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Multiple Classes of Transcription Factors Regulate the Expression of *VASCULAR-RELATED NAC-DOMAIN7*, a Master Switch of Xylem Vessel Differentiation

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The secondary cell walls of xylem cells, including vessel elements, provide mechanical strength and contribute to the conduction of water and minerals. VASCULAR-RELATED NAC-DOMAIN7 (VND7) is a NAC-domain transcription factor that regulates the expression of genes required for xylem vessel element formation. Transient expression assays using 68 transcription factors that are expressed during xylem vessel differentiation showed that 14 transcription factors, including VND1–VND7, are putative positive regulators of *VND7* expression. Electrophoretic mobility shift assays revealed that all seven VND proteins bound to the *VND7* promoter region at its SMBE/TERE motif, indicating that VND7 is a direct target of all of the VND transcription factors. Overexpression of *VND1–VND5*, *GATA12* and *ANAC075*, newly identified transcription factors that function upstream of *VND7*, resulted in ectopic xylem vessel element formation. These data suggest that *VND7* transcription is a regulatory target of multiple classes of transcription factors.

Keywords: Secondary cell wall • Transcription factor • Transcriptional network • Vascular development • Xylem vessel formation.

Abbreviations: ASL, ASYMMETRIC LEAVES2-LIKE; bHLH, basic helix–loop–helix; CDS, coding sequence; CESA, CELLULOSE SYNTHASE A; HD-Zip, homeodomain-leucine zipper; CaMV, *Cauliflower mosaic virus*; Col-0, Columbia; EMSA, electrophoresis mobility shift assay; FBH, FLOWERING BHLH; fLUC, firefly luciferase; GUS, β-glucuronidase; GWRFC, GATEWAY Reading Frame Cassette; KNAT, KNOTTED1-LIKE HOMEODOMAIN PROTEIN; LBD, LATERAL ORGAN BOUNDARIES DOMAIN; LUC, luciferase; MBP, maltose-binding protein; MCS, multicloning site; NAC, no apical meristem (NAM), Arabidopsis transcription activation factor (ATAF1/2) and cup-shaped cotyledon (CUC); NST, NAC SECONDARY WALL THICKENING PROMOTING FACTOR, PHB, PHABOULOSA; RAFL, RIKEN Arabidopsis full-length; REV, REVOLUTA; rLUC, *Renilla reniformis* luciferase, SMB, SOMBRERO; SNBE, secondary wall NAC-binding element; SND, SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN; TERE, tracheary element-regulating *cis*-element; VND, VASCULAR-RELATED NAC-DOMAIN7; VNI2, VND-INTERACTING2; XCP, XYLEM CYSTEINE PEPTIDASE; YFP–NLS, yellow fluorescent protein fused to the SV40 nuclear localization signal.

Introduction

The thick secondary cell walls of xylem cells of the plant vascular system contribute to the conduction of water and minerals throughout the plant body and provide mechanical strength that supports the entire plant. Cell walls are composed mainly of polysaccharides, such as cellulose and hemicellulose, and of the phenolic polymer lignin. Because these components can be converted into biofuels and biomaterials, secondary cell walls represent an alternative energy source to fossil fuels (Simmons et al. 2008, Yang et al. 2013).

We previously established an in vitro system for xylem vessel element transdifferentiation of *Arabidopsis thaliana* Columbia (Col-0) suspension-cultured cells (Kubo et al. 2005). Through transcriptome analysis, a number of genes that exhibit significant changes in expression during xylem vessel element transdifferentiation were isolated, including genes 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*), which encodes a NAC [no apical meristem (NAM),

