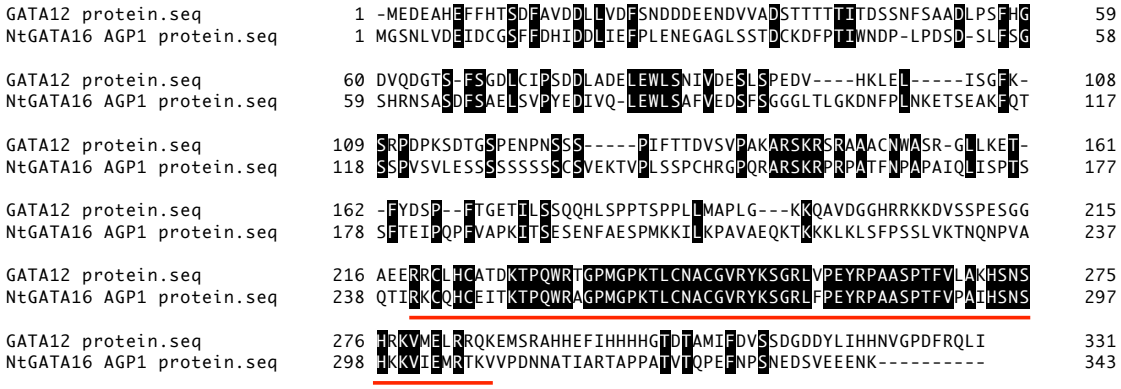


Fig. 13. Amino acid alignment of GATA12 and AGP1. Conserved amino acid sequences are indicated by black boxes with white letters. The conserved AGP domain (putative DNA-binding motif; Sugimoto et al. 2003) is indicated by red underlining.

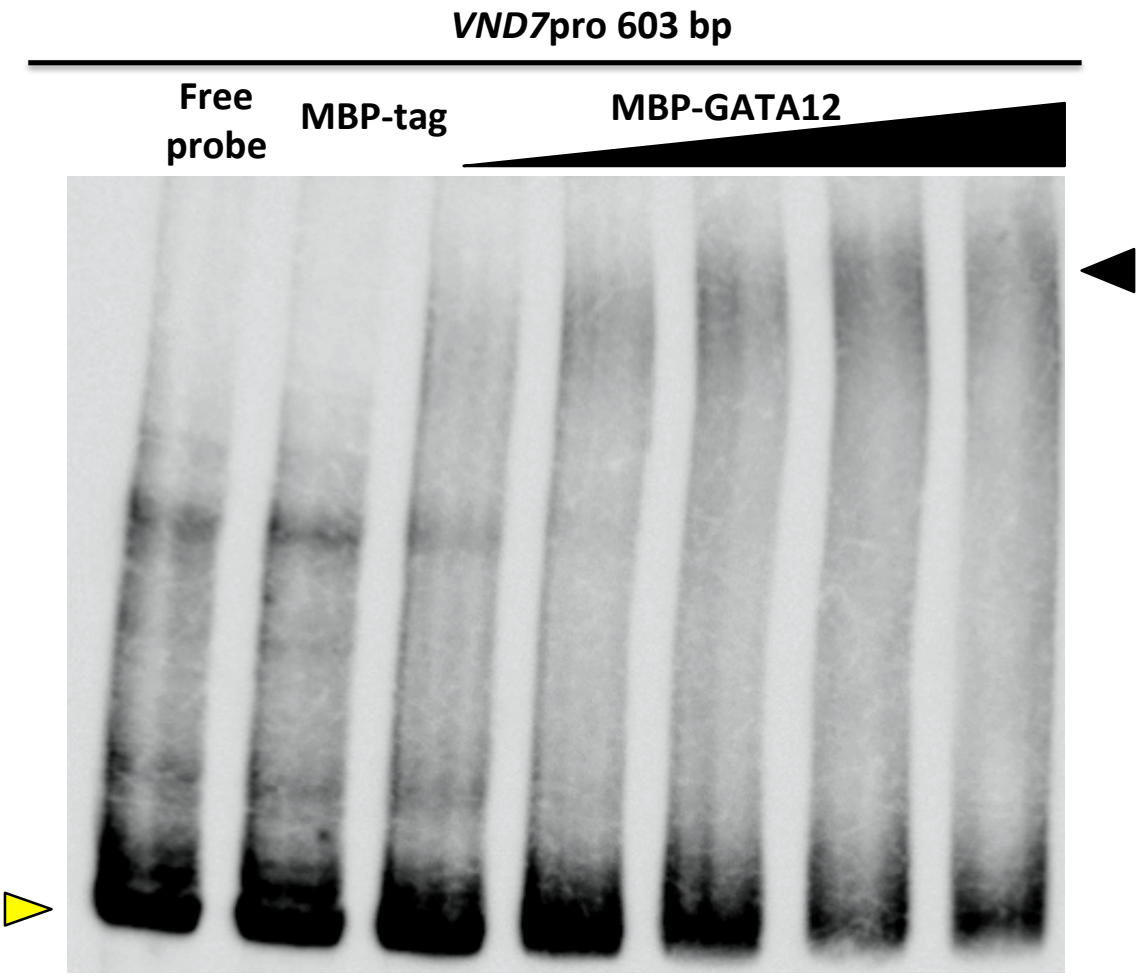


Fig. 14. GATA12 binds to the *VND7* promoter in a concentration-dependent manner. EMSA was performed using MBP-tagged GATA12 proteins. Biotin-labeled *VND7* promoter fragment (-1 to -603 bp) was incubated with (+) or without (-) MBP or with MBP-tagged GATA12 proteins with a concentration of 1, 2, 4, 6, and 8 pmol. Yellow and black arrowheads indicate free probe and possible shifted bands, respectively.

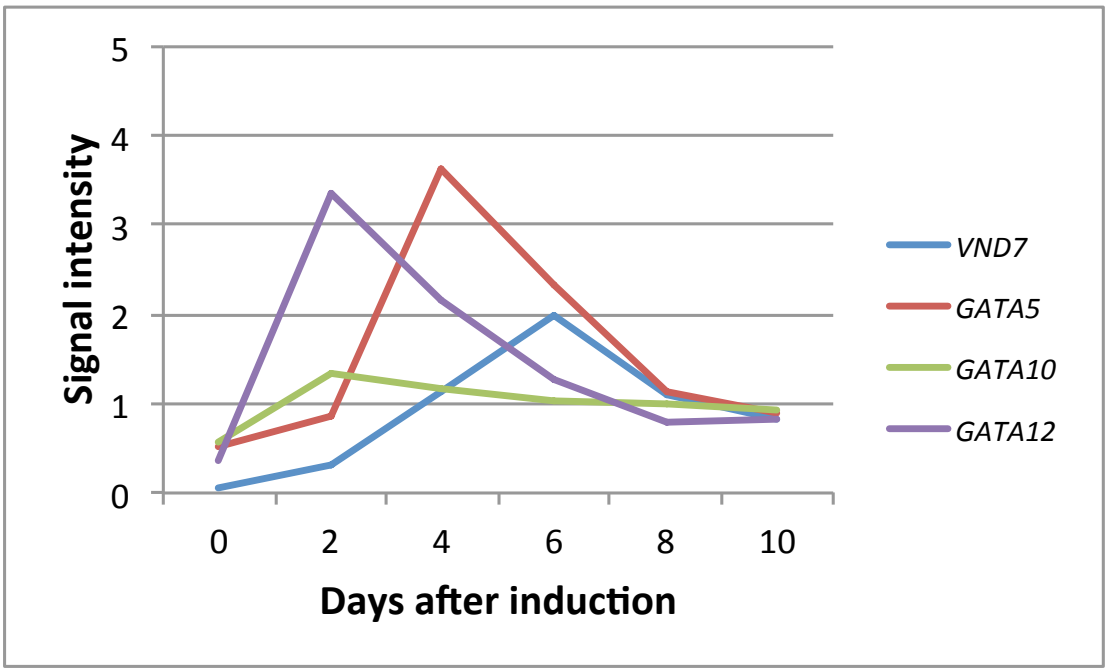


Fig. 15. Expression patterns of *VND7*, *GATA5*, *GATA10*, and *GATA12* during *in vitro* xylem vessel element differentiation, as revealed by microarray analysis (Kubo et al. 2005).

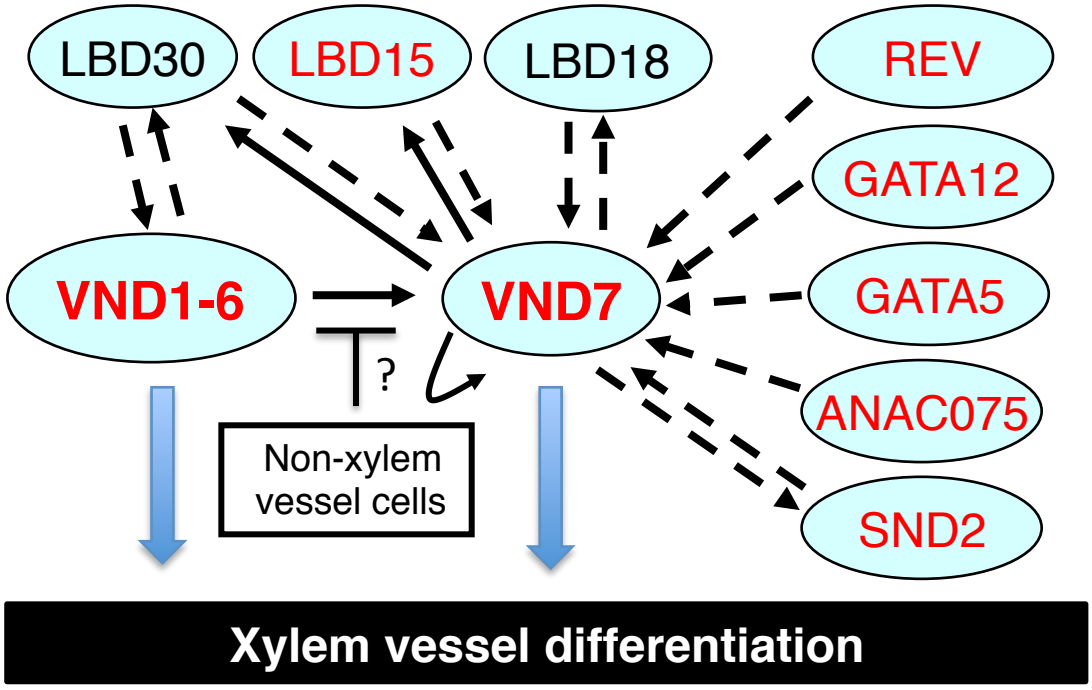


Fig. 16. Schematic model of the local transcriptional network that controls *VND7* expression. Transcription factors in red were isolated in this study. Arrows with solid lines indicate that direct transcriptional regulation has been confirmed. Upregulation of *VND7* expression by VND transcription factors was strongly repressed (T-bar) in non-xylem cells. VND1 to VND6 promote xylem vessel differentiation independently of *VND7* expression (blue arrows).

Results and Discussion

Part II

Epigenetic control of *VND7* expression

Results II

2-1. The *VND7* genomic region is marked by H3K27me3 and DNA methylation in seedlings

In Part I of this study, I showed that several transcription factors induce *VND7* promoter activity in a transient expression assay using *Arabidopsis* leaves, while endogenous *VND7* expression was not markedly upregulated in transgenic plants overexpressing the transcription factors (Table 1, 3, Fig. 8). Furthermore, I showed that all VNDs and GATA12 directly bind to the *VND7* promoter region (Fig. 10, 11, 13). Based on these findings, I speculated that *VND7* is regulated by certain additional elements/factors that strongly repress its expression in non-xylem cells (Fig. 16). One possible mechanism to explain this phenomenon is the epigenetic control of the *VND7* genomic region. Recent studies demonstrated that epigenetic phenomena in plants and animals are mediated by stable chromatin modification and DNA methylation (Feng et al. 2010, Feil and Fraga 2012). In eukaryotes, these genomic modifications form a physical barrier that blocks gene transcription (Lauria and Rossi 2011). Therefore, to assess whether the *VND7* genomic region undergoes chromatin modification and/or DNA methylation, I examined the epigenetic status of *VND7* using the genome browser created by the Genome Bioinformatics Group of UC Santa Cruz (The UCSC Genome Browser; http://genome.ucsc.edu). I found that the *VND7* genomic region has a relatively high histone H3 lysine K27 trimethylation (H3K27me3) signal on the promoter region and about half of the genic region (Fig. 17A). To confirm that these marks are indeed present in *Arabidopsis* seedlings, I conducted a chromatin immunoprecipitation-qPCR (ChIP-qPCR) using 5-day-old Col-0 seedlings, anti-trimethylation-histone H3 (Lys27) antibody (Helliwell et al. 2006), and nine primer sets (Fig. 18A) based on the information of H3K27me3 marks on the *VND7* genomic region in the UCSC Genome Browser (Fig. 17A). I detected a strong enrichment of H3K27me3 around 2-kb upstream of the genic region and between the first intron and second exon of the *VND7* genic region (Fig. 18B). As controls, I also used primer sets to amplify *Flowering Locus C* (*FLC*) and *FUSCA3* (*FUS3*) genomic regions, because these loci are targeted by polycomb-group proteins (PcG) (Makarevich et al. 2006, Jiang et al. 2008, Buzas et al. 2011). As reported, both of these loci were enriched in H3K27me3 (Fig. 18B). By

contrast, the genome browser indicated that the genomic region of *VND6* was not marked by H3K27me3 (Fig. 17B). ChIP-qPCR analysis confirmed that enrichment of the H3K27me3 mark on the *VND6* genomic region was relatively low compared with all other regions tested (Fig. 18B). The genic region of *VND6* could thus be used as a control not marked by H3K27me3. These data suggest that the *VND7* genomic region is indeed broadly marked by H3K27me3 in seedlings.

A strong DNA methylation signal exists around the second exonic region of the *VND7* genomic region, while there is little, if any, DNA methylation signal at the *VND7* locus in *met1-3*, a T-DNA insertion null mutant of the gene encoding DNA cytosine methyltrasferase1 (MET1) (Saze et al. 2003; Fig. 17A). Therefore, I examined the methylation status of the second exonic region of *VND7* using genomic DNA, which was extracted from 7-day-old Col-0 Arabidopsis seedlings after a 6-day treatment with or without 5-adC, a DNA methylation inhibitor, using McrBC-PCR. In this method, genomic DNA is digested with McrBC, a restriction enzyme that specifically cleaves DNA-containing methyl cytosine preceded by a purine nucleotide, and then subjected to PCR analysis. For this assay, I designed primers to amplify the first and second exonic regions of *VND7* as well as the promoter region of *CACTA1*, a DNA-type transposon known to be silenced by CpG methylation, as a control (Fig. 19A; Kato et al. 2003, Kato et al. 2004, Ikeda et al. 2011). All samples not subjected to McrBC digestion yielded PCR products (Fig. 19B). By contrast, the second exonic region of *VND7* and the promoter region of *CACAT1* of mock-treated seedlings yielded much lower levels of amplification product when digested with McrBC than did those of plants treated with 5-adC, while the first exonic region of *VND7* showed no difference between the mock- and 5-adC-treated samples (Fig. 19B). This result indicated that the second exonic region of *VND7* is CpG methylated and that this methylation can be blocked by 5-adC treatment of seedlings (Fig. 19B).

Because DNA CpG methylation is known to affect a wide range of processes by altering gene expression patterns (Chan et al. 2005), I next tested whether the expression of endogenous *VND7* is affected by the DNA demethylation treatment. Wild-type Col-0 plants were treated with different concentrations of 5-adC (0.1, 1.0, 10 μg/ml) for 24 h. Then, RNA was extracted, cDNA was prepared, and the samples were subjected to quantitative RT-PCR analysis (Fig. 19C). The expression of endogenous *VND7* was significantly upregulated by the application of 0.1 and 1.0 μg/ml of 5-adC,

indicating that endogenous *VND7* expression is affected when DNA methylation is inhibited by 5-adC treatment (Fig. 19C). A previous study also reported that treatment with 4 μM (approx. 0.91 μg/ml) 5-adC mimicked the *met1-3* mutant phenotype (Mathieu et al. 2007), and I thus used 1 μg/ml of 5-adC for all the following analyses.

2-2. Endogenous *VND7* expression is repressed by H3K27me3 and DNA methylation

Polycomb Repressive Complex 2 (PRC2) is known to be essential for silencing specific genes, such as *FLC* and *Class I KNOX* genes, via H3K27me3 (Xu et al. 2008, Ikeda 2012). ChIP analysis of the *VND7* genomic region (Fig. 18) suggests that *VND7* is also likely to be targeted by the PRC2. To test this possibility, I obtained a *Fertilization Independent Endosperm* (*FIE*) mutant, one of the components of PRC2. In Arabidopsis, at least three types of PRC2 complexes are known to control transitions between the vegetative and the reproductive phases of development. *FIE* is a single copy gene and is therefore expected to be present in all PRC2 complexes (Bouyer et al. 2011, Butenko et al. 2011). The null *fie-1* mutant was shown to have a mutation in the first intron that causes an aberrant splicing reaction, and the maternal null *fie-1* allele was shown to be embryonic lethal (Ohad et al. 1996, 1999, Kinoshita et al. 2001). Therefore, to examine the postembryonic phenotypes of homozygous *fie-1* mutant plants, Kinoshita et al. (2001) established a *fie-1* mutant carrying a modified *FIE* transgene (pFIE::FIE-GFP) that suppresses the embryonic lethality (*fie-1*/*pFIE*::*FIE-GFP*, hereafter called *fie*). Furthermore, Kinoshita et al. (2001) showed that wild-type plants transgenically expressing the *pFIE*::*FIE-GFP* transgene developed normally and embryos and seedlings of *fie* activate the expression of floral induction genes that are normally repressed in embryos and seedlings of wild-type plants (Kinoshita et al. 2001).

The ectopic expression of *VND7* causes cells to transdifferentiate into xylem vessel elements, even in the case of mature cells, such as trichomes, epidermal, guard, and mesophyll cells (Yamaguchi et al. 2010a). Therefore, I observed the phenotype of *fie* plants in detail to check whether ectopic xylem vessel formation occurs in *fie*, since the miss-expression of *VND7* should result in ectopic xylem vessel element formation. The observation revealed no ectopic xylem vessel element formation in aerial tissues of 15-day-old seedlings (Table 6; Fig. 20E). Auxin, cytokinin, and brassinosteroids are

known to play important roles in xylem vessel formation (Fukuda 2004) and endogenous *VND7* expression was shown to be upregulated in cultured hypocotyls by treatment with a combination of these phytohormone (Kubo et al. 2005). I thus treated the *fie* mutant seedlings with a combination of cytokinin (Kinetin; K), auxin (2,4-dichlorophenoxyacetic acid; D), and brassinosteroid (Brassinolide; B) (Table 4-6, Fig. 20, 21). I subjected wild type Col-0 and Ler to the same treatment because the "*fie"* mutant was generated by crossing *fie-1* (Ler background) with the *pFIE::FIE-GFP* transgenic Col-0 plant (Kinoshita et al. 2001). The treatments rarely induced ectopic xylem vessel element formation in the Col-0 or Ler wild-type plants, except for roots treated with a combination of cytokinin and auxin (Table 4, 5). By contrast, the "*fie*" mutant showed a marked induction of ectopic xylem vessel element formation in response to various combinations of the three phytohormones, especially all three phytohormones together (Table 6), which never induced ectopic xylem vessel element formation in wild-type plants (Table 4-6, Fig. 20, 21).

Since endogenous *VND7* expression is enhanced by the 5-adC treatment (Fig. 19C), I investigated the effect of 5-adC treatment on the ectopic xylem vessel element formation induced by phytohormone treatment in the *fie* mutant. I first confirmed that the 5-adC treatment alone did not induce any ectopic xylem element formation in this plant. The 5-adC treatment drastically enhanced the ectopic xylem element formation induced by treatment with the three phytohormones (Table 7, Fig. 22-25).

To investigate whether the application of 5-adC and phytohormones can upregulate the endogenous expression of *VND* genes, I monitored the expression of *VND* genes by quantitative RT-PCR using seedlings of the *fie* mutant and Col-0. RNA was extracted from 5-day-old seedlings treated with or without 5-adC and/or the three phytohormones for 3 days (Fig. 27). In Col-0, only *VND1* and *VND2* were upregulated more than 2-fold compared with the mock samples treated with the phytohormones or 5-adC plus the phytohormones (Fig. 27, Table 8, 9). By contrast, in the *fie* mutant, expression of *VND1*, *VND2*, *VND3*, *VND4,* and *VND5* was upregulated by the phytohormone treatment in the presence or absence of 5-adC treatment (Fig. 27G-K), while *VND7* was only upregulated when the 5-adC and phytohormone treatments were combined (Fig. 27F, L, Table 8, 9).

2-3. Some transgenic plants harboring *VND7pro*::*VND7* in the *vnd4*/*vnd7* mutant background showed ectopic xylem element formation

The T-DNA insertion mutant, which *vnd4* and *vnd7* were crossed (*vnd4*/*vnd7*) was generated by Dr. Masatoshi Yamaguchi of Saitama University and he observed that the *vnd4*/*vnd7* mutant showed growth defects and discontinuous formation of protoxylem vessels in the roots (unpublished data). Several homozygous transgenic lines carrying *VND7*pro::*VND7* (a genomic fragment harboring a 1-kb 5' upstream fragment from the start codon of *VND7* and genomic region of *VND7*) in the *vnd4*/*vnd7* background were kindly provided by Mr. Yoshito Ogawa of the Nara Institute of Science and Technology and Dr. Masatoshi Yamaguchi (unpublished data). Careful observation of the transgenic lines that contain only a single copy of the transgene revealed that some lines exhibited ectopic xylem vessel element formation in the pericycle cells of roots with higher expression levels of the *VND7* transgene (Yoshito Ogawa, unpublished data). These data prompted me to speculate that this phenomenon is caused by *VND7* transgene fragments that are not epigenetically repressed. To evaluate this possibility, I selected two independent transgenic lines, 1-2A and 1-3D, which lacked and exhibited ectopic xylem vessel element formation, respectively, in roots (Fig. 28B). Five-day-old seedlings of these lines, Col-0, and the *vnd4*/*vnd7* double mutant were treated with 5-adC and/or phytohormones for 5 days (Table 7-10). None of the seedlings of Col-0 or the double mutant showed ectopic xylem vessel element formation in roots or hypocotyls under any conditions tested (Table 10, 11, Fig. 29A-H, Fig. 31A-H). By contrast, 1-2A and 1-3D showed ectopic xylem vessel element formation under several conditions (Table 10, 11, Fig. 29I-P, 30D, 31I-P, 32C, D). It is noteworthy that, in line 1-3D, the ectopic xylem vessel element formation in the hypocotyl induced by phytohormone treatment was enhanced by 5-adC treatment (Table 13, Fig. 30D). In addition, the roots of both lines, especially of line 1-3D, exhibited ectopic xylem vessel element formation upon treatment with 5-adC in the absence of phytohormone treatment (Table 12, 13, Fig. 31I, J, M, N, 32C, D). These data suggest that the *VND7* genomic fragment introduced into the 1-2A and 1-3D lines is much more sensitive to 5-adC and phytohormone treatment than is the endogenous *VND7* genomic region.

Discussion II

In Results and Discussion I, I demonstrated that *VND7* expression is tightly regulated by multiple transcription factors together with unidentified additional mechanism(s)/factor(s). Here I sought to reveal the nature of the unidentified factor(s) that regulate *VND7* expression. Several reports showed that Polycomb-repressive complexes (PRCs) define the correct spatiotemporal expression of numerous key developmental regulators in plants, such as *WUSCHEL* and several members of the class I *KNOTTED1-like homeobox* (*KNOX*) gene family (Köhler and Hennig 2010, Liu et al. 2011, Bemer et al. 2012, Lodha et al. 2013). I hypothesized that *VND7* expression is also regulated by such a mechanism. To assess this possibility, I examined whether the *VND7* genomic region has chromatin and/or DNA modifications that are mediated by PRCs or other elements involved in epigenetic control. By consulting the publicly available Genome browser data, I found that the *VND7* genomic region is marked by H3K27me3 in its promoter and genic region and that the second exonic region of *VND7* is DNA methylated in seedlings (Fig. 17A). Indeed, ChIP-PCR analysis of the *VND7* genomic region using anti-H3K27me3 antibody showed relatively strong enrichment of H3K27me3 at the promoter region and the second exonic region of *VND7* (Fig. 18B). Furthermore, McrBC-PCR analysis confirmed that the second exonic region of *VND7* is CpG-methylated (Fig. 19B) and 5-adC treatment of Col-0 seedlings upregulated the expression of endogenous *VND7* at a concentration known to mimic the phenotype of a cytosine-DNA-methyl-transferase mutant, *met1-3* (Fig. 19C; Saze et al. 2003). My analysis of the *fie* mutant demonstrated that simultaneous treatment with 5-adC and three phytohormones drastically enhanced both the frequency of ectopic xylem vessel element formation and the expression of *VND* genes (Table 7, 8, 9, Fig. 22-27). Treatment with both 5-adC and phytohormones elevated the expression of all *VND* genes in *fie* to a greater extent than any other condition tested (Fig 27, Table 8, 9). These data suggest that not only *VND7* but also the other *VND* genes are marked by H3K27me3 and/or DNA methylation, both of which act as repressive marks and loss of these marks is a prerequisite to activate the expression of *VND* genes in the xylem vessel cells. Genome-wide analyses suggested that the presence of H3K27me3 marks in the genomic region and DNA methylation in the genic region is linked to genic transcription (Zilberman et al. 2007, Lister et al .2008, Shu et al. 2012) and there is a

report that a member of the polycomb group, MEDEA, interacts with MET1 to methylate histones and DNA of certain target genes to repress endosperm development (Schmidt et al. 2013). However, there is no direct evidence showing that a key transcription factor, such as *VND7,* involved in the transdifferentiation of specific cell types is tightly regulated by both H3K27me3 and DNA methylation during postembryonic development. My study suggests that *VND7* together with some of the other *VND* genes are possible targets of PRC2 and that somehow the DNA methylation acts as a mark to repress their expression and that the repression is canceled in certain cells such as xylem precursor cells. However, it is possible that other factors regulate this phenomenon, since the application of cytokinin, auxin, and brassinosteroids, as well as a DNA de-methylation chemical, broadly affect the expression of numerous genes (Fukuda 2004, Chang and Pikaard 2005). Systems such as INTACT or FACS (Taylor-Teeples et al. 2011) that enable the enrichment of a specific cell type can be used to monitor changes in genomic status and global gene expression during xylem precursor or xylem cell formation, and such studies would definitively reveal whether the *VND* genomic regions are indeed marked by DNA methylation and/or H3K27me3 in a cell-specific manner.

Some of the transgenic lines harboring *VND7*pro::*VND7* in the *vnd4*/*vnd7* background showed ectopic xylem vessel element formation. Treatment of the transgenic lines 1-2A and 1-3D with 5-adC and/or phytohormones enhanced ectopic xylem vessel element formation (Table 10-13, Fig. 29-32), while Col-0 and *vnd4*/*vnd7* showed no ectopic xylem formation in the roots or hypocotyls, suggesting that the genome regions harboring the *VND7* transgenes in 1-2A and 1-3D are more sensitive to phytohormone and 5-adC treatment than is the endogenous *VND7* genomic region. The finding that almost all cells in the 1-3D root stele were transdifferentiated into xylem vessel elements in response to 5-adC treatment (Fig. 32D) strongly indicates that the 1-kb region of the *VND7* promoter in the genomic region is sufficient to respond to endogenous levels of signal molecules (phytohormones) to activate the expression of *VND7* when DNA of the genomic region is de-methylated. This observation raises two hypotheses: (1) the promoter and genic regions of *VND7* introduced in the *vnd4*/*vnd7* background lack some elements responsible for the repression or silencing of the transgene; (2) the introduced *VND7* genomic fragment can escape the epigenetic regulatory mechanism that represses the expression of endogenous *VND7*. The latter

hypothesis can be interpreted as a position effect, where the expression of a transgene is influenced by the chromosome location, which has been well documented in plants (Singh et al. 2008, Lisch 2009). My study showed that there is a difference in sensitivity to signaling molecules among the transgenic lines tested, which suggests that the expression level of the *VND7* transgene in *vnd4*/*vnd7* is influenced by the position effect (Table 12, 13, Fig. 29-32). These data also support the idea that the expression of endogenous *VND7* is controlled by epigenetic regulation *in planta*.

In Part II of my study, I showed that the *VND7* genomic region is enriched in H3K27me3 and DNA methylation and that both marks seem to repress its expression. Moreover, I showed that some of the other *VND* family members are also regulated by the H3K27me3 marks. Based on these findings, I propose that expression of endogenous *VND7* is silenced by H3K27me3 and DNA methylation in non-xylem cells, while loss of the H3K27me3 mark, DNA de-methylation, and the presence of signaling molecules such as cytokinin, auxin, and brassinosteroids activate *VND7* expression (Fig. 33). Also, I believe that this study provides important and significant clues into the relationship between H3K27me3 and DNA methylation in the epigenetic control of gene expression, and provides strong evidence that hormonal crosstalk controls the master regulator of xylem vessel formation.

Table 4. Efficiency of ectopic xylem vessel element fomation induction by hormone treatment

	col-0[a]					
	1			2		
	leaf	hypocotyl	root	leaf	hypocotyl	root
Mock[b]	−	−	−	−	−	−
K	−	−	−	−	−	−
D	−	−	−	−	−	−
B	−	−	−	−	−	−
KD	−	−	+	−	−	+
KB	−	−	−	−	−	−
DB	−	−	−	−	−	−
KDB	−	−	−	−	−	−

++ several
+ few
− none

Number of ectopic xylem vessel element formation: ++ orange, several; + yellow, few; - white, none.

[a]Two independet 6-day-old plants were treated for 5 days.

[b](Mock) hormone free; (K) 50 ng/mL kinetin; (D) 500 ng/mL 2,4-dichlorophenoxyacetic acid; (B) 1 μM brassinolide.

Table 5. Efficiency of ectopic xylem vessel element fomation induction by hormone treatment

	Ler[a]					
	1			2		
	leaf	hypocotyl	root	leaf	hypocotyl	root
Mock[b]	−	−	−	−	−	−
K	−	−	−	−	−	−
D	−	−	−	−	−	−
B	−	−	−	−	−	−
KD	−	−	−	−	−	+
KB	−	−	−	−	−	−
DB	−	−	−	−	−	−
KDB	−	−	−	−	−	−

++ several
+ few
− none

Number of ectopic xylem vessel element formation: ++ orange, several; + yellow, few; - white, none.

[a]Two independet 6-day-old plants were treated for 5 days.

[b](Mock) hormone free; (K) 50 ng/mL kinetin; (D) 500 ng/mL 2,4-dichlorophenoxyacetic acid; (B) 1 μM brassinolide.

Table 6. Efficiency of ectopic xylem vessel element fomation induction by hormone treatment

	fie-1/pFIE::FIE-GFP (fie)[a]					
	1			2		
	leaf	hypocotyl	root	leaf	hypocotyl	root
Mock[b]	−	−	−	−	−	−
K	−	−	−	−	−	−
D	−	−	+	−	−	−
B	−	−	−	−	−	−
KD	+	−	+	+	−	−
KB	+	−	−	−	−	−
DB	+	−	−	+	−	−
KDB	++	+	+	+	+	−

++ several
+ few
− none

Number of ectopic xylem vessel element formation: ++ orange, several; + yellow, few; - white, none.

[a]Two independet 6-day-old plants were treated for 5 days.

[b](Mock) hormone free; (K) 50 ng/mL kinetin; (D) 500 ng/mL 2,4-dichlorophenoxyacetic acid; (B) 1 μM brassinolide.

Table 7. Efficiency of ectopic xylem vessel elemen fomation induction by chemical and hormone treatment

	fie-1/pFIE::FIE-GFP (fie)[a]				
	1	2	3	4	5
Mock[b]	–	–	–	–	–
5-adC	–	–	–	–	–
KDB	++	+	++	+	+
5adC + KDB	+++	+++	++	+	+++

+++	many
++	several
+	few
–	none

Number of ectopic xylem vessel element formation:+++ red, many; ++ orange, several; + yellow, few; - white, none.

[a]Five independet 15-day-old platns were treated for 5days.

[b](Mock) chemical free; (5-adC) 1 μg/mL 5-aza-dC; (K) 50 ng/mL kinetin;

(D) 500 ng/mL 2,4-dichlorophenoxyacetic acid; (B) 1 μM brassinolide.

Table 8. Sumarry of gene expression changes relative to value of mock treatment sample

	col-0[a]		
	5-adC[b]	KDB[b]	5adC + KDB[b]
VND1	1.3	3.4	3.0
VND2	0.9	2.2	2.3
VND3	0.9	0.4	0.5
VND4	0.7	0.4	0.3
VND5	1.0	0.3	0.5
VND7	0.9	0.4	0.6

upregulated
no significance
downregulated

Greater or equal to 2 fold = red; upregulated

Less than 2 and greater or equal to 0.5 fold = no significance; gray

Less than 0.5 fold = downregulated; blue

[a] Five independet 6-day-old platns were treated for 5 days.

[b](5-adC) 1 μg/mL 5-aza-dC; (K) 50 ng/mL kinetin;

(D) 500 ng/mL 2,4-dichlorophenoxyacetic acid; (B) 1 μM brassinolide.

Table 9. Sumarry of gene expression changes relative to value of mock treatment sample

	fie-1/pFIE::FIE-GFP (*fie*)[a]		
	5-adC[b]	KDB[b]	5adC + KDB[b]
VND1	1.0	5.6	5.3
VND2	0.8	7.7	10.0
VND3	0.9	2.0	2.3
VND4	1.0	1.7	2.9
VND5	1.4	2.2	4.6
VND7	0.8	0.6	2.2

upregulated
no significance
downregulated

Greater or equal to 2 fold = red; upregulated

Less than 2 and greater or equal to 0.5 fold = no significance; gray

Less than 0.5 fold = downregulated; blue

[a] Five independet 6-day-old platns were treated for 5 days.

[b](5-adC) 1 μg/mL 5-aza-dC; (K) 50 ng/mL kinetin;

(D) 500 ng/mL 2,4-dichlorophenoxyacetic acid; (B) 1 μM brassinolide.

Table 10. Efficiency of ectopic xylem vessel elemen fomation induction by chemical and hormone treatment

	col-0[a]					
	1		2		3	
	hypocotyl	root	hypocotyl	root	hypocotyl	root
Mock[b]	–	–	–	–	–	–
5-adC	–	–	–	–	–	–
KDB	–	–	–	–	–	–
5-adC + KDB	–	–	–	–	–	–

+++	many
++	several
+	few
–	none

Number of ectopic xylem vessel element formation:+++ red, many; ++ orange, several; + yellow, few; - white, none.

[a]Three independet 6-day-old platns were treated for 5days.

[b](Mock) chemical free; (5-adC) 1 μg/mL 5-aza-dC; (K) 50 ng/mL kinetin; (D) 500 ng/mL 2,4-dichlorophenoxyacetic acid; (B) 1 μM brassinolide.

Table 11. Efficiency of ectopic xylem vessel elemen fomation induction by chemical and hormone treatment

	vnd4/vnd7[a]					
	1		2		3	
	hypocotyl	root	hypocotyl	root	hypocotyl	root
Mock[b]	–	–	–	–	–	–
5-adC	–	–	–	–	–	–
KDB	–	–	–	–	–	–
5-adC + KDB	–	–	–	–	–	–

+++	many
++	several
+	few
–	none

Number of ectopic xylem vessel element formation:+++ red, many; ++ orange, several; + yellow, few; - white, none.

[a]Three independet 6-day-old platns were treated for 5days.

[b](Mock) chemical free; (5-adC) 1 μg/mL 5-aza-dC; (K) 50 ng/mL kinetin; (D) 500 ng/mL 2,4-dichlorophenoxyacetic acid; (B) 1 μM brassinolide.

Table 12. Efficiency of ectopic xylem vessel elemen fomation induction by chemical and hormone treatment

	1-2A[a]					
	1		2		3	
	hypocotyl	root	hypocotyl	root	hypocotyl	root
Mock[b]	–	–	–	–	–	–
5-adC	–	+	–	–	–	+
KDB	–	–	–	–	+	–
5-adC + KDB	–	–	+	–	+	–

+++	many
++	several
+	few
–	none

Number of ectopic xylem vessel element formation:+++ red, many; ++ orange, several; + yellow, few; - white, none.

[a]Three independet 6-day-old platns were treated for 5days.

[b](Mock) chemical free; (5-adC) 1 μg/mL 5-aza-dC; (K) 50 ng/mL kinetin; (D) 500 ng/mL 2,4-dichlorophenoxyacetic acid; (B) 1 μM brassinolide.