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Arabidopsis transcription activation factor (ATAF1/2) and cup-shaped cotyledon (CUC)]-domain transcription factor, was found to be important for xylem vessel element differentiation: overexpression of VND7 induces the ectopic differentiation of xylem vessel elements; overexpression of dominant-negative forms of VND7 inhibits the normal differentiation of xylem vessel elements; and *VND7* is strongly expressed in all types of xylem vessel cells (Kubo et al. 2005, Yamaguchi et al. 2008, Yamaguchi et al. 2010a, Ohtani et al. 2011). Further studies showed that VND7 influences the expression of a number of genes encoding transcription factors, such as those in the MYB, NAC domain and LATERAL ORGAN BOUNDARIES DOMAIN (LBD)/ASYMMETRIC LEAVES2-LIKE (ASL) families and KNOTTED1-LIKE HOMEODOMAIN PROTEIN7 (KNAT7) (Zhong et al. 2010, Yamaguchi et al. 2011). For instance, *MYB46* and its close homolog *MYB83*, which regulate the expression of many genes related to secondary cell wall formation by activating biosynthetic pathways for cellulose, hemicellulose and lignin, are directly up-regulated by VND7 (Ko et al. 2009, McCarthy et al. 2009, Zhong et al. 2010, Zhong et al. 2011, Yamaguchi et al. 2011, Kim et al. 2012, Kim et al. 2013). KNAT7 was proposed to be a regulatory module that represses secondary cell wall biosynthesis to fine-tune metabolic commitment to secondary cell wall deposition during development (Li et al. 2011, Li et al. 2012).

VND7 is subjected to proteasome-mediated proteolysis (Yamaguchi et al. 2008), and its transcriptional activation activity is negatively regulated by the NAC transcription factor VND-INTERACTING2 (VNI2) (Yamaguchi et al. 2010b). Recent research revealed that xylem vessel formation is regulated by a complex transcriptional network (Demura and Fukuda 2007, Caño-Delgado et al. 2010, Ohashi-Ito and Fukuda 2010, Yamaguchi and Demura 2010, Brady et al. 2011, Miyashima et al. 2012). Several Class III homeodomain-leucine zipper (HD-Zip) transcription factors, such as *ATHB-8* and *PHABULOSA* (*PHB*), determine vascular patterning, which suggests their possible ability to regulate *VND7* expression (Carlsbecker et al. 2010, Miyashima et al. 2011, Furuta et al. 2012). Soyano et al. (2008) reported that the LBD transcription factors, LBD18/ASL20 and LBD30/ASL19, are involved in a positive feedback loop that possibly boosts *VND7* expression, while the expression of these LBD/ASL transcription factors in turn depends on VND7 (Soyano et al. 2008). Therefore, our knowledge of the upstream transcription factors that regulate *VND7* expression is still relatively limited.

To better understand the molecular mechanisms underlying the transcriptional control of *VND7* expression, we sought to isolate the transcription factors that regulate the expression of *VND7*. We selected 68 transcription factors known to be expressed during transdifferentiation of xylem vessel elements (Kubo et al. 2005). A dual luciferase (LUC) assay showed that 14 of these transcription factors, including members of the VND family, ANAC075, the GATA transcription factors GATA5 and GATA12, and the LBD/ASL transcription factors LBD15/ASL11 and LBD30/ASL19, induced *VND7* promoter activity. Promoter reporter analysis revealed that *ANAC075*, *GATA5* and *GATA12* are preferentially expressed in the vascular cylinder of the root tip region. Moreover, electrophoretic mobility shift assays (EMSAs) revealed that VND1–VND7 bind directly to the SNBE/TERE-like motif (Pyo et al. 2007, Zhong et al. 2010) on the *VND7* promoter region. These results reveal that the expression of *VND7* is potentially regulated by multiple transcription factors. We also showed that overexpression of *VND1–VND5*, *GATA12* or *ANAC075* induced ectopic differentiation of xylem vessel elements, accompanied by secondary cell wall deposition. Moreover, we noticed that the ectopic transdifferentiation into vessel elements occurred in the absence of the immediate up-regulation of endogenous *VND7*, indicating that additional undefined factors are required for the tight regulation of *VND7* expression in vivo.