Table 13. Efficiency of ectopic xylem vessel elemen fomation induction by chemical and hormone treatment

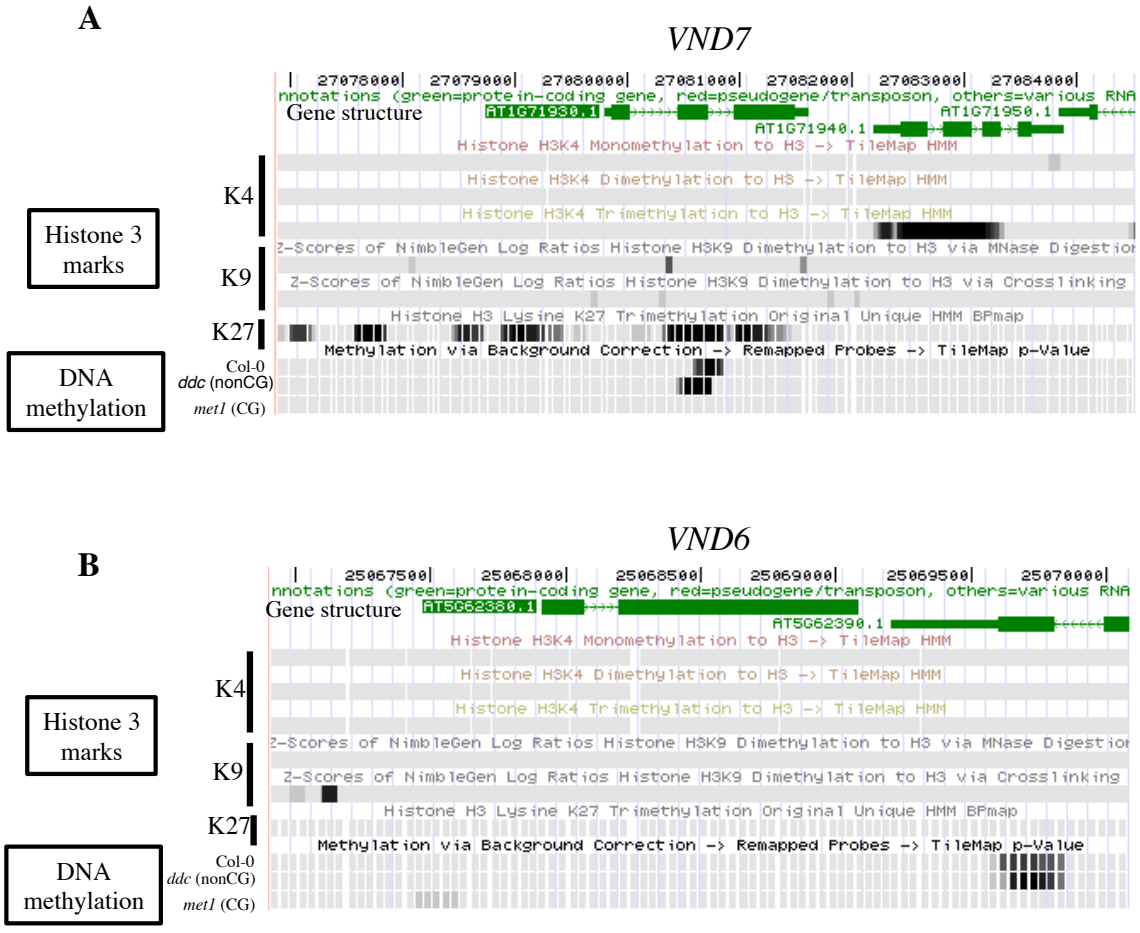
	1-3D[a]					
	1		2		3	
	hypocotyl	root	hypocotyl	root	hypocotyl	root
Mock[b]	–	++	–	+	–	+
5-adC	–	+++	–	+++	–	+++
KDB	++	+	++	+	++	+
5-adC + KDB	+++	+	+++	–	+++	–

+++	many
++	several
+	few
–	none

Number of ectopic xylem vessel element formation:+++ red, many; ++ orange, several; + yellow, few; - white, none.

[a]Three independet 6-day-old platns were treated for 5days.

[b](Mock) chemical free; (5-adC) 1 μg/mL 5-aza-dC; (K) 50 ng/mL kinetin; (D) 500 ng/mL 2,4-dichlorophenoxyacetic acid; (B) 1 μM brassinolide.



(http://epigenomics.mcdb.ucla.edu/cgi-bin/hgTracks 2013.01.14)

Fig. 17. Genomic status of the *VND7* (A1G71930.1) and *VND6* (AT5G62380.1) loci, as determined using the UCSC Genome Browser. Green bars and black boxes show gene structure and signal intensity of each genomic status, respectively. The darker the shade of the box, the greater the relative signal strength of each status. The *VND7* genic region exhibits relatively strong signals for H3K27me3 and DNA methylation, while the promoter region has H3K27me3 marks.

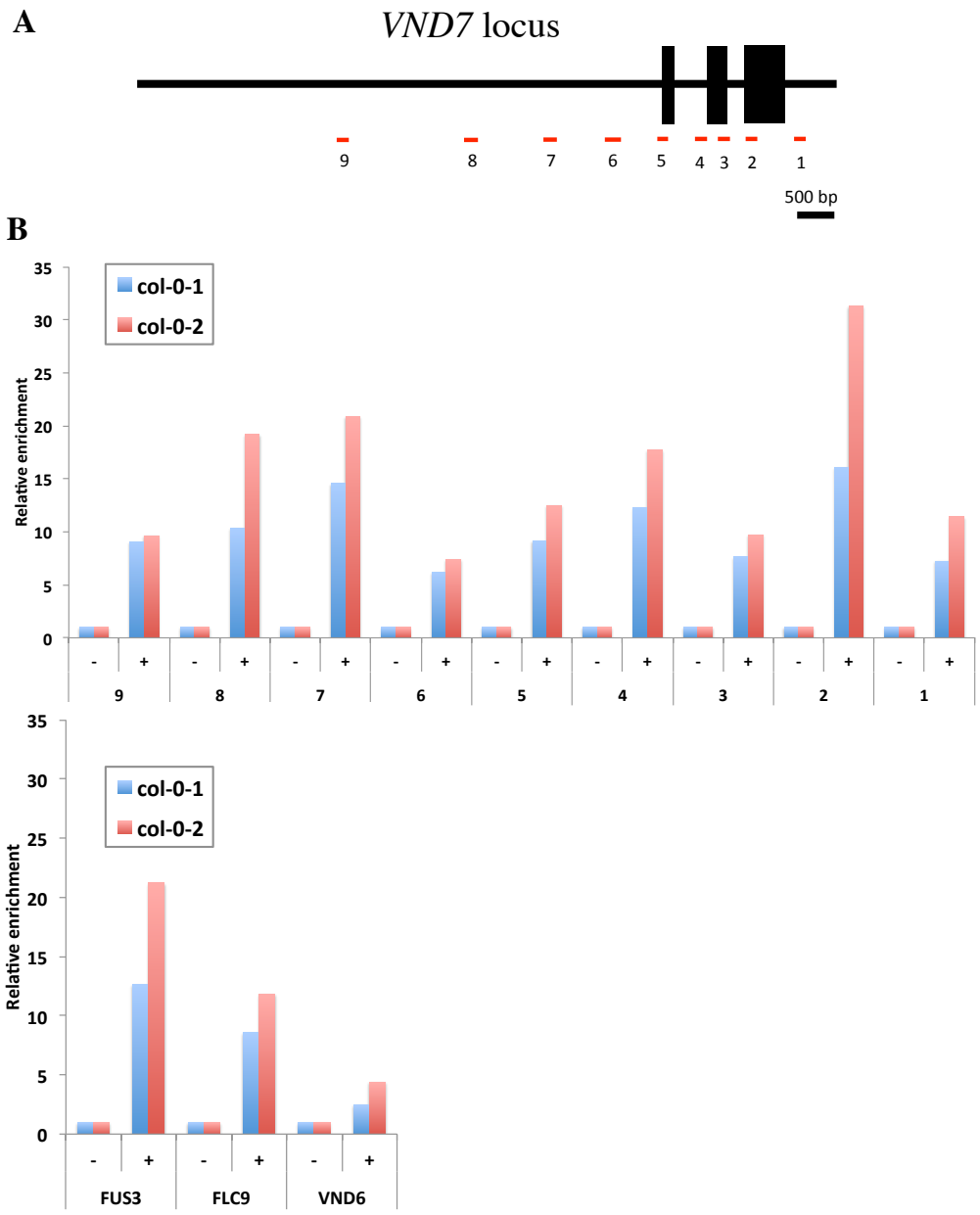


Fig. 18. H3K27me3 enrichment in the *VND7* genomic region of Col-0 seedlings.
(A) Positions of PCR amplicons 1–9 relative to the *VND7* locus are shown. Scale = 500 bp. (B) Relative enrichment of H3K27me3 at the *VND7*, *FUS3*, *FLC*, and *VND6* loci, determined by ChIP-qPCR. Data are ChIP with (+) or without (-) antibody to show that amplicons are enriched in the ChIP assay. Two independent biological replicates for 5-day-old Col-0 seedlings, Col-0-1, and Col-0-2 were analyzed.

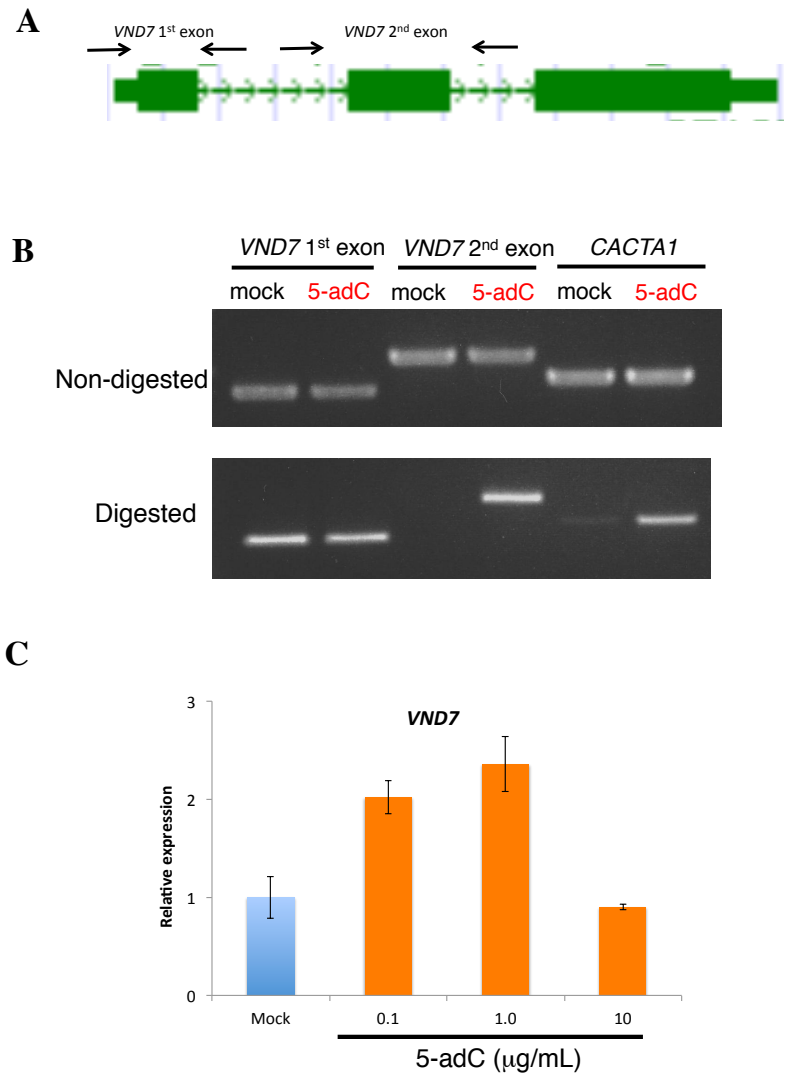


Fig. 19. The *VND7* genic region is DNA methylated. (A) Positions of the PCR primers designed to amplify the *VND7* genomic region are shown. The green box and green line indicate the exon and intron of *VND7* genes, respectively. (B) Genomic DNA was extracted from 7-day-old seedlings treated with or without 1 μg/ml 5-adC for 6 days. Analysis of DNA methylation levels of *VND7* and *CACTA1* after genomic DNA was digested or not with McrBC, a 5-methylcytosine-specific restriction enzyme. The first and second exons of *VND7* and the *CACTA* promoter region were amplified by PCR. (C) Expression analysis of endogenous *VND7*. Quantitative RT-PCR was performed for the indicated treatment. Eleven-day-old Col-0 seedlings were treated with or without 5-adC (0.1, 1.0. or 10 μg/mL) for 24 h. mRNA levels for each gene were normalized to *UBQ10 mRNA*. Error bars indicate SD (n = 3).

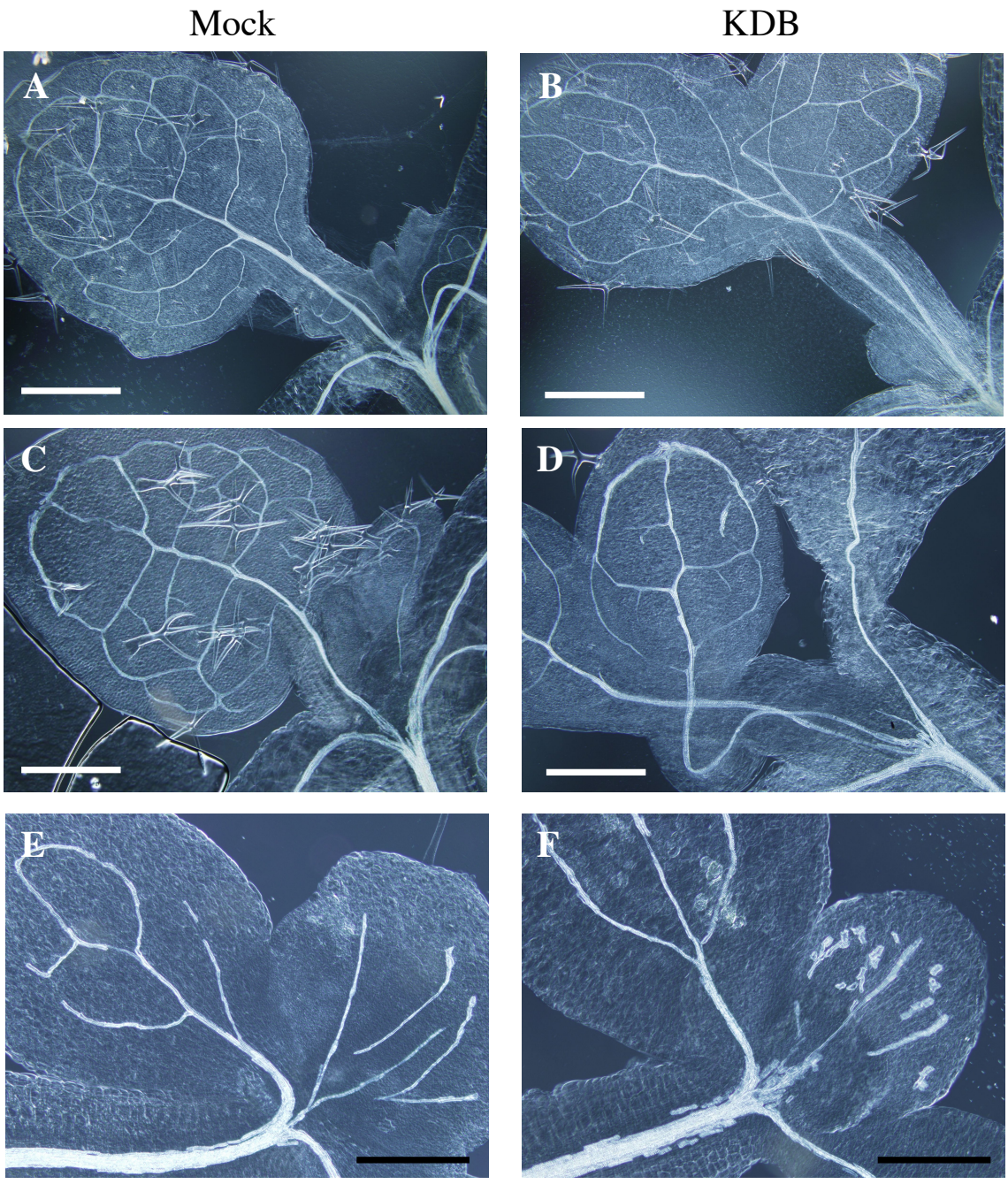


Fig. 20. Phytohormone treatment induces ectopic xylem vessel elements in *fie* mutant. DIC images of 11-day-old (A, B) Col-0, (C, D) Ler, and (E, F) *fie* seedlings treated with (B, D, F) or without (A, C, E) phytohormones for 5 days. (Mock) hormone free; (K) 50 ng/mL kinetin; (D) 500 ng/mL 2,4-dichlorophenoxyacetic acid; (B) 1 μM brassinolide. White bar = 500 μm. Black bar = 200 μm.

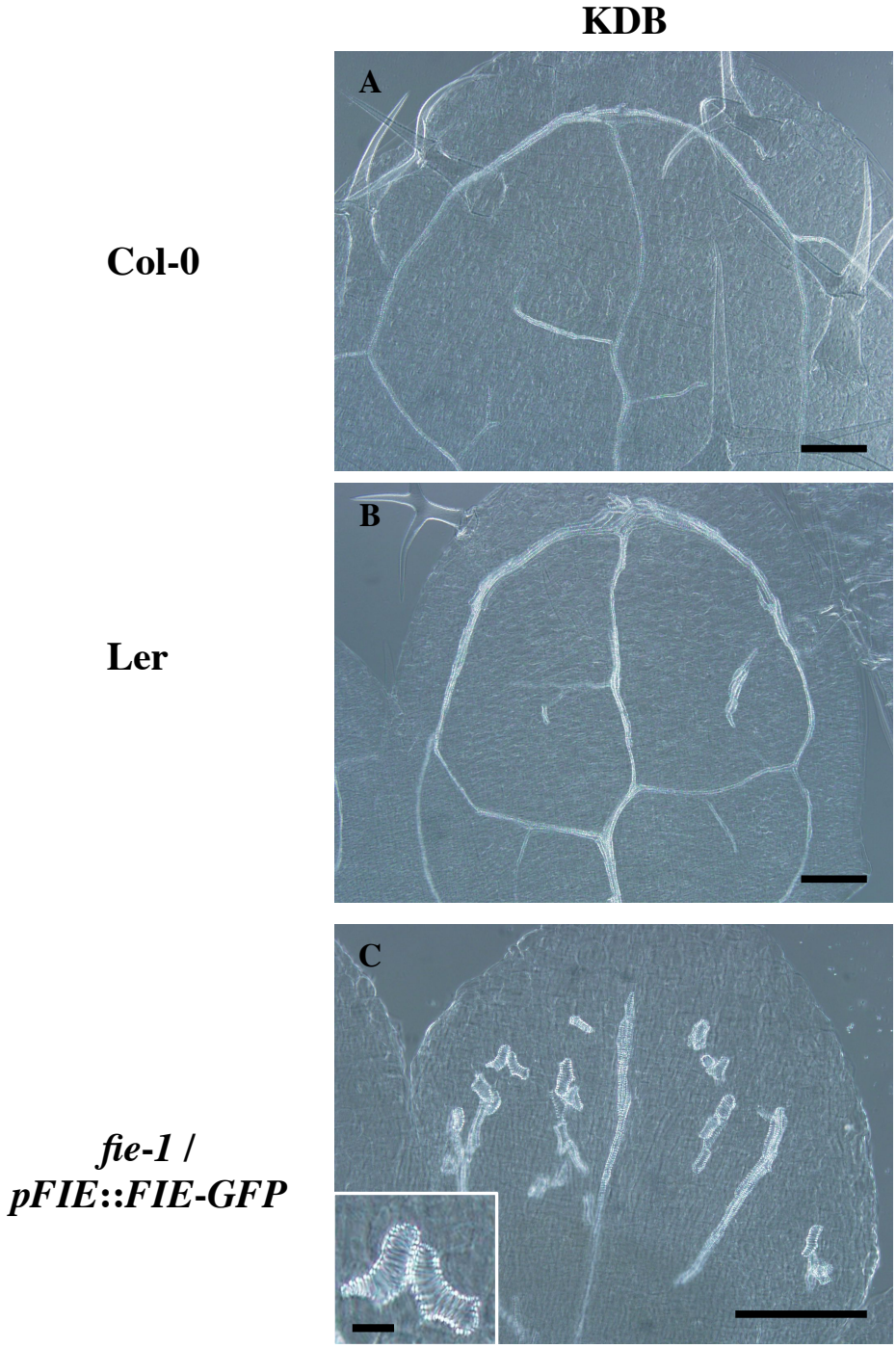


Fig. 21. Phytohormone treatment induces ectopic xylem vessel element with patterned secondary cell wall in *fie* mutant. DIC images of 11-day-old (A) Col-0 (B) Ler, and (C) *fie* seedlings treated with phytohormones for 5 days. Inset in C is showing ectopic xylem vessel elements with patterned secondary cell wall. KDB, 50 ng/mL kinetin; 500 ng/mL 2,4-dichlorophenoxyacetic acid; and 1 μM brassinolide. Black bar = 200 μm; inset in C, 10 μm.