Results

Isolation of transcription factors that induce *VND7* promoter activity

To elucidate the upstream mechanisms that regulate *VND7* expression, we selected 68 transcription factors that met the criteria of having more than double the normalized signal intensity at 2–6 d after induction when compared with the expression data of the non-induced, 0 day sample, or that at 4–6 d after induction when compared with the expression data at 2 d after induction, in the Arabidopsis in vitro xylem vessel element formation system (see the Materials and Methods for details; Kubo et al. 2005). The transcription factor genes were subcloned into a transient expression vector downstream of the *Cauliflower mosaic virus* (CaMV) *35S* promoter and the resulting vectors were used as effector constructs in a dual LUC reporter assay (Mitsuda et al. 2009).

A 1,000 bp promoter region of *VND7* flanked by a 9 bp coding region was sufficient to induce xylem vessel-specific expression in the root (**Supplementary Fig. S1A**), and was linked to the firefly LUC (*fLUC*) gene to generate a reporter construct (**Supplementary Fig. S1B**). We delivered this reporter construct together with an effector construct containing the coding sequence (CDS) of each of the candidate transcription factors and a reference construct harboring the *Renilla reniformis LUC* (*rLUC*) gene downstream of the CaMV *35S* promoter into Arabidopsis rosette leaves by particle bombardment. We found that 14 transcription factors significantly up-regulated the fLUC activity (by >3-fold compared with the control, P-value <0.1; **Table 1**). Expression patterns of the 14 transcription factors during the in vitro xylem vessel element formation are shown in **Supplementary Fig. S2**. Among the candidates, *LBD15/ASL11*, *LBD30/ASL19*, *SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN 2* (*SND2*) and *REVOLUTA* (*REV*) are known to be involved in xylem tissue formation (Soyano et al. 2008, Zhong et al. 2008, Ohathis-Ito et al. 2010, Zhong et al. 2010, Carlsbecker et al. 2010, Yamaguchi et al. 2011). In addition, our assay identified novel transcription factors that potentially induce *VND7* expression, including a NAC domain-containing transcription factor *ANAC075* and the GATA domain-containing transcription factors *GATA5* and *GATA12* (**Table 1**). These results were confirmed by