Mock

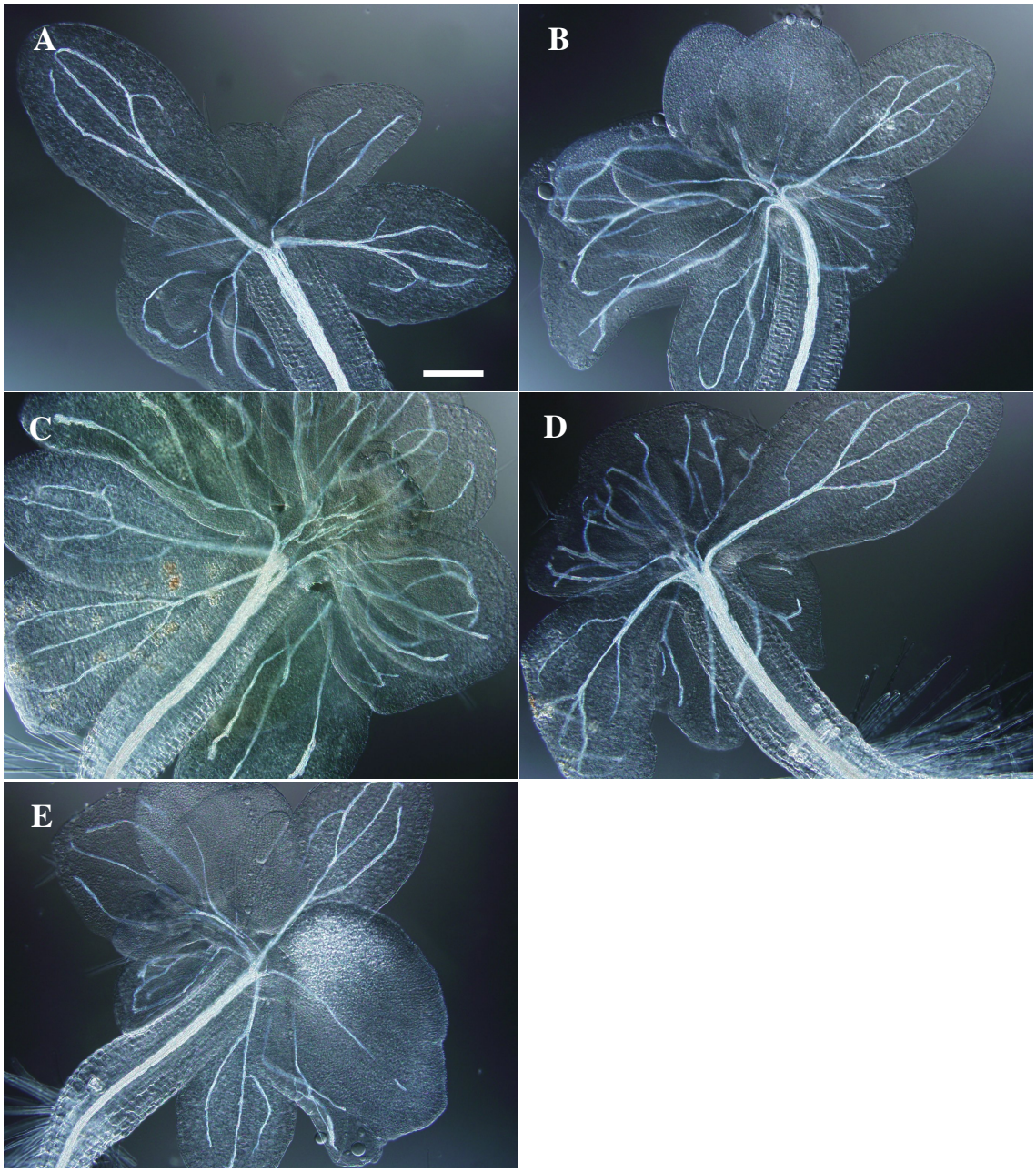


Fig. 22. DIC images of five individual 20-day-old *fie* mutants. (A-E) *fie* 1-5 plants incubated without chemicals (Mock) for 5 days. Bar = 200 μm.

5-adC

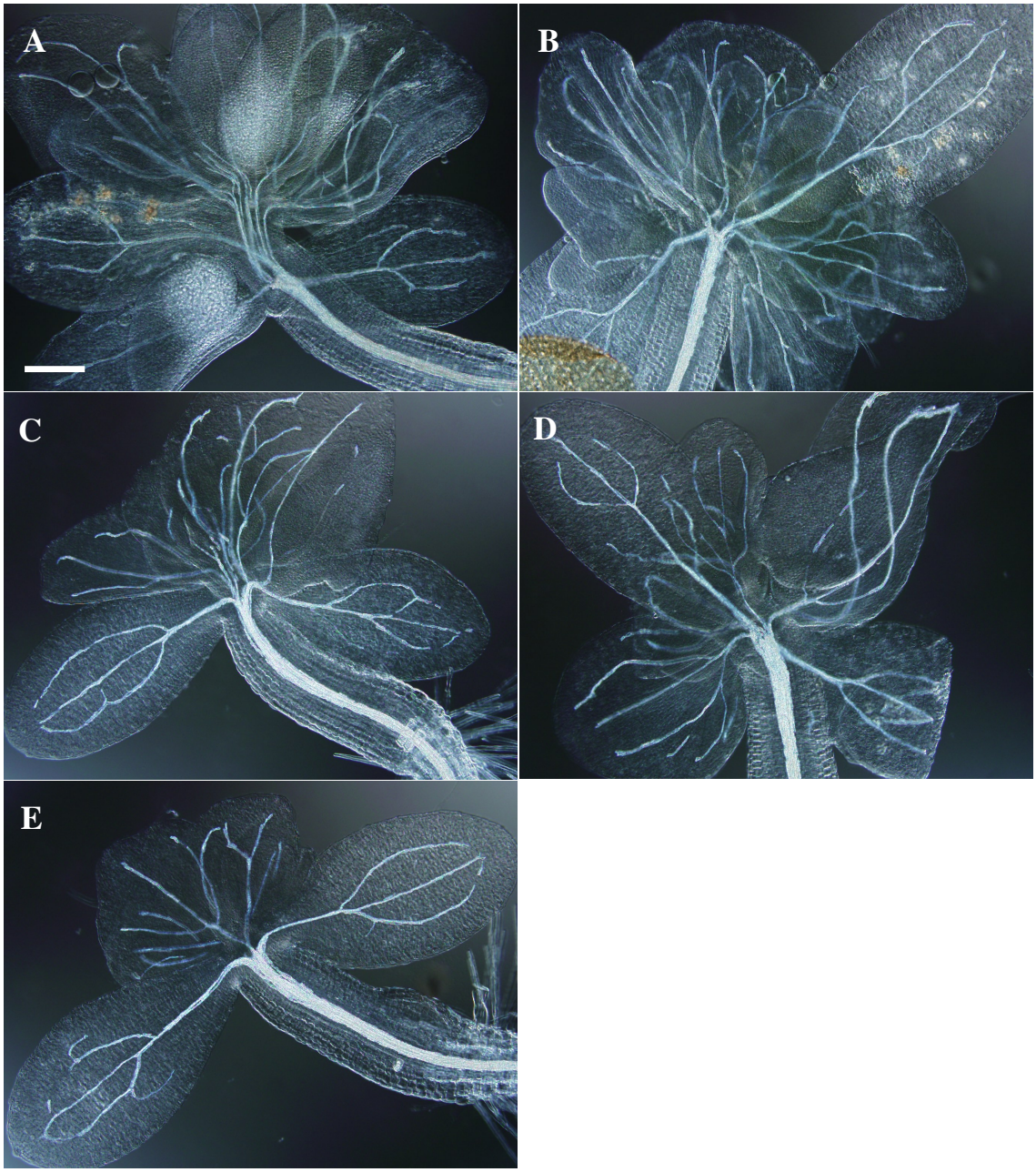


Fig. 23. DIC images of five individual 20-day-old *fie* mutants treated with 5-adC. (A-E) *fie* 1-5 plants treated with 5-adC for 5 days. 5-adC, 1 μg/mL 5-aza-dC. Bar = 200 μm.

KDB

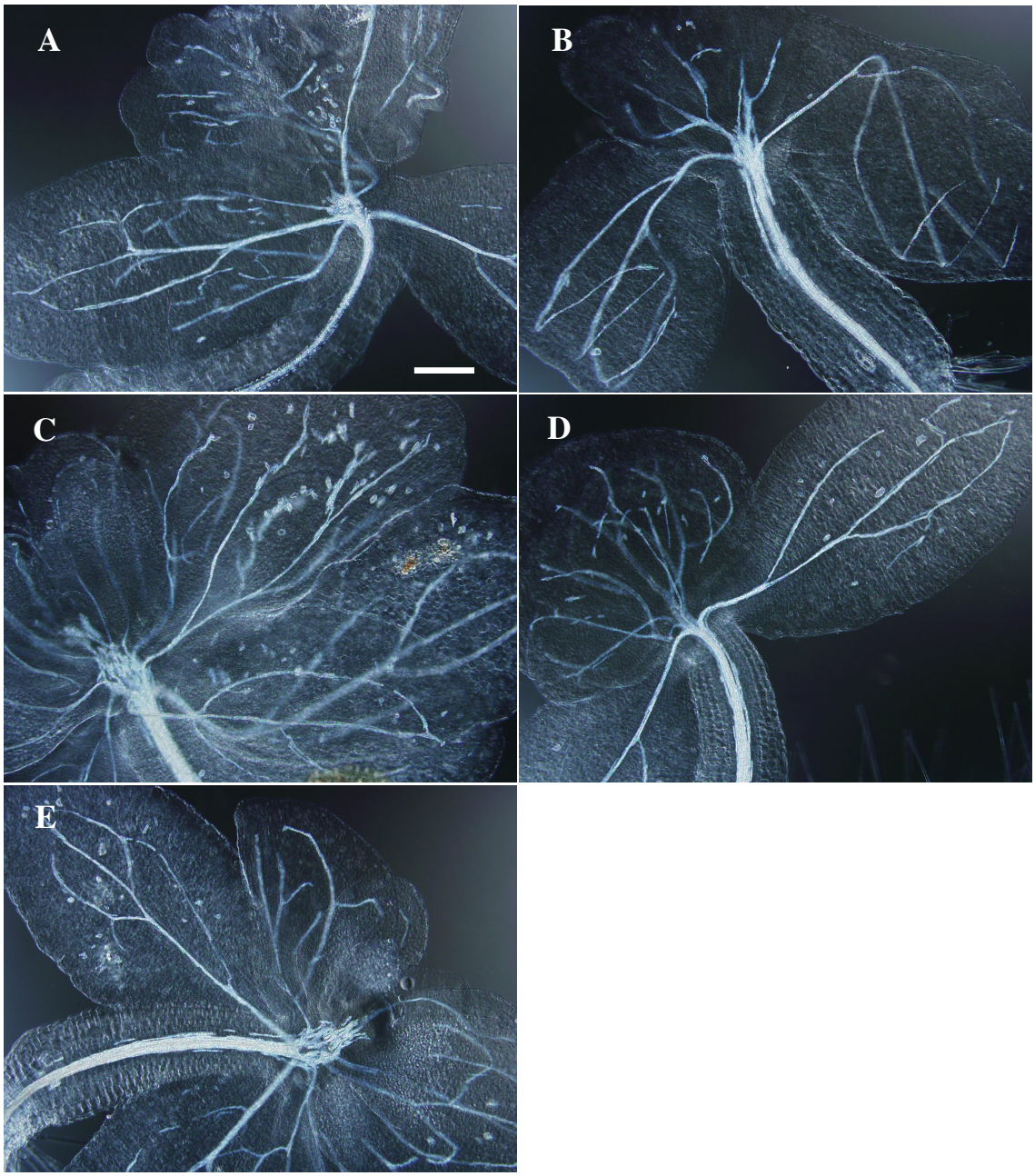


Fig. 24. DIC images of five individual 20-day-old *fie* mutants treated with phytohormones. (A-E) *fie* 1-5 plants treated with phytohomornes for 5 days. KDB, 50 ng/mL kinetin; 500 ng/mL 2,4-dichlorophenoxyacetic acid; and 1 μM brassinolide. Bar = 200 μm.

5-adC + KDB

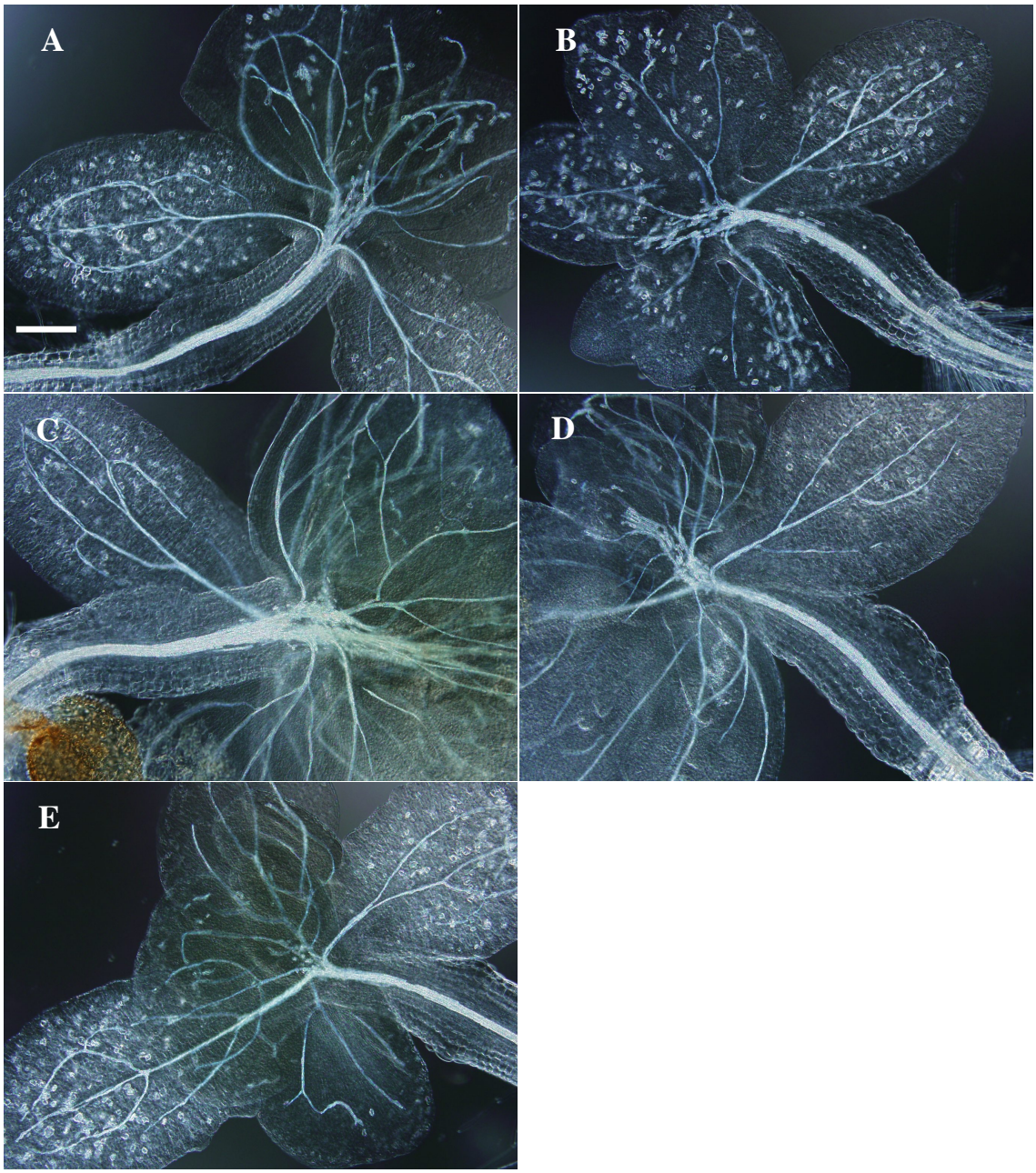


Fig. 25. DIC images of five individual 20-day-old *fie* mutants treated with 5-adC and phytohormones. (A-E) *fie* 1-5 plants treated with 5-adC and phytohormones for 5 days. (5-adC) 1 μg/mL 5-aza-dC; KDB, 50 ng/mL kinetin; 500 ng/mL 2,4-dichlorophenoxyacetic acid; and 1 μM brassinolide. Bar = 200 μm.