Table 1 List of transcription factors used in the dual luciferase assay

Category	AGI No.	Description	Fold change[a]	SD[b]	*P*-value[c]	Vector[d]
AP2	At5g05410	DREB2	1.00	0.50	0.9527	B
	At5g25190	AP2/ERF	1.01	0.49	0.9549	B
	At5g61600	AP2/ERF	1.02	0.24	0.9426	B
bHLH	At1g63650	EGL1/EGL3	1.50	0.50	0.3793	A
	At1g35460	bHLH	0.82	0.23	0.4692	A
	At1g68810	TMO5-like1	2.11	1.11	0.2203	A
	At1g51140	bHLH	0.84	0.14	0.5339	A
	At1g29950	bHLH	1.49	0.27	0.1796	A
	At3g26744	ICE1/SCRM	0.92	0.50	0.9034	B
	At3g56980	ORG3	0.95	0.24	0.3812	A
	At4g36540	BEE2	0.96	0.44	0.8836	B
	At5g64340	SAC51	0.74	0.22	0.4092	A
	At5g09460	SAC51like	0.83	0.09	0.5466	A
bZIP	At5g49450	ATBZIP1	2.64	0.36	0.0082	A
	At5g15830	ATBZIP3	0.74	0.35	0.3106	B
CCAT	At4g14540	NF-YB3	2.01	1.60	0.3889	A
GATA	At5g66320	GATA5	10.32	1.39	0.0056	A
	At5g25830	GATA12	7.93	2.72	0.0454	A
Homeobox	At1g62990	KNAT7	0.92	0.14	0.7769	A
	At5g06710	HAT14	1.43	0.05	0.0587	A
	At4G32880	AtHB8	2.00	0.70	0.1578	A
	At1g52150	AtHB15	2.30	0.90	0.1141	A
	At2g34710	PHB	1.55	0.46	0.1631	A
	At1g30490	PHV	1.82	0.27	0.0176	A
	At5g60690	REV	3.80	0.90	0.0150	A
	At5g41410	BEL1	0.49	0.11	0.0478	B
LBD	At2g40470	LBD15	12.27	4.34	0.0459	A
	At2g45420	LBD18	5.92	3.24	0.1182	A
	At4g00220	LBD30	7.10	2.00	0.0224	A
	At1g31320	LBD4	1.24	0.26	0.4532	A
MADS	At3g02310	SEPALLATA2	0.93	0.36	0.8619	B
MYB	At1g22640	MYB3	0.67	0.08	0.3961	B
	At1g69580	MYB like	1.11	0.54	0.7851	A
	At3g10760	MYB	0.98	0.23	0.9253	A
	At5g16600	MYB43	1.08	0.17	0.8869	B
NAC	At4g28500	SND2	3.39	0.59	0.0088	A
	At1g28470	SND3	0.45	0.07	0.0408	B
	At1g34190	ANAC017	0.39	0.04	0.0399	B
	At1g77450	ANAC032	0.64	0.07	0.1010	B
	At3g04420	ANAC048	0.63	0.09	0.0839	B
	At4g28530	ANAC074	0.93	0.23	0.6943	B
	At5g39610	ANAC092	0.73	0.39	0.3824	B
	At5g13180	VNI2	0.83	0.25	0.6366	B
	At4g29230	ANAC075	6.70	1.71	0.0277	A
	At5g64530	XND1	2.09	0.46	0.0419	A
	At2g18060	VND1	61.33	10.37	0.0095	A
	At4g36160	VND2	30.42	4.44	0.0067	A
	At5g66300	VND3	41.87	21.19	0.0789	A
	At1g12260	VND4	14.73	6.87	0.0730	A

(continued)

Table 1 Continued

Category	AGI No.	Description	Fold change[a]	SD[b]	*P*-value[c]	Vector[d]
	At1g62700	VND5	62.70	30.50	0.0348	A
	At5g62380	VND6	47.71	18.89	0.0502	A
	At1g71930	VND7	32.85	0.94	0.0000	A
NF-YC2	At1g56170	HAP5B	0.60	0.19	0.1361	A
TCP	At2g31070	TCP10	1.29	0.81	0.6210	B
TUB	At1g43640	ATTLP5	0.80	0.53	0.6048	A
WRKY	At2g30590	WRKY21	1.00	0.41	0.9663	B
	At3g04670	WRKY39	0.49	0.27	0.1282	B
	At2g46400	WRKY46	0.57	0.17	0.1639	B
zf-C2H2	At1g66140	ZFP4	0.60	0.21	0.2716	B
	At5g03510	Zinc finger C2H2	0.47	0.16	0.1288	A
zf-C3HC4	At1g26800	Zinc finger C3HC4	0.52	0.29	0.1449	B
	At1g72220	RING-H2Zinc finger	1.82	0.9	10.2445	A
	At3g23060	Zinc finger C3HC4	0.57	0.39	0.2310	B
	At5g08750	Zinc finger C3HC4	0.36	0.09	0.0810	B
	At5g17600	RING-H2 zinc finger	0.82	0.18	0.3283	A
	At5g55970	MDA7.1	0.66	0.22	0.2819	A
zf-Dof	At1g64620	Dof-type zinc finger	0.83	0.08	0.6224	B
	At5g60200	TMO6/Dof5.3	1.57	0.54	0.2352	A

[a] Relative luciferase activity compared with control effector.
[b] Standard deviation of three replicates.
[c] Welch's *t*-test showing differences from the values for the control effector.
[d] Vectors used for subcloning of transcription factors: A, pA35G; B, pBIG2113SF (see the Materials and Methods for detailed information)

transient expression assay using the transgenic plant *VND7pro:β-glucuronidase* (*GUS*) leaves, in which the 2,000 bp promoter region of *VND7* was linked to the *GUS* gene (Yamaguchi et al. 2008); particle bombardment of the candidate transcription factors induced the GUS activity (**Supplementary Fig. S3**, **Supplementary Table S1**).

Moreover, we found that all *VND* genes strongly up-regulated the *VND7* promoter activity (**Table 1**; **Supplementary Fig. S3**, **Supplementary Table S1**). 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, we performed dual LUC assays using all combinations of *VND* promoters as reporters and *VND* genes as effectors. Although the reporter gene expression driven by the *VND3*, *VND5* or *VND6* promoter was somewhat elevated by some of the *VND* genes, the up-regulation of *VND7* promoter activity by *VND* genes was most striking (**Fig. 1**).

VND proteins bind to the *VND7* promoter at its SNBE/TERE motif in vitro

VND6 and VND7 are reported to regulate downstream gene expression through the *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). The *VND7* promoter region used in our dual LUC assay contained both an SNBE- and a TERE-like motif (SNBE/TERE-like motif, −425 to −408) (**Fig. 2A**; **Supplementary Fig. S4**; Pyo et al. 2007, Zhong et al. 2010). Thus, to test the possibility that VND1–VND7 directly bind to the *VND7* promoter via the SNBE/TERE-like motif, EMSAs were performed. A biotinylated *VND7* promoter fragment (−1 to −603 bp, relative to the ATG start codon) was used as a probe, and maltose-binding protein (MBP)-tagged N-terminal regions of VND proteins that include whole NAC domains (MBP–VND) were prepared (**Supplementary Table S2**). When the probe was incubated with the MBP–VND, shifted bands were detected for all cases (**Fig. 2**). These shifted band signals were markedly, but not completely, decreased by the application of excess amounts (×200) of a 33 bp DNA fragment containing the SNBE/TERE-like motif as a competitor (−428 to −396 bp relative to the *VND7* ATG start codon) (**Fig. 2B–H**; **Supplementary Fig. S4**). Moreover, most of the samples showed multiple bands (**Fig. 2**). These data indicate that VND proteins can bind to the 33 bp sequence of the *VND7* promoter and also to other regions of the *VND7* promoter. The multiple bands may reflect the formation of different complex combinations between the probe and VND proteins, which can exist as either monomers or homodimers (Yamaguchi et al. 2008).

To examine further if VND proteins bind to the SNBE/TERE-like motif, the 33 bp fragment of the *VND7* promoter was biotinylated for EMSA. MBP–VND3 protein was used as a representative of VND1–VND5, and MBP–VND7 was used as a positive control known to bind to SNBE and TERE (Zhong et al. 2010, Yamaguchi et al. 2011). A shifted band was observed for both MBP–VND3 and MBP–VND7, and the intensity of the