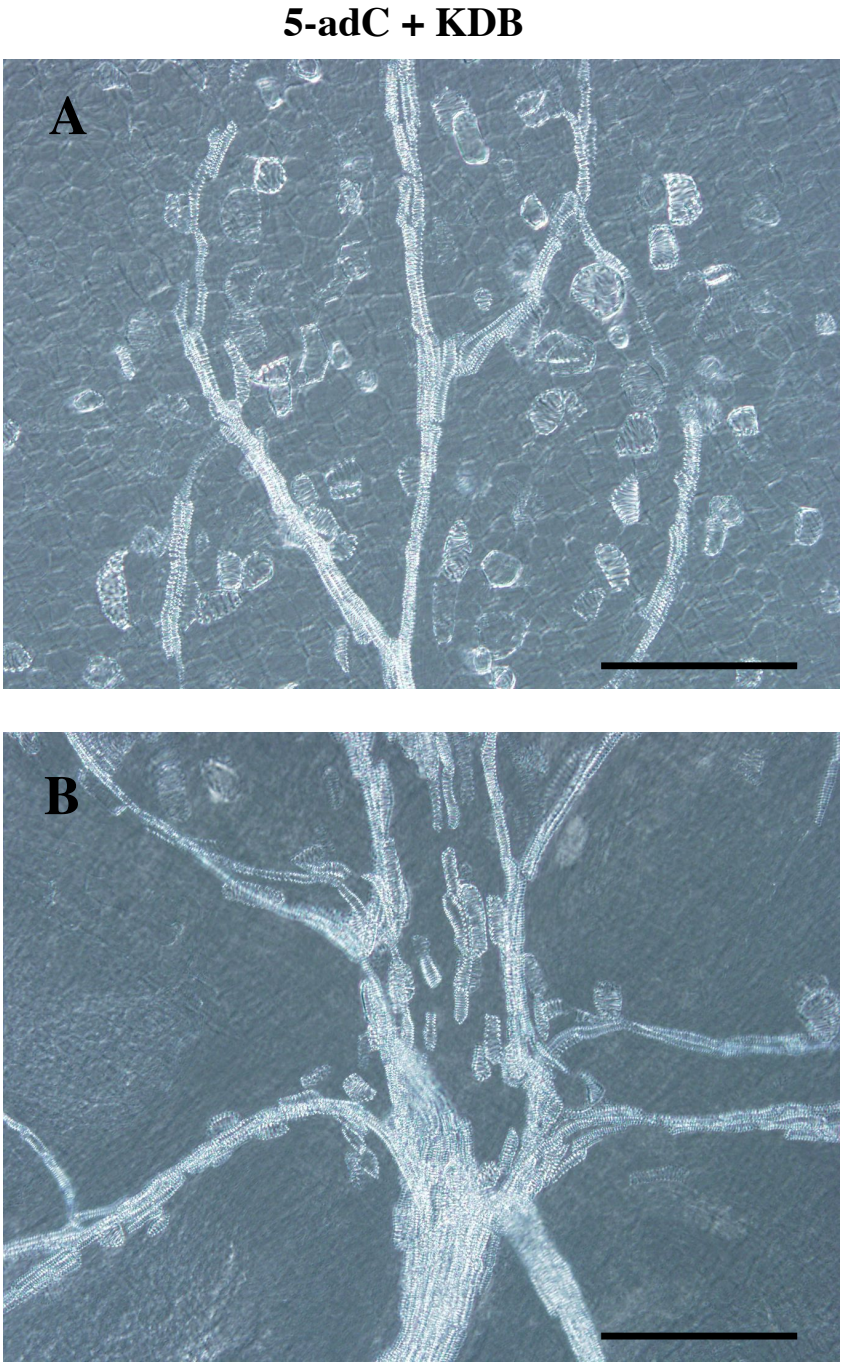


Fig. 26. DIC images of a 20-day-old *fie* 2 plant treated with 5-adC and phytohormones. (A) Leaf. (B) Hypocotyl. Plant treated with 5-adC and phytohormones for 5 days. 5-adC, 1 μg/mL 5-aza-dC; KDB, 50 ng/mL kinetin; 500 ng/mL 2,4-dichlorophenoxyacetic acid; and 1 μM brassinolide. Bar = 200 μm.

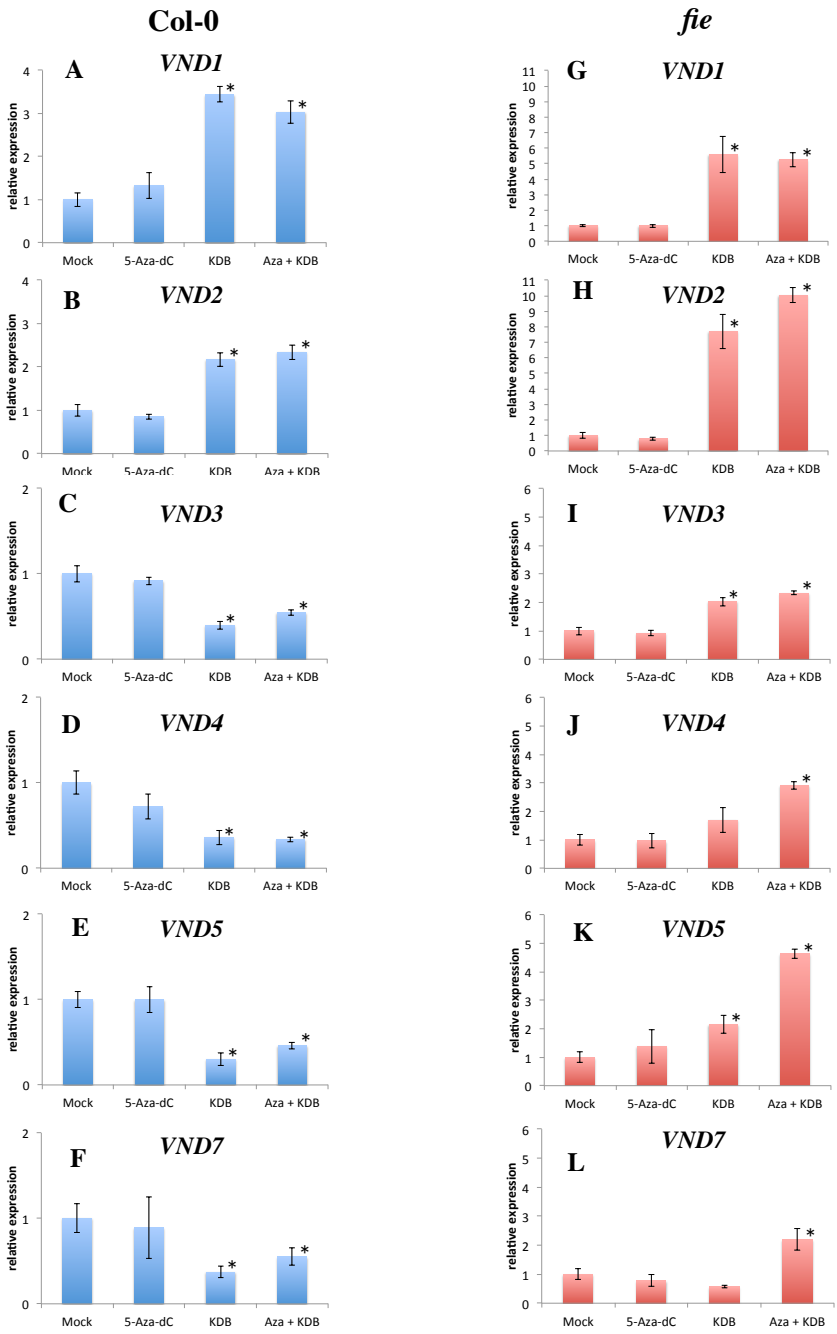


Fig. 27. Expression analysis of *VND1* to *VND5* and *VND7*. Quantitative RT-PCR was performed to analyze the expression of the indicated genes. Five six-day-old seedlings of (A-F) Col-0 and (G-L) *fie* plants were treated with or without 5-adC, phytohormones, or a combination of 5-adC and phytohormones for 3 days. mRNA levels for each gene were normalized to *UBQ10 mRNA*. Error bars indicate SD (n = 3). Asterisks indicate statistically significant differences (Welch's t-test; *P < 0.05) compared with the values of the mock treatment.

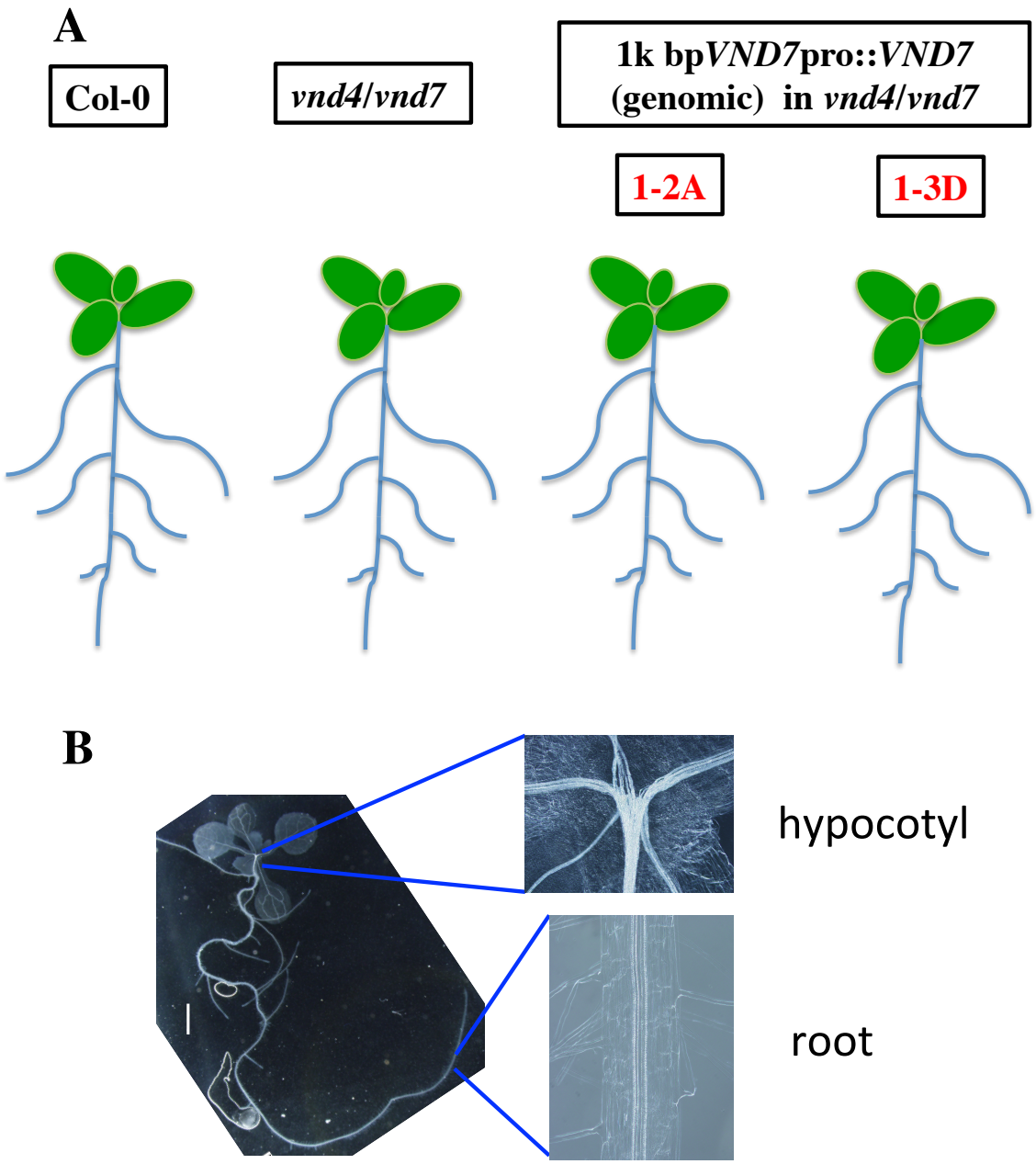


Fig. 28. Schematic diagram of transgenic plants harboring *VND7pro*::*VND7* in the *vnd4*/*vnd7* double mutant background. (A) Col-0, *vnd4*/*vnd7* double mutant, and two independent lines, 1-2A and 1-3D, were selected for analysis. (B) Images of the part of the seedling observed in this study. Bar = 1 cm.

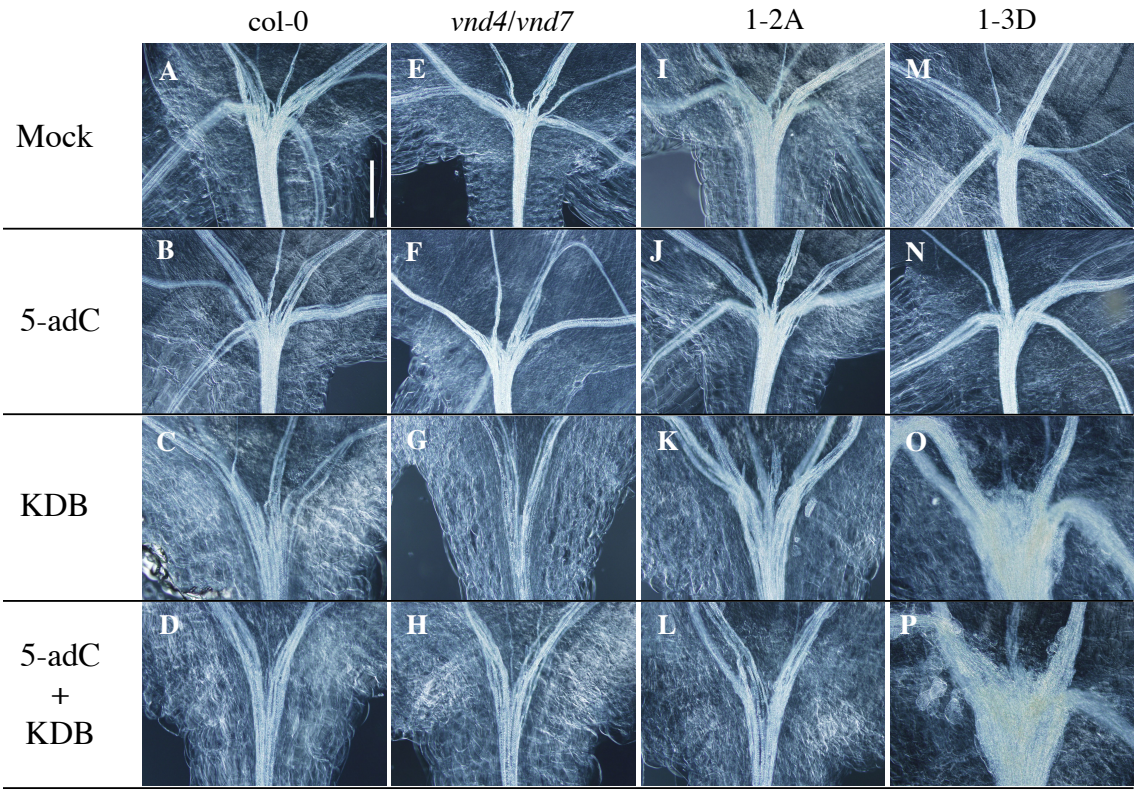


Fig. 29. DIC images of the hypocotyls of Col-0, *vnd4*/*vnd7*, 1-2A, and 1-3D plants treated with chemicals. Six-day-old (A-D) Col-0, (E-H) *vnd4*/*vnd7*, (I-L) 1-2A, and (M-P) 1-3D plants treated with (B, F, J, M) 5-adC, (C, G, K, O) phytohormones (KDB), (D, H, L, P) a combination of 5-adC and phytohormones (5-adC + KDB), or (A, E, I, M) without any treatment (Mock) for 5 days. (5-adC) 1 μg/mL 5-aza-dC; KDB, 50 ng/mL kinetin; 500 ng/mL 2,4-dichlorophenoxyacetic acid; and 1 μM brassinolide. Bar = 100 μm.

5-adC + KDB

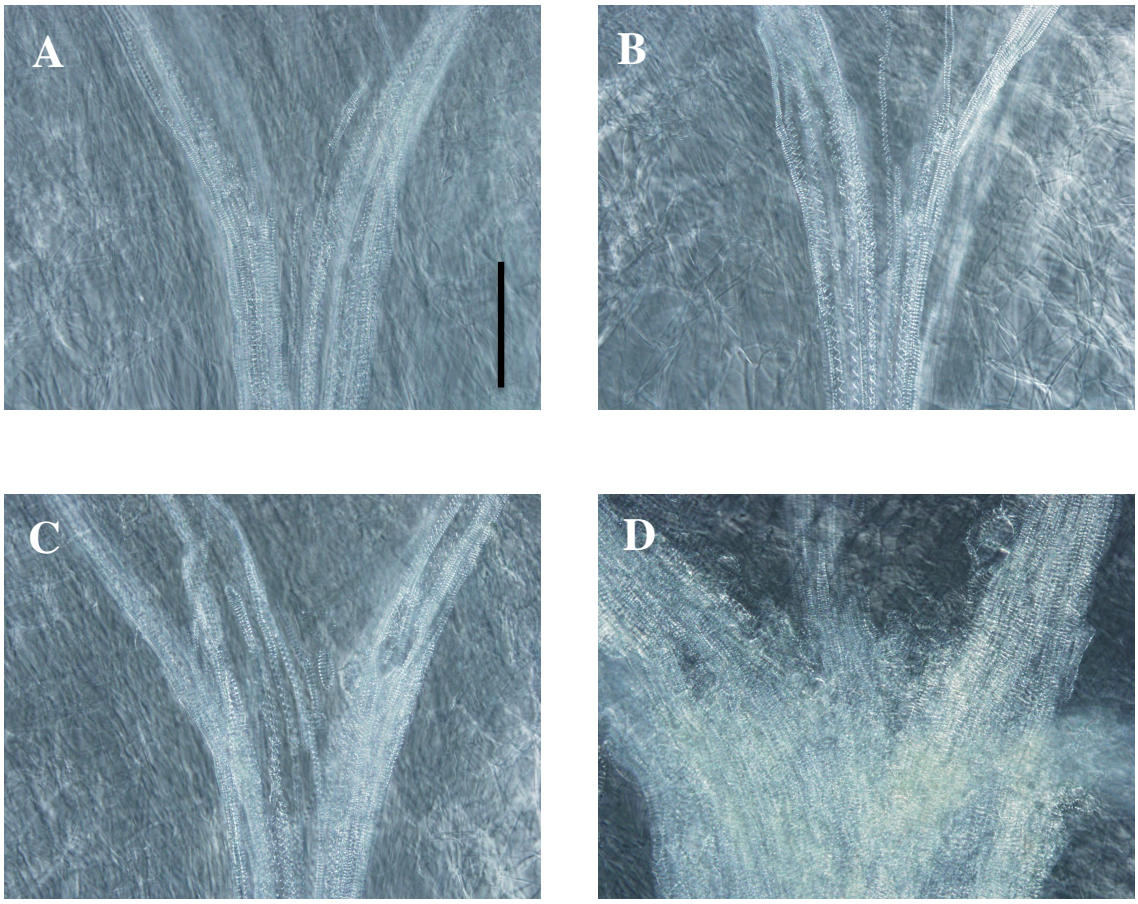


Fig. 30. Magnified DIC images of hypocotyl of Col-0, *vnd4*/*vnd7*, 1-2A, and 1-3D plants. Six-day-old (A) Col-0, (B) *vnd4*/*vnd7*, (C) 1-2A, and (D) 1-3D plants treated with a combination of 5-adC and phytohormones (5-adC + KDB) for 5 days. (5-adC) 1 μg/mL 5-aza-dC; KDB, 50 ng/mL kinetin; 500 ng/mL 2,4-dichlorophenoxyacetic acid; and 1 μM brassinolide. Bar = 100 μm.

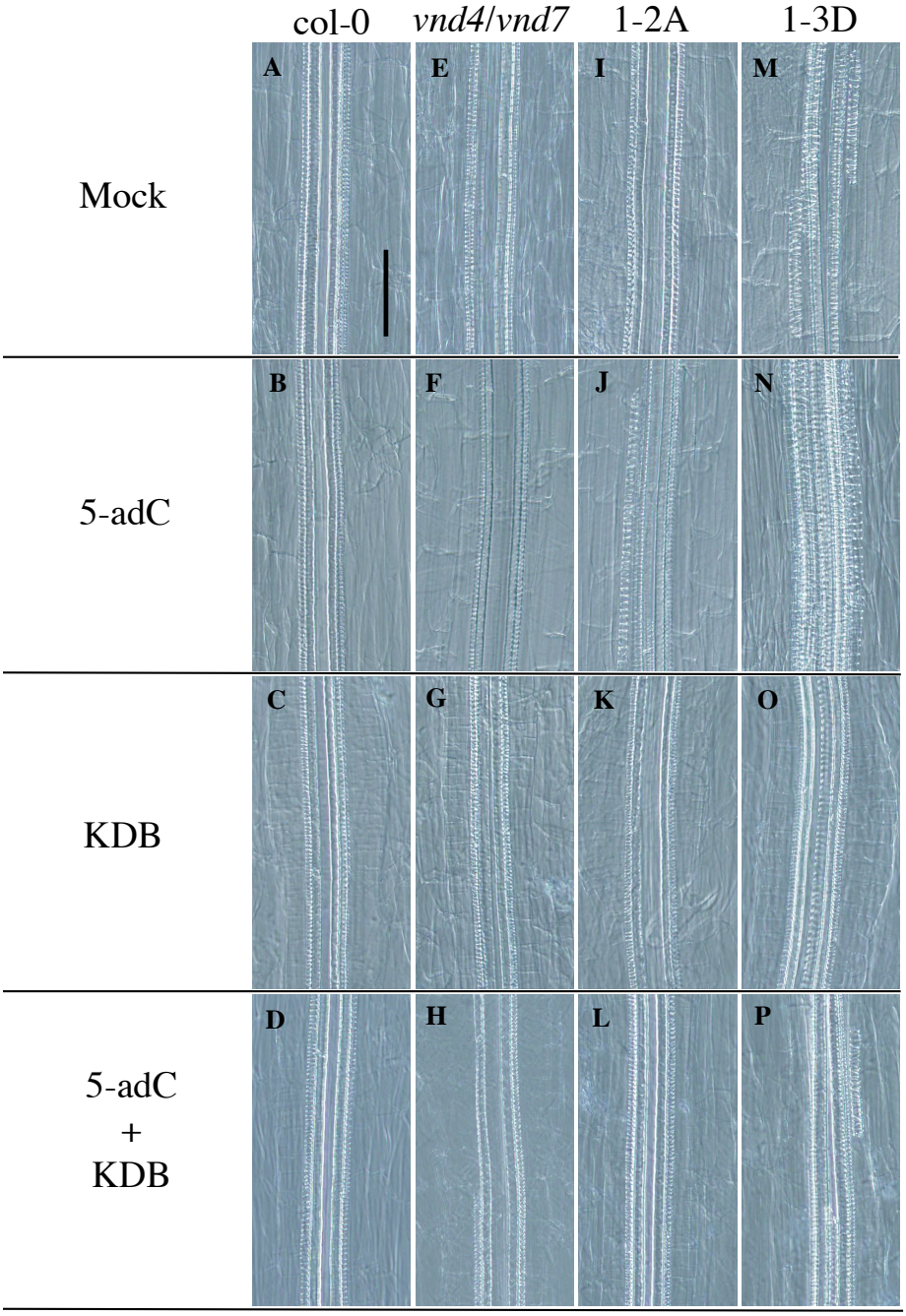


Fig. 31. DIC images of the roots of Col-0, *vnd4*/*vnd7*, 1-2A, and 1-3D plants treated with chemicals. Six-day-old (A-D) Col-0, (E-H) *vnd4*/*vnd7*, (I-L) 1-2A, and (M-P) 1-3D seedlings treated with (B, F, J, M) 5-adC, (C, G, K, O) phytohormones (KDB), (D, H, L, P) a combination of 5-adC and phytohormones (5-adC + KDB), or (A, E, I, M) without any treatment (Mock) for 5 days. (5-adC) 1 μg/mL 5-aza-dC; KDB, 50 ng/mL kinetin; 500 ng/mL 2,4-dichlorophenoxyacetic acid; 1 μM brassinolide. Bar = 50 μm.

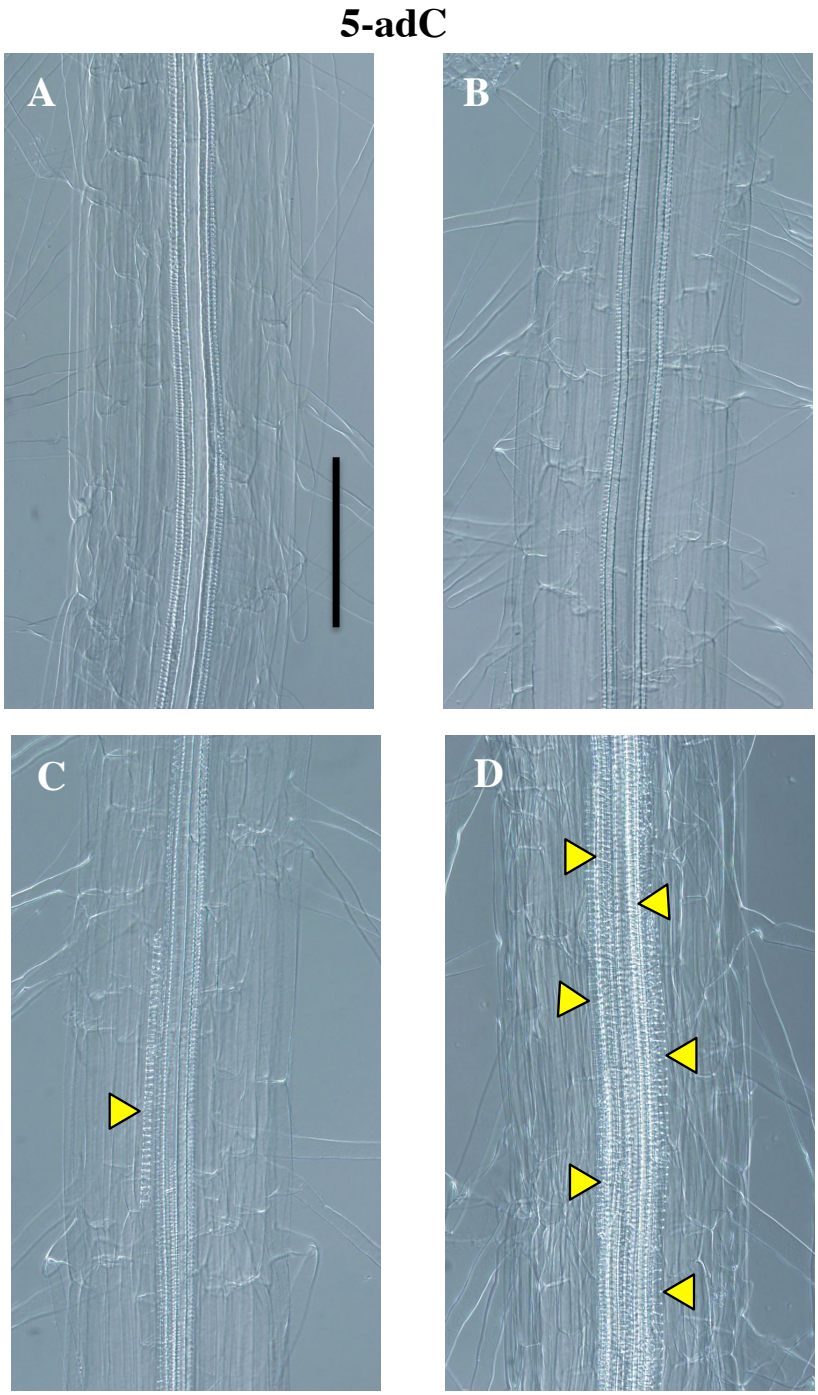


Fig. 32. Magnified DIC images of the roots of Col-0, *vnd4*/*vnd7*, 1-2A, and 1-3D plants treated with 5-adC. Six-day-old (A) Col-0, (B) *vnd4*/*vnd7*, (C) 1-2A, and (D) 1-3D plants treated with 5-adC for 5 days. (5-adC) 1 μg/mL 5-aza-dC. Yellow arrowheads indicate ectopic xylem vessel element. Bar = 100 μm.

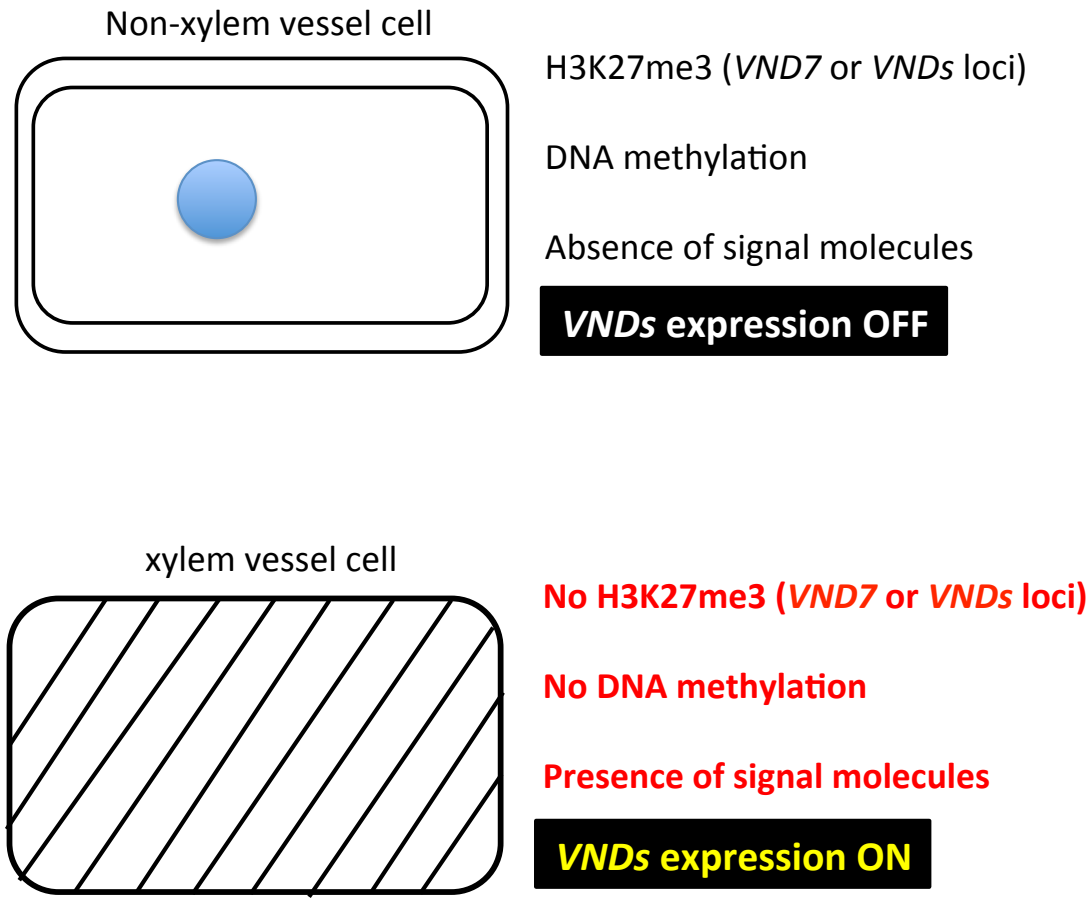


Fig. 33. Model of the transcriptional regulation mechanism underlying *VND* gene expression in xylem and non-xylem cells. The blue circle in the non-xylem cell indicates the nucleus. The spiral patterns in the xylem cell indicate the secondary cell wall deposition typically formed in xylem vessel cells.

Conclusions and perspectives

In this study, I aimed to address the question of how the expression of *VND7*, a master transcriptional switch for the formation of xylem vessels, is spatio-temporally regulated during plant development. Firstly, to decipher the regulatory mechanism underlying *VND7* expression, I tried to reveal the transcription factors that act upstream of *VND7*. I succeeded in identifying several potential transcription factors that positively regulate the expression of *VND7*. However, this study also revealed another unknown potential mechanism that contributes to the tight control of *VND7* expression in planta. To explore this possible mechanism, I secondly focused on the epigenetic control of *VND* genes as epigenetic control is a well-studied mechanism for the tight regulation of certain genes in plants and animals. I found that the *VND7* locus is marked by at least two epigenetic marks, histone H3 lysine 27 tri-methylation (H3K27me3) and DNA methylation. In particular, both epigenetic marks seemed to repress *VND7* expression. Although phytohoromes such as auxin, cytokinin, and brassinosteroid are known to regulate xylem vessel formation principally by controlling the expression of transcription factors, the contribution of epigenetic control to xylem vessel formation had not previously been reported. Therefore, this is the first study showing that xylem vessel formation is coordinately regulated by phytohormone signaling together with the epigenetic control of master transcriptional switches such as *VND7* and/or other *VND* genes. Taken all together, I propose that differentiation of xylem vessel formation is precisely controlled by the spatio-temporal regulation of *VND* gene expression by both phytohormone signaling and epigenetic mechanisms.

To evaluate this possibility, we need to establish a system or method that can monitor changes in epigenetic status and phytohoromone concentration in intact xylem precursor cells. One possible system might involve live imaging techniques that simultaneously trace the epigenetic status of a locus or loci of interest and the level of endogenous phytohormones at the single cell level. For this ambitious endeavor, it will be critical to establish a labeling system that reports the epigenetic status. I believe that my study has precipitated the need to establish such a system, not only for studies of xylem vessel formation, but also for studies that aim to advance our understanding of developmental biology.

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transient assay(pA35G Vectors)

Table S1. transient assay Repoter = VND7pro, Effecter = Transcription Factors (TFs)

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pA35G-MCS		pAGL-VND7proB	284	31135	0.009	1013	56270	0.018	89	22753	0.004	462	36719	0.010	487	17442	0.007	1.00	0.70
pA35G-VND4	At1g12260	pAGL-VND7proB	4360	22626	0.193	8107	12213	0.664	3165	8460	0.374	5211	14433	0.410	2578	7339	0.238	41.00	23.80
pA35G-VND5	At1g62700	pAGL-VND7proB	11203	24608	0.455	35613	36385	0.979	11465	25581	0.448	19427	28858	0.627	14018	6537	0.305	62.70	30.50
pA35G-LBD30	At4g00220	pAGL-VND7proB	332	6555	0.051	615	6797	0.090	339	4703	0.072	429	6018	0.071	161	1146	0.020	7.10	2.00
pA35G-AtHB8	At4G32880	pAGL-VND7proB	668	24981	0.027	405	18999	0.021	287	21474	0.013	453	21818	0.020	195	3006	0.007	2.00	0.70
pA35G-AtHB15	At1g52150	pAGL-VND7proB	2163	65765	0.033	514	25110	0.020	509	29171	0.017	1062	40015	0.023	953	22392	0.009	2.30	0.90
pA35G-REV	At5g60690	pAGL-VND7proB	481	16926	0.028	812	18032	0.045	883	21515	0.041	725	18824	0.038	215	2395	0.009	3.80	0.90
pA35G-pE7(bHLH)	At1g63650	pAGL-VND7proB	367	29831	0.012	273	20338	0.013	646	30636	0.021	429	26935	0.015	194	5727	0.005	1.50	0.50

Table S2. transient assay Repoter = VND7pro, Effecter = Transcription Factors (TFs)

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pA35G-MCS		pAGL-VND7proB	239	18554	0.013	282	27643	0.010	411	45267	0.009	311	30488	0.011	90	13582	0.002	1.00	0.18
pA35G-VND7	At1g71930	pAGL-VND7proB	10030	26619	0.377	7747	14906	0.520	4162	22829	0.182	7313	21451	0.360	2958	5977	0.170	32.73	15.46
pA35G-PHB	At2g34710	pAGL-VND7proB	962	45218	0.021	474	44389	0.011	515	26666	0.019	650	38758	0.017	271	10480	0.005	1.55	0.46
pA35G-PHV	At1g30490	pAGL-VND7proB	245	13010	0.019	612	27109	0.023	304	17823	0.017	387	19314	0.020	197	7167	0.003	1.82	0.27
pA35G-pE10(z-f)	At1g72220	pAGL-VND7proB	285	14057	0.020	242	8066	0.030	373	37852	0.010	300	19992	0.020	67	15755	0.010	1.82	0.91
pA35G-pE17(z-f)	At5g17600	pAGL-VND7proB	953	98991	0.010	474	76193	0.006	586	60234	0.010	671	78473	0.009	251	19479	0.002	0.82	0.18
pA35G-pE22(XND1)	At5g64530	pAGL-VND7proB	255	12332	0.021	538	18280	0.029	466	24591	0.019	420	18401	0.023	147	6130	0.005	2.09	0.46
pA35G-pE26(BZIP1)	At5g49450	pAGL-VND7proB	1043	31006	0.034	543	21267	0.026	570	21014	0.027	719	24429	0.029	281	5697	0.004	2.64	0.36

Table S3. transient assay Repoter = VND7pro, Effecter = Transcription Factors (TFs)

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pA35G-MCS		pAGL-VND7proB	491	3657	0.134	127	1870	0.068	317	4213	0.075	312	3247	0.092	182	1224	0.036	1.00	0.39
pA35G-NF-YB3	At4g14540	pAGL-VND7proB	339	967	0.351	750	5711	0.131	229	3146	0.073	439	3275	0.185	275	2375	0.147	2.01	1.60
pA35G-KNAT7	At1g62990	pAGL-VND7proB	153	1616	0.095	131	1463	0.090	193	2706	0.071	159	1928	0.085	31	678	0.013	0.92	0.14
pA35G-VND6	At5g62380	pAGL-VND7proB	4522	5218	0.867	1012	3560	0.284	5472	9066	0.604	3669	5948	0.585	2349	2825	0.292	6.36	3.17
pA35G-VND3	At5g66300	pAGL-VND7proB	1245	2182	0.571	2955	3462	0.854	3329	4368	0.762	2510	3337	0.729	1111	1098	0.144	7.92	1.57
pA35G-z-fC2H2	At5g03510	pAGL-VND7proB	350	9388	0.037	1722	28766	0.060	686	21300	0.032	919	19818	0.043	715	9774	0.015	0.47	0.16
pA35G-MDA7.1	At5g55970	pAGL-VND7proB	176	2708	0.065	164	4082	0.040	318	4001	0.079	219	3597	0.061	86	771	0.020	0.66	0.22

Table S4. transient assay Repoter = VND7pro, Effecter = Transcription Factors (TFs)

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pA35G-MCS		pAGL-VND7proB	617	13932	0.044	492	8660	0.057	476	15419	0.031	528	12670	0.044	77	3552	0.013	1.00	0.30
pA35G-GATA	At5g66320	pAGL-VND7proB	2552	4859	0.525	2488	6031	0.413	1937	4553	0.425	2326	5148	0.454	338	780	0.061	10.32	1.39
pA35G-bHLH	At1g35460	pAGL-VND7proB	613	21145	0.029	847	26791	0.032	699	14625	0.048	720	20854	0.036	118	6088	0.010	0.82	0.23
pA35G-SND2	At4g28500	pAGL-VND7proB	1349	9089	0.148	903	5168	0.175	528	4310	0.123	927	6189	0.149	411	2548	0.026	3.39	0.59
pA35G-VND1	At2g18060	pAGL-VND7proB	8170	6069	1.346	4499	3079	1.461	5858	6921	0.846	6176	5356	1.218	1856	2018	0.327	27.68	7.43

Table S5. transient assay Repoter = VND7pro, Effecter = Transcription Factors (TFs)