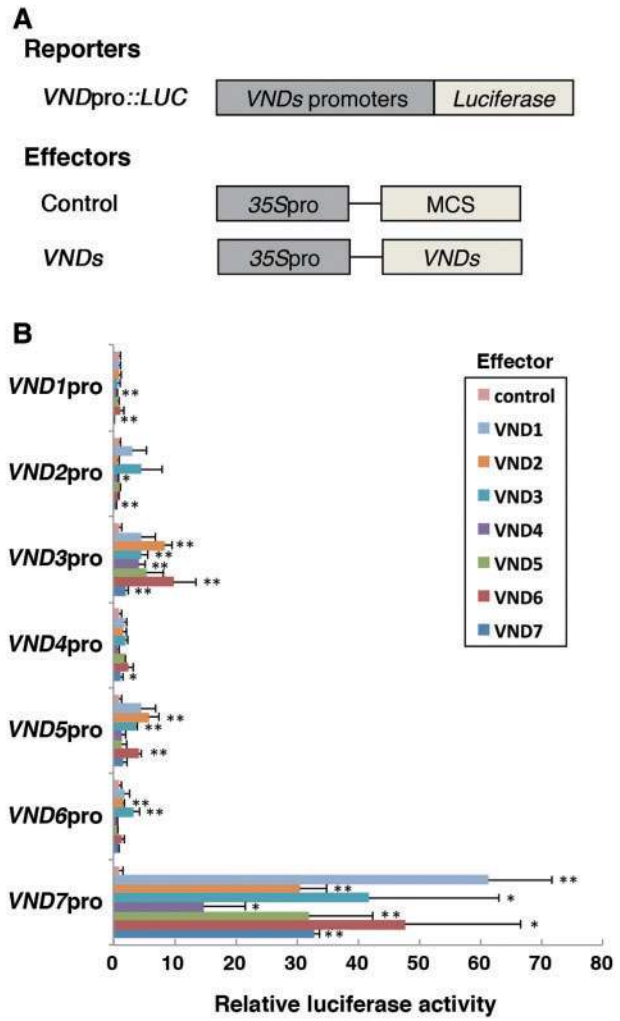

Fig. 1 VND family proteins induce *VND7* promoter activity in vitro. (A) Schematic diagrams of the effector and reporter plasmids used in luciferase transactivation assays. The reporter plasmids contain the promoters of *VND1–VND7* upstream of the firefly luciferase reporter gene. The effector plasmids contain *VND1–VND7* genes or a multi-cloning site (MCS; control) downstream of the CaMV *35S* promoter. (B) Result of transient expression assays. The reporter gene activity was normalized by the activity of *Renilla* luciferase. Error bars indicate the SD ($n = 3$). Asterisks indicate statistically significant differences (Welch's *t*-test; $^{*}P < 0.1$, $^{**}P < 0.05$) from the values for the control effector.

shifted band was reduced upon application of excess amounts ($\times 200$) of unlabeled fragment (**Fig. 3**). When a mutated 33 bp fragment with three substituted nucleotides in the SNBE/TERE-like motif was used as a competitor, the shifted bands remained clearly detectable (**Fig. 3**). These results suggest that VND proteins bind to the SNBE/TERE motif located in the promoter region of *VND7*.

GATA5, *GATA12* and *ANAC075* are expressed in the vascular cylinder of the root

To monitor the expression patterns of three transcription factors, newly identified in this study, i.e. *GATA5*, *GATA12* and *ANAC075*, we expressed constructs consisting of yellow fluorescent protein fused to the SV40 nuclear localization signal (*YFP-NLS*) under the control of the *GATA5*, *GATA12*, *ANAC075* or *VND7* promoter in Arabidopsis. As previously reported by Yamaguchi et al. (2008, 2010b), the *VND7pro::YFP-NLS* signal was specifically detected along differentiating xylem vessels in the root stele region (**Fig. 4A**; **Supplementary Figs. S1A**, **S5**). *GATA5pro::YFP-NLS*, *GATA12pro::YFP-NLS* and *ANAC075pro::YFP-NLS* signals were detected in the differentiation zone of the root stele, as was *VND7pro::YFP-NLS* (**Fig. 4B–D**), while it seemed that they were much more widely expressed in the root than *VND7*. (**Supplementary Fig. S5**). Confocal microscopy observations revealed that these 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. 4**). Together with previous data showing that *VND1–VND6*, *LBD18/ASL20* and *LBD30/ASL19* are expressed in the root stele (Kubo et al. 2005, Soyano et al. 2008, Yamaguchi et al. 2008), these results suggest that multiple classes of transcription factors are expressed during xylem vessel element differentiation for the co-ordinated regulation of *VND7* expression.

Overexpression of *VND1–VND7*, *GATA12* and *ANAC075* induced the formation of ectopic xylem vessel-like elements

To investigate the molecular functions of the isolated transcription factors, we generated transgenic plants overexpressing *GATA5*, *GATA12*, *ANAC075* and *VND1–VND7* under the control of an estrogen receptor-mediated inducible gene expression system (Zuo et al. 2000). Upon application of 17-β-estradiol, xylem vessel-like cells with thickened secondary cell walls were induced in transgenic plants overexpressing *VND1–VND7*, *GATA12* or *ANAC075* (**Fig. 5**), but not in the *GATA5* overexpressor. Safranin-O staining revealed that these secondary cell walls were lignified (**Supplementary Fig. S6B–J**). The xylem vessel element-like cells were found in both the leaves and roots in inducible lines for *VND* genes and *ANAC075*, while these cells were detected only infrequently in the roots in the *GATA12* inducible lines (**Fig. 5B–J**; **Supplementary Fig. S6B–J**), suggesting that GATA12 has a lower ability to induce transdifferentiation of xylem vessel elements than do the other tested transcription factors.

Expression of xylem vessel formation-related genes was up-regulated by overexpression of isolated transcription factors

To investigate changes in mRNA levels of xylem vessel formation-related genes in transgenic plants by inducible expression of *VND1–VND7*, *GATA12* or *ANAC075*, we analyzed the expression levels of *VND7* and its downstream target genes, *LBD30/ASL19*, *MYB46*, *XYLEM CYSTEINE PEPTIDASE1* (*XCP1*) and *CELLULOSE SYNTHASE A7* (*CESA7*) (Zhong et al. 2010, Yamaguchi et al. 2011), by quantitative real-time PCR (**Fig. 6**). The mRNA levels of the downstream genes were significantly