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pA35G-MCS		pAGL-VND7proB	743	13541	0.055	945	10014	0.094	1396	18375	0.076	1028	13977	0.075	334	4197	0.020	1.00	0.27
pA35G-VND1	At2g18060	pAGL-VND7proB	8303	5295	1.568	9150	6300	1.452	4610	3797	1.214	7354	5131	1.411	2414	1260	0.180	18.81	2.40
pA35G-VND4	At1g12260	pAGL-VND7proB	7611	13918	0.547	6155	16338	0.377	3691	6335	0.583	5819	12197	0.502	1981	5219	0.110	6.69	1.47
pA35G-GATA5	At5g66320	pAGL-VND7proB	1173	3859	0.304	1503	2855	0.526	1820	4918	0.370	1499	3877	0.400	324	1032	0.114	5.33	1.52
pA35G-LBD18	At2g45420	pAGL-VND7proB	1798	3823	0.470	2019	3003	0.672	671	3544	0.189	1496	3457	0.444	723	417	0.243	5.92	3.24
pA35G-LBD30	At4g00220	pAGL-VND7proB	330	1873	0.176	490	2704	0.181	429	1196	0.359	416	1924	0.239	81	755	0.104	3.19	1.39
pA35G-ATBZIP1	At5g49450	pAGL-VND7proB	426	8264	0.052	908	8282	0.110	1010	11346	0.089	781	9297	0.084	312	1774	0.029	1.12	0.39
pA35G-REV	At5g60690	pAGL-VND7proB	715	8073	0.089	1135	10128	0.112	1123	8549	0.131	991	8917	0.111	239	1076	0.021	1.48	0.28

Table S6. transient assay Repoter = VND7pro, Effecter = Transcription Factors (TFs)

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pA35G-MCS		pAGL-VND7proB	825	14730	0.056	1394	18542	0.075	466	5685	0.082	895	12986	0.071	468	6604	0.013	1.00	0.18
pA35G-REV	At5g60690	pAGL-VND7proB	449	9832	0.046	271	7448	0.036	478	11758	0.041	399	9679	0.041	112	2159	0.005	0.58	0.07
pA35G-REVabv	At5g60690	pAGL-VND7proB	459	5188	0.088	215	7904	0.027	442	7765	0.057	372	6952	0.057	136	1530	0.031	0.80	0.44
pA35G-GATA5	At5g66320	pAGL-VND7proB	3264	4164	0.784	1055	4851	0.217	1720	7780	0.221	2013	5598	0.407	1133	1920	0.326	5.73	4.59
pA35G-VND1	At2g18060	pAGL-VND7proB	3509	5830	0.602	3720	6397	0.582	4024	8156	0.493	3751	6794	0.559	259	1213	0.058	7.87	0.82

Table S7. transient assay Repoter = VND7pro / XCP1pro, Effecter = Transcription Factors (TFs)

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pA35G-MCS		pAGL-VND7proB	243	5382	0.045	1077	7601	0.142	132	5685	0.023	484	6223	0.070	517	1203	0.063	1.00	0.90
pA35G-REV	At5g60690	pAGL-VND7proB	263	3115	0.084	293	3947	0.074	114	11758	0.010	223	6273	0.056	96	4768	0.040	0.80	0.57
pA35G-REVabv	At5g60690	pAGL-VND7proB	140	2473	0.057	227	3688	0.062	631	7765	0.081	333	4642	0.067	262	2772	0.013	0.96	0.19
pA35G-GATA5	At5g66320	pAGL-VND7proB	840	1027	0.818	888	590	1.505	730	7780	0.094	819	3132	0.806	81	4031	0.706	11.51	10.09

Table S8. transient assay Repoter = VND7pro, Effecter = Transcription Factors (TFs)

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pA35G-MCS		pAGL-VND7proB	1996	36979	0.054	471	19987	0.024	752	22017	0.034	1073	26328	0.037	812	9280	0.015	1.00	0.41
pA35G-pE3(bHLH)	At1g35460	pAGL-VND7proB	988	34220	0.029	757	39574	0.019	3488	95333	0.037	1744	56376	0.028	1514	33844	0.009	0.76	0.24
pA35G-bHLH(29950)	At1g29950	pAGL-VND7proB	906	15677	0.058	808	18497	0.044	447	7095	0.063	720	13756	0.055	242	5939	0.010	1.49	0.27
pA35G-Dof5.3	At5g60200	pAGL-VND7proB	1471	20817	0.071	577	16538	0.035	942	13981	0.067	997	17112	0.058	450	3454	0.020	1.57	0.54
pA35G-bHLH(68810)	At1g68810	pAGL-VND7proB	679	11287	0.060	1126	9090	0.124	276	5647	0.049	694	8675	0.078	425	2843	0.041	2.11	1.11
pA35G-bHLH(51140)	At1g51140	pAGL-VND7proB	1748	67846	0.026	1352	38886	0.035	1381	44921	0.031	1494	50551	0.031	221	15279	0.005	0.84	0.14

Table S9. transient assay Repoter = VND7pro, Effecter = Transcription Factors (TFs)

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pA35G-MCS		pAGL-VND7proB	215	2671	0.080	189	1245	0.152	388	3081	0.126	264	2332	0.119	108	964	0.036	1.00	0.30
pA35G-TLP5	At1g43640	pAGL-VND7proB	127	771	0.165	137	3169	0.043	87	1115	0.078	117	1685	0.095	26	1297	0.063	0.80	0.53
pA35G-bHLH038	At3g56970	pAGL-VND7proB	102	707	0.144	153	3257	0.047	180	3308	0.054	145	2424	0.082	40	1487	0.054	0.69	0.45
pA35G-bHLH039	At3g56980	pAGL-VND7proB	135	1039	0.130	134	1661	0.081	105	813	0.129	125	1171	0.113	17	439	0.028	0.95	0.24
pA35G-Dof5.8	At5g66940	pAGL-VND7proB	188	1647	0.114	198	3296	0.060	297	8097	0.037	228	4347	0.070	60	3351	0.040	0.59	0.34
pA35G-TLP1	At1g76900	pAGL-VND7proB	269	1899	0.142	298	7844	0.038	221	2701	0.082	263	4148	0.087	39	3226	0.052	0.73	0.44
pA35G-MYB(2g38090)	At2g38090	pAGL-VND7proB	111	1291	0.086	192	3198	0.060	240	10700	0.022	181	5063	0.056	65	4974	0.032	0.47	0.27
pA35G-HAP5B	At1g56170	pAGL-VND7proB	109	1180	0.092	145	1978	0.073	127	2668	0.048	127	1942	0.071	18	745	0.022	0.60	0.19

Table S10. transient assay Repoter = VND7pro, Effecter = Transcription Factors (TFs)

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pA35G-MCS		pAGL-VND7proB	185	2672	0.069	108	3159	0.034	66	1875	0.035	120	2569	0.046	60	648	0.020	1.00	0.44
pA35G-SAC51	At5g64340	pAGL-VND7proB	93	2048	0.045	85	2895	0.029	73	2730	0.027	84	2558	0.034	10	449	0.010	0.74	0.22
pA35G-SAClike	At5g09460	pAGL-VND7proB	164	3992	0.041	85	2173	0.039	103	3137	0.033	117	3101	0.038	41	910	0.004	0.83	0.09
pA35G-LBD4	At1g31320	pAGL-VND7proB	162	2499	0.065	61	1391	0.044	65	1035	0.063	96	1642	0.057	57	764	0.012	1.24	0.26
pA35G-LBD15	At2g40470	pAGL-VND7proB	242	1536	0.158	197	909	0.217	177	1405	0.126	205	1283	0.167	33	331	0.046	3.63	1.00
pA35G-MP	At1g19850	pAGL-VND7proB	113	2630	0.043	66	1792	0.037	86	2082	0.041	88	2168	0.040	24	426	0.003	0.87	0.07

Table S11. transient assay Repoter = VND7pro, Effecter = Transcription Factors (TFs)

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pA35G-MCS		pAGL-VND7proB	354	6444	0.055	356	5274	0.068	366	8020	0.046	359	6579	0.056	6	1378	0.011	1.00	0.20
pA35G-MYB	At3g10760	pAGL-VND7proB	421	8098	0.052	557	7992	0.070	331	7508	0.044	436	7866	0.055	114	315	0.013	0.98	0.23
pA35G-ZFP6	At1g67030	pAGL-VND7proB	566	3972	0.142	373	4058	0.092	413	3879	0.106	451	3970	0.113	102	90	0.026	2.02	0.46
pA35G-HAT14	At5g06710	pAGL-VND7proB	139	1761	0.079	228	2915	0.078	175	2103	0.083	181	2260	0.080	45	593	0.003	1.43	0.05
pA35G-MYB	At1g69580	pAGL-VND7proB	561	6307	0.089	673	9844	0.068	391	13682	0.029	542	9944	0.062	142	3689	0.030	1.11	0.54
pA35G-ANAC075	At4g29230	pAGL-VND7proB	1857	3942	0.471	3286	8797	0.374	1747	6259	0.279	2297	6333	0.375	859	2428	0.096	6.70	1.71
pA35G-LBD15	At2g40470	pAGL-VND7proB	1551	2248	0.690	1640	1767	0.928	1395	3156	0.442	1529	2390	0.687	124	705	0.243	12.27	4.34
pA35G-GATA5	At5g66320	pAGL-VND7proB	2827	3152	0.897	1307	3564	0.367	1614	3256	0.496	1916	3324	0.587	804	214	0.276	10.48	4.93

Table S12. transient assay Repoter = VND7pro, Effecter = Transcription Factors (TFs)

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pA35G-MCS		pAGL-VND7proB	112	1217	0.092	103	2398	0.043	102	1552	0.066	106	1722	0.067	6	609	0.025	1.00	0.37
pA35G-GATA5	At5g66320	pAGL-VND7proB	1425	703	2.027	542	679	0.798	931	747	1.246	966	710	1.357	443	34	0.622	20.25	9.28
pA35G-GATA12	At5g25830	pAGL-VND7proB	253	724	0.349	83	156	0.532	1651	2316	0.713	662	1065	0.531	860	1120	0.182	7.93	2.72

Table S13. transient assay Repoter = VND1pro, Effecter = VND1 ~ VND7

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pA35G-MCS		pAGL-VND1pro	4037	9529	0.424	4071	12254	0.332	5997	11471	0.523	4702	11085	0.426	1122	1403	0.096	1.00	0.23
pA35G-VND1	At2g18060	pAGL-VND1pro	1514	3275	0.462	1360	4008	0.339	2508	5374	0.467	1794	4219	0.423	623	1065	0.073	0.99	0.17
pA35G-VND2	At4g36160	pAGL-VND1pro	3343	6017	0.556	1605	4913	0.327	4605	12654	0.364	3184	7861	0.416	1506	4187	0.123	0.98	0.29
pA35G-VND3	At5g66300	pAGL-VND1pro	2543	10871	0.234	1830	3745	0.489	5635	18284	0.308	3336	10967	0.344	2023	7270	0.131	0.81	0.31
pA35G-VND4	At1g12260	pAGL-VND1pro	2119	12831	0.165	866	2940	0.295	922	6227	0.148	1302	7333	0.203	708	5037	0.080	0.48	0.19
pA35G-VND5	At1g62700	pAGL-VND1pro	967	2677	0.361	1647	9283	0.177	3058	8171	0.374	1891	6710	0.304	1067	3537	0.110	0.71	0.26
pA35G-VND6	At5g62380	pAGL-VND1pro	1968	5424	0.363	2463	5614	0.439	4218	5537	0.762	2883	5525	0.521	1182	96	0.212	1.22	0.50
pA35G-VND7	At1g71930	pAGL-VND1pro	310	4081	0.076	274	4388	0.062	336	6889	0.049	307	5119	0.062	31	1540	0.014	0.15	0.03

Table S14. transient assay Repoter = VND2pro, Effecter = VND1 ~ VND7

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pA35G-MCS		pAGL-VND2pro	5353	16138	0.332	11035	23567	0.468	15292	41408	0.369	10560	27038	0.390	4986	12988	0.070	1.00	0.18
pA35G-VND1	At2g18060	pAGL-VND2pro	2101	4613	0.455	7773	3514	2.212	3894	4210	0.925	4589	4112	1.197	2899	556	0.910	3.07	2.33
pA35G-VND2	At4g36160	pAGL-VND2pro	3908	9766	0.400	3863	12726	0.304	2506	12665	0.198	3426	11719	0.301	797	1692	0.101	0.77	0.26
pA35G-VND3	At5g66300	pAGL-VND2pro	6377	1933	3.299	13442	11013	1.221	9600	11194	0.858	9806	8047	1.793	3537	5295	1.317	4.60	3.38
pA35G-VND4	At1g12260	pAGL-VND2pro	3525	16503	0.214	1766	5142	0.343	2323	11584	0.201	2538	11076	0.253	899	5697	0.079	0.65	0.20
pA35G-VND5	At1g62700	pAGL-VND2pro	4607	9891	0.466	3864	7887	0.490	2953	6200	0.476	3808	7993	0.477	828	1848	0.012	1.22	0.03
pA35G-VND6	At5g62380	pAGL-VND2pro	1742	5753	0.303	4895	15891	0.308	4037	27434	0.147	3558	16359	0.253	1630	10848	0.092	0.65	0.24
pA35G-VND7	At1g71930	pAGL-VND2pro	2011	11612	0.173	1805	11476	0.157	1806	7973	0.227	1874	10354	0.186	119	2063	0.037	0.48	0.10

Table S15. transient assay Repoter = VND3pro, Effecter = VND1 ~ VND7

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pA35G-MCS		pAGL-VND3pro	327	4802	0.068	701	8735	0.080	220	5996	0.037	416	6511	0.062	253	2016	0.022	1.00	0.36
pA35G-VND1	At2g18060	pAGL-VND3pro	2317	5199	0.446	1052	5843	0.180	712	3163	0.225	1360	4735	0.284	846	1399	0.142	4.58	2.29
pA35G-VND2	At4g36160	pAGL-VND3pro	1192	2195	0.543	3013	6777	0.445	4357	7466	0.584	2854	5479	0.524	1588	2865	0.071	8.45	1.15
pA35G-VND3	At5g66300	pAGL-VND3pro	3330	10507	0.317	2010	10338	0.194	3262	10223	0.319	2867	10356	0.277	743	143	0.072	4.47	1.16
pA35G-VND4	At1g12260	pAGL-VND3pro	1103	5893	0.187	3240	9838	0.329	2788	11719	0.238	2377	9150	0.251	1126	2973	0.072	4.05	1.16
pA35G-VND5	At1g62700	pAGL-VND3pro	4578	8741	0.524	14085	47966	0.294	3731	19202	0.194	7465	25303	0.337	5749	20312	0.169	5.44	2.73
pA35G-VND6	At5g62380	pAGL-VND3pro	3097	4783	0.648	5053	6174	0.818	2267	6084	0.373	3472	5680	0.613	1430	778	0.225	9.89	3.63
pA35G-VND7	At1g71930	pAGL-VND3pro	761	4924	0.155	526	4601	0.114	416	4120	0.101	568	4548	0.123	176	405	0.028	1.98	0.45

Table S16. transient assay Repoter = VND4pro, Effecter = VND1 ~ VND7

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pA35G-MCS		pAGL-VND4pro	7370	7833	0.941	9009	5213	1.728	14582	14536	1.003	10320	9194	1.224	3781	4808	0.438	1.00	0.36
pA35G-VND1	At2g18060	pAGL-VND4pro	4944	2437	2.029	8024	5341	1.502	9640	7664	1.258	7536	5147	1.596	2386	2619	0.394	1.30	0.32
pA35G-VND2	At4g36160	pAGL-VND4pro	12879	6381	2.018	33542	12399	2.705	4145	3970	1.044	16855	7583	1.922	15097	4341	0.835	1.57	0.68
pA35G-VND3	At5g66300	pAGL-VND4pro	10736	5596	1.919	40676	13767	2.955	14751	8804	1.675	22054	9389	2.183	16251	4117	0.680	1.78	0.56
pA35G-VND4	At1g12260	pAGL-VND4pro	5006	9898	0.506	2158	4524	0.477	9234	15763	0.586	5466	10062	0.523	3560	5621	0.056	0.43	0.05
pA35G-VND5	At1g62700	pAGL-VND4pro	6297	8535	0.738	7824	6031	1.297	5860	4842	1.210	6660	6469	1.082	1031	1885	0.301	0.88	0.25
pA35G-VND6	At5g62380	pAGL-VND4pro	7618	4864	1.566	2894	1132	2.557	6269	3597	1.743	5594	3198	1.955	2433	1898	0.529	1.60	0.43
pA35G-VND7	At1g71930	pAGL-VND4pro	2582	5499	0.470	2453	6332	0.387	3756	5479	0.686	2930	5770	0.514	718	487	0.154	0.42	0.13

Table S17. transient assay Repoter = VND5pro, Effecter = VND1 ~ VND7

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pA35G-MCS		pAGL-VND5pro	1196	2363	0.506	4557	15811	0.288	2266	7862	0.288	2673	8679	0.361	1717	6761	0.126	1.00	0.35
pA35G-VND1	At2g18060	pAGL-VND5pro	6106	2385	2.560	6284	4024	1.562	7429	8063	0.921	6606	4824	1.681	718	2922	0.826	4.66	2.29
pA35G-VND2	At4g36160	pAGL-VND5pro	7627	2754	2.769	12761	7544	1.692	11658	6189	1.884	10682	5496	2.115	2703	2469	0.574	5.86	1.59
pA35G-VND3	At5g66300	pAGL-VND5pro	11438	8047	1.421	7103	5304	1.339	11225	8835	1.271	9922	7395	1.344	2444	1854	0.075	3.72	0.21
pA35G-VND4	At1g12260	pAGL-VND5pro	6925	9291	0.745	3660	18798	0.195	3565	7769	0.459	4717	11953	0.466	1913	5977	0.275	1.29	0.76
pA35G-VND5	At1g62700	pAGL-VND5pro	5284	10992	0.481	2031	7623	0.266	9726	12061	0.806	5680	10225	0.518	3863	2316	0.272	1.44	0.75
pA35G-VND6	At5g62380	pAGL-VND5pro	6233	4389	1.420	33363	24506	1.361	16329	9712	1.681	18642	12869	1.487	13712	10423	0.170	4.12	0.47
pA35G-VND7	At1g71930	pAGL-VND5pro	2960	3613	0.819	1877	5103	0.368	3261	5449	0.598	2699	4722	0.595	728	976	0.226	1.65	0.63

Table S18. transient assay Repoter = VND6pro, Effecter = VND1 ~ VND7

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pA35G-MCS		pAGL-VND6pro	445	7702	0.058	180	4789	0.038	269	8294	0.032	298	6928	0.043	135	1876	0.014	1.00	0.33
pA35G-VND1	At2g18060	pAGL-VND6pro	524	4319	0.121	508	7874	0.065	258	5472	0.047	430	5888	0.078	149	1814	0.039	1.81	0.91
pA35G-VND2	At4g36160	pAGL-VND6pro	339	4862	0.070	287	4407	0.065	235	2970	0.079	287	4080	0.071	52	988	0.007	1.65	0.16
pA35G-VND3	At5g66300	pAGL-VND6pro	599	6193	0.097	2226	12627	0.176	1731	11151	0.155	1519	9990	0.143	834	3370	0.041	3.33	0.95
pA35G-VND4	At1g12260	pAGL-VND6pro	218	7612	0.029	350	15529	0.023	264	9808	0.027	277	10983	0.026	67	4087	0.003	0.61	0.07
pA35G-VND5	At1g62700	pAGL-VND6pro	420	12346	0.034	218	6626	0.033	181	5466	0.033	273	8146	0.033	129	3683	0.001	0.77	0.02
pA35G-VND6	At5g62380	pAGL-VND6pro	783	11023	0.071	1094	15673	0.070	252	8267	0.030	710	11654	0.057	426	3743	0.023	1.33	0.54
pA35G-VND7	At1g71930	pAGL-VND6pro	208	5685	0.037	386	9869	0.039	147	4319	0.034	247	6624	0.037	124	2892	0.003	0.86	0.07