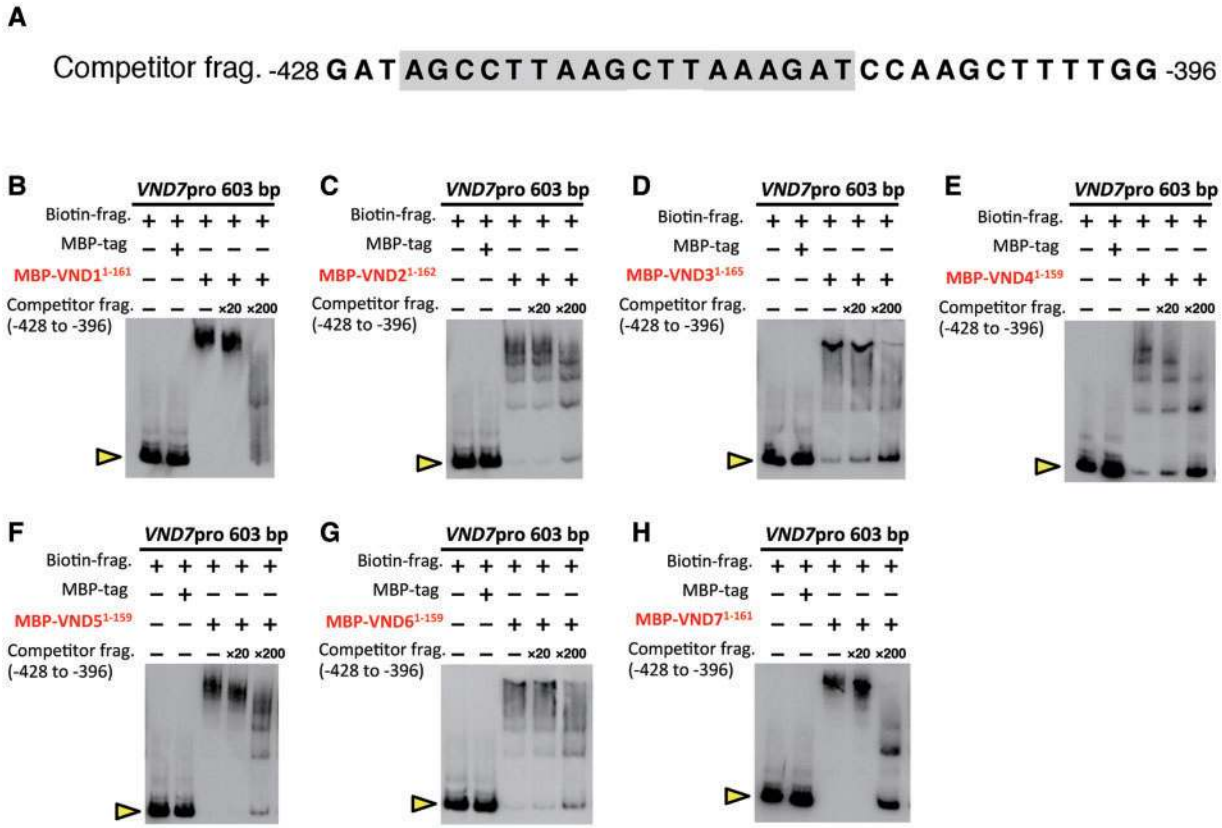

Fig. 2 VND proteins bind to the *VND7* promoter. (A) The 33 bp *VND7* promoter fragment (−428 to −396 bp) was used for competitive analysis. The gray box represents the SNBE/TERE-like motif. (B–H) EMSA was performed using MBP-tagged VND1–VND7 proteins. A biotin-labeled *VND7* promoter fragment (−1 to −603 bp, relative to the ATG start codon) was incubated with (+) or without (−) MBP or MBP-tagged VND proteins (in red). The 33 bp *VND7* promoter fragment was used as the competitor (×20 or ×200). The yellow arrowheads indicate free probe.

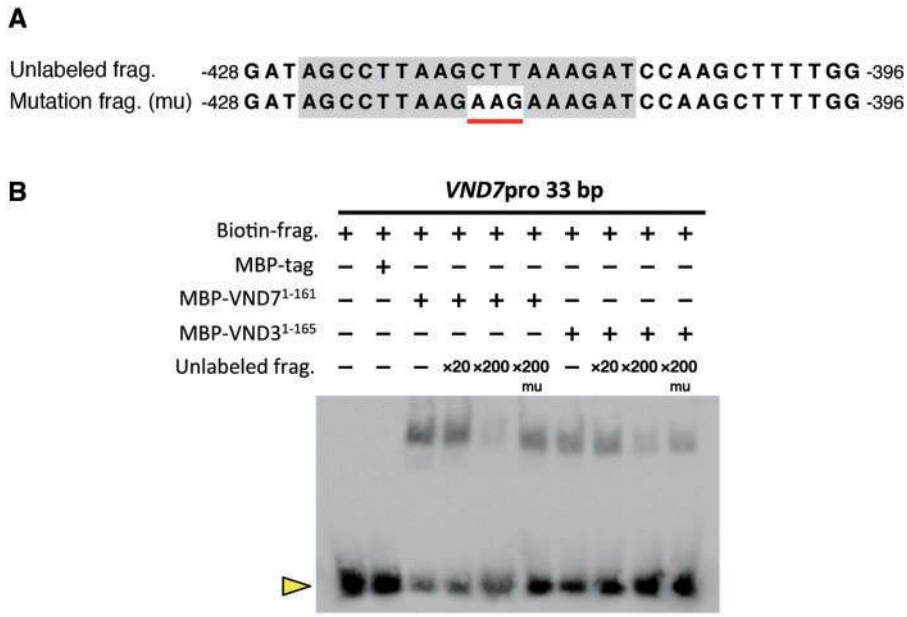

Fig. 3 VND proteins bind to the 33 bp *VND7* promoter