Table S19. transient assay Repoter = VND7pro, Effecter = VND1 ~ VND7

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pA35G-MCS		pAGL-VND7proB	396	11190	0.035	1008	11326	0.089	327	10625	0.031	577	11047	0.052	375	372	0.032	1.00	0.62
pA35G-VND1	At2g18060	pAGL-VND7proB	22622	6020	3.758	22867	8516	2.685	23435	7500	3.125	22975	7345	3.189	417	1255	0.539	61.33	10.37
pA35G-VND2	At4g36160	pAGL-VND7proB	9262	5019	1.845	7836	5264	1.489	5497	3895	1.411	7532	4726	1.582	1901	730	0.231	30.42	4.44
pA35G-VND3	At5g66300	pAGL-VND7proB	24072	7000	3.439	8580	5087	1.687	23945	17026	1.406	18866	9704	2.177	8908	6412	1.102	41.87	21.19
pA35G-VND4	At1g12260	pAGL-VND7proB	10149	16026	0.633	11400	9740	1.170	4585	9286	0.494	8711	11684	0.766	3628	3767	0.357	14.73	6.87
pA35G-VND5	At1g62700	pAGL-VND7proB	12225	10132	1.207	28019	18274	1.533	25416	11240	2.261	21887	13215	1.667	8468	4416	0.540	32.06	10.39
pA35G-VND6	At5g62380	pAGL-VND7proB	18866	14002	1.347	15797	5213	3.030	17234	5623	3.065	17299	8279	2.481	1536	4960	0.982	47.71	18.89
pA35G-VND7	At1g71930	pAGL-VND7proB	11899	7129	1.669	11305	6413	1.763	37168	21956	1.693	20124	11833	1.708	14764	8774	0.049	32.85	0.94

*MCS=Multi Cloning Site(Negative control)
*firefly= luciferase activity
*Renilla= renilla activity (reference)
*SD=Standard Deviation
*Ratio=firefly/Renilla
*Relative Ave.= relative average compared with MCS value
*VND7proB=VND7promoter(-1000 - +9 bp)

transient assay(pBIG2113SF vector)

Table S20. transient assay Repoter = VND7pro, Effecter = Transcription Factors (TFs)

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pBIG2113SF		pAGL-VND7proB	317	15150	0.021	271	14341	0.019	565	19579	0.029	384	16357	0.023	158	2820	0.005	1.00	0.22
pBIG-1a(BEE2)	At4g36540	pAGL-VND7proB	628	20440	0.031	303	24857	0.012	501	22094	0.023	477	22464	0.022	164	2232	0.010	0.96	0.44
pBIG-3h(ATBZIP3)	At5g15830	pAGL-VND7proB	353	14011	0.025	319	20608	0.015	324	32445	0.010	332	22355	0.017	18	9340	0.008	0.74	0.35

Table S21. transient assay Repoter = VND7pro, Effecter = Transcription Factors (TFs)

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pBIG2113SF		pAGL-VND7proB	1634	23787	0.069	752	17152	0.044	435	7912	0.055	940	16284	0.056	621	7973	0.013	1.00	0.23
pBIG-1d(ANAC092)	At5g39610	pAGL-VND7proB	1467	24963	0.059	710	14657	0.048	356	20549	0.017	844	20056	0.041	568	5171	0.022	0.73	0.39
pBIG-2f(ANAC048)	At3g04420	pAGL-VND7proB	325	8154	0.040	438	13152	0.033	579	18882	0.031	447	13396	0.035	127	5368	0.005	0.63	0.09
pBIG-2g(ANAC017)	At1g34190	pAGL-VND7proB	766	34009	0.023	715	33013	0.022	409	20779	0.020	630	29267	0.022	193	7368	0.002	0.39	0.04
pBIG-2h(SND3)	At1g28470	pAGL-VND7proB	791	27575	0.029	466	18057	0.026	608	29427	0.021	622	25020	0.025	163	6101	0.004	0.45	0.07
pBIG-2i(ANAC032)	At1g77450	pAGL-VND7proB	779	23272	0.033	767	18756	0.041	248	7171	0.035	598	16400	0.036	303	8305	0.004	0.64	0.07
pBIG-2j(ANAC074)	At4g28530	pAGL-VND7proB	370	9279	0.040	402	6166	0.065	430	8623	0.050	401	8023	0.052	30	1641	0.013	0.93	0.23

Table S22. transient assay Repoter = VND7pro, Effecter = Transcription Factors (TFs)

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pBIG2113SF		pAGL-VND7proB	230	31829	0.007	347	17032	0.020	177	19627	0.009	251	22829	0.012	87	7901	0.007	1.00	0.58
pBIG-2a(DREB2)	At5g05410	pAGL-VND7proB	1057	54254	0.019	190	19486	0.010	410	51646	0.008	552	41795	0.012	451	19364	0.006	1.00	0.50
pBIG-1i(ICE1)	At3g26744	pAGL-VND7proB	238	20765	0.011	196	33682	0.006	145	8733	0.017	193	21060	0.011	47	12477	0.006	0.92	0.50
pBIG-1b(VNI2)	At5g13180	pAGL-VND7proB	129	13745	0.009	149	11809	0.013	215	31604	0.007	164	19053	0.010	45	10913	0.003	0.83	0.25
pBIG-4i(zDof)	At1g64620	pAGL-VND7proB	320	33238	0.010	314	34973	0.009	242	24203	0.010	292	30805	0.010	43	5783	0.001	0.83	0.08
pBIG-4j(MYB3)	At1g22640	pAGL-VND7proB	96	13137	0.007	197	26851	0.007	211	23701	0.009	168	21230	0.008	63	7183	0.001	0.67	0.08
pBIG-1g(MYB43)	At5g16600	pAGL-VND7proB	129	11948	0.011	653	42802	0.015	94	7705	0.012	292	20818	0.013	313	19156	0.002	1.08	0.17

Table S23. transient assay Repoter = VND7pro, Effecter = Transcription Factors (TFs)

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pBIG2113SF		pAGL-VND7proB	450	5386	0.084	104	2036	0.051	426	5709	0.075	327	4377	0.070	193	2034	0.017	1.00	0.24
pBIG-1c(ERF)	At5g25190	pAGL-VND7proB	803	7275	0.110	283	5376	0.053	183	3599	0.051	423	5417	0.071	333	1838	0.034	1.01	0.49
pBIG-3i(BEL1)	At5g41410	pAGL-VND7proB	105	2422	0.043	162	5383	0.030	118	4251	0.028	128	4019	0.034	30	1494	0.008	0.49	0.11

Table S24. transient assay Repoter = VND7pro, Effecter = Transcription Factors (TFs)

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pBIG2113SF		pAGL-VND7proB	640	10041	0.064	461	17396	0.027	798	23806	0.034	633	17081	0.042	169	6888	0.020	1.00	0.48
pBIG-2b(ERF)	At5g61600	pAGL-VND7proB	1330	39607	0.034	752	18898	0.040	1094	20080	0.054	1059	26195	0.043	291	11630	0.010	1.02	0.24
pBIG-2e(SEPALLATA)	At3g02310	pAGL-VND7proB	167	6191	0.027	415	7354	0.056	256	7567	0.034	279	7037	0.039	126	741	0.015	0.93	0.36
pBIG-3b(TCP10)	At2g31070	pAGL-VND7proB	522	12012	0.043	324	3531	0.092	304	11378	0.027	383	8974	0.054	121	4724	0.034	1.29	0.81
pBIG-4b(WRKY21)	At2g30590	pAGL-VND7proB	556	23311	0.024	1117	20060	0.056	441	9371	0.047	705	17581	0.042	362	7293	0.017	1.00	0.41
pBIG-4c(ZFP4)	At1g66140	pAGL-VND7proB	312	13780	0.023	416	12081	0.034	172	10260	0.017	300	12040	0.025	122	1760	0.009	0.60	0.21

Table S25. transient assay Repoter = VND7pro, Effecter = Transcription Factors (TFs)

			1st			2nd			3rd			Average			SD			Relative luciferase activity	
Effector		Reporter	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	firefly	Renilla	Ratio	Relative Ave.	Relative SD
pBIG2113SF		pAGL-VND7proB	1440	11599	0.124	1608	11969	0.134	6869	30044	0.229	3306	17871	0.162	3087	10544	0.058	1.00	0.36
pBIG-1f(WRKY46)	At2g46400	pAGL-VND7proB	845	13120	0.064	823	6901	0.119	991	10201	0.097	886	10074	0.093	91	3111	0.028	0.57	0.17
pBIG-3c(WRKY39)	At3g04670	pAGL-VND7proB	468	9506	0.049	711	11750	0.061	897	6841	0.131	692	9366	0.080	215	2458	0.044	0.49	0.27
pBIG-4d(zfC3HC4)	At1g26800	pAGL-VND7proB	727	9568	0.076	1127	8415	0.134	438	10752	0.041	764	9578	0.084	346	1169	0.047	0.52	0.29
pBIG-4f(zfC3HC4)	At3g23060	pAGL-VND7proB	598	11298	0.053	1488	9001	0.165	1567	26247	0.060	1218	15515	0.093	538	9365	0.063	0.57	0.39
pBIG-4g(zfC3HC4)	At5g08750	pAGL-VND7proB	946	14027	0.067	466	11261	0.041	914	13962	0.065	775	13083	0.058	268	1579	0.014	0.36	0.09

*pBIG2113SF= empty vector(negative control)
*firefly= luciferase activity
*Renilla= renilla activity (refarence)
*SD=Standard Deviation
*Ratio=firefly/Renilla
*Relative Ave.= relative average compared with pBIG2113SF value
*VND7proB=VND7promoter(-1000 - +9 bp)

Supplementary Table S27. Amino acid sequence of VND proteins used in this study.

Gene	Number of amino acids	Sequence
VND1 (1-483 + stopTGA)	161	MEPMESCSVPPGFRFHPTDEELVGYYLRKKIASQKIDLDVI RDIDLYRIEPWDLQEQCRIGYEEQNEWYFFSHKDKKYPTG TRTNRATMAGFWKATGRDKAVYDKTKLIGMRKTLVFYKG RAPNGKKSDWIMHEYRLESDENAPPQEEGWVVCRAFKKR AT
VND2 (1-486 + stopTGA)	162	MESVDQSCSVPPGFRFHPTDEELVGYYLRKKVASQKIDLD VIRDIDLYRIEPWDLQESCRIGYEERNEWYFFSHKDKKYPT GTRTNRATMAGFWKATGRDKAVYDKSKLIGMRKTLVFYK GRAPNGQKTDWIMHEYRLESDENAPPQEEGWVVCRAFK KKPM
VND3 (1-495 + stopTAA)	165	MMKVDQDYSCSIPPGFRFHPTDEELVGYYLKKKIASQRID LDVIREIDLYKIEPWDLQERCRIGYEEQTEWYFFSHRDKKY PTGTRTNRATVAGFWKATGRDKAVYLNSKLIGMRKTLVFY RGRAPNGQKSDWIIHEYYSLESHQNSPPQEEGWVVCRAFK KRTT
VND4 (1-477 + stopTGA)	159	MNSFSHVPPGFRFHPTDEELVDYYLRKKVASKRIEIDFIKDI DLYKIEPWDLQELCKIGHEEQSDWYFFSHKDKKYPTGTRT NRATKAGFWKATGRDKAIYLRHSLIGMRKTLVFYKGRAP NGQKSDWIMHEYRLETDENGTPQEEGWVVCRVFKKRLA
VND5 (1-477 + stopTAG)	159	MNSFSQVPPGFRFHPTDEELVDYYLRKKVASKRIEIDIIKDV DLYKIEPCDLQELCKIGNEEQSEWYFFSHKDKKYPTGTRTN RATKAGFWKATGRDKAIYIRHSLIGMRKTLVFYKGRAPNG QKSDWIMHEYRLETSENGTPQEEGWVVCRVFKKKLA
VND6 (1-477 + stopTAA)	159	MESLAHIPPGYRFHPTDEELVDYYLKNKVAFPGMQVDVIK DVDLYKIEPWDIQELCGRGTGEEREWYFFSHKDKKYPTGT RTNRATGSGFWKATGRDKAIYSKQELVGMRKTLVFYKGR APNGQKSDWIMHEYRLETDENGPPHEEGWVVCRAFKKKL T
VND7 (1-483 + stopTAA)	161	MDNIMQSSMPPGFRFHPTEEELVGYYLDRKINSMKSALDV IVEIDLYKMEPWDIQARCKLGYEEQNEWYFFSHKDRKYPT GTRTNRATAAGFWKATGRDKAVLSKNSVIGMRKTLVYYK GRAPNGRKSDWIMHEYRLQNSELAPVQEEGWVVCRAFR KPIP

Supplementary Table S26. List of primer sequences.

primer name	AGI No.	Primer sequences (5'-3')	Primer sequences (5'-3')
For promoter			
VND1	At2g18060	CACCCATAACTTTGTGACATAAACGTTATTAGG	TGGCTCCATTATTTCTACACCAACAAAATG
VND2	At4g36160	CACCGAACTACTTAAACCTAGTCCTTGTATATG	CGATTCCATTATTTCTGCATAACACCAATA
VND3	At5g66300	CACCGGTGAAATCCGAGAAACAACAATCAAAATA	ATCAACCTTCATCATATTGATAGTTATTTTCTAGG
VND4	At1g12260	CACCGATCATACACTGTTTGTCTCTTGATGATTT	TGAATTCATGTTTGTCTTCTTGATCGGTTA
VND5	At1g62700	CACCGTGTGTTGTTAATGTTGTTATATATGGGTC	CGAATTCATTCTTTATTCTTTGTTTTTCCGAG
VND6	At5g62380	CACCGAGATAATTAGTACACTACCTTATTAGGGC	GTGTGCGAGACTTTCCATTTGATCTTTTTA
VND7 (1000 bp)	At1g71930	CACCCTTGAATAGTATACATGTGTGTGGTCCTGT	ATTATCCATCCACGATGATCCTATAAACGT
GATA5	At5g66320	CACCGGCTAGTTTTGTCATTTAATATTTAG	TTGTTCCATTTTCCTATCGAGAAAAAAAC
GATA12	At5g25830	CACCTTATTACATTAGTAATTCTCATTTCGT	ATCTTCCATAAGTTTCGTTGATTAAAAC
ANAC075	At4g29230	CACCTTACACTTCATTCGAGAATTTTAAGTG	CTTGTTCATCTCAATCTCGAATATCT
For EMSA			
VND7 (603 bp)	At1g71930	GCATCGTTCGGTATGTAGAAGGCG	ATTATCCATCCACGATGATCCTATAAACGT
VND7 (33 bp)	At1g71930	GATAGCCTTAAGCTTAAAGATCCAAGCTTTTGG	CCAAAAGCTTGGATCTTTAAGCTTAAGGCTATC
VND7 (mutated 33 bp)	At1g71930	GATAGCCTTAAGaggAAAGATCCAAGCTTTTGG	CCAAAAGCTTGGATCTTTcctCTTAAGGCTATC
For qRT-PCR			
VND1	At2g18060	CAACAATGATGTGGAGATGGATTCGTC	GTACTCAAGAATTCACTGACGAACCTG
VND2	At4g36160	CGGTCATTACAATAACGAAGAGAGC	CATGTAAATCCCTATATAAGTCATAGTC
VND3	At5g66300	CAGCTCGAGAGCCCCTCTCTTCCGTCGG	CTCATCAAAAATTGAGACGCCACGAAC
VND4	At1g12260	GGCTGCCACAGCTTCTGCATCTATACAG	GATCAATCTGACAACTCGAAGAAGTAG
VND5	At1g62700	GGTTTCTTCTTCTTCTCATCAAAACAACG	CAGCATGAGCATTTGAATACTCTTCTCC
VND6	At5g62380	CGCTTGACAAGTTTGTTGCTTCTCAGC	GGAAGAAGCATTCATCGAAACCATTG
VND7	At1g71930	CACCATGCATCAATATGGCAACATTGAG	TAGTGTTCTCCAATCCACACAGTT
GATA12	At5g25830	ACCATCACGGTACGGACACT	CAACGTTGTGGTGGATCAAG
LBD30	At4g00220	CTATCTACGGCTGCGTCTCTCACATCGT	TAGAGATCCTGAAGATGACACCGGAAC
MYB46	AT5g12870	GAATGTGAAGAAGGTGATTGGTACA	CGAAGGAACCTCAGTGTTCATCA
XCP1	AT4g35350	TTGACCCATGAAGAGTTCAAAGGAAGA	GAAAGCGAACTCAGATTCCCTGTTG
CESA7	AT5g17420	GGCAAACTCAAGTGGCTTGAGCG	TAACTCCGCTCCATCTCAATTCC
ANAC075	AT4G29230	GCAACTACGTCAAGAAGGAGAAGAAG	GCTTCTTGTTGGTTCCCCATTGATGATG
UBQ10	At5g57860	AACTTTGGTGGTTTGTGTTTTGG	TCGACTTGTCATTAGAAAGAAAGAGATAA
For ChIP-PCR			
region 9	At1g71930	TGTTTTATGTTGGCGGTTTG	CGTTTACATGGGTCCATACTGA
region 8	At1g71930	CCTTAAGCCGAAAGGTGATG	CTCCCATTGTCTGGTTTGGT
region 7	At1g71930	CTGCCGGTAAAGTGGAGAAG	ATGTGGGTGCAAAATGATGA
region 6	At1g71930	TCCAACCTCAGTGACTTCCA	TAAGGGTTTGTTTCGCCTTC
region 5	At1g71930	CTCTGCCACTTCTCCATCTTG	CCCACAAGCTCTTCCTCTGT
region 4	At1g71930	CCGGTTGAAAAGTATAATCATATCAC	CACCTCGCTGCAAATGTAAA
region 3	At1g71930	AAAACAGTGTCATCGGAATGC	TGAATCGCGTATCAGTCAGAG
region 2	At1g71930	GGTGGTGTGTCGAGCATTTA	TGATGAAGATGCACCGATATG
region 1	At1g71930	GTTGAATTTCACAAAGGTGGATT	AAGTTCTCTTAACTAATAGGCCACCA
FUS3T	AT3G26790	ACTTTTGCTACACTTGTTCACCA	CGCAACAAGATCTAATGCCACT
FLC	AT5G10140	AAGCCAGCGCTATCACTAAACTTT	TCGGCAGATTGAAAATGACATT
VND6	At5g62380	TGCACGAATACCGTCTTGAG	TACCATTCCCCACATCCATT
For McrBC-PCR			
VND7-2ndex-McrBC	At1g71930	CCGGTTGAAAAGTATAATCATATCAC	CATTATTGAATCAAATGTCGTCAAAC
VND7-1stex-McrBC	At1g71930	CTCTAACAATTTTCCAAATTAAATAC	CACTCCGTTTATGAACATACAAGTC
CACTA1pro		CAGTACTCATTCTCACATGATACATCA	GAATTTCTGGCCAGCGACAGATCTT

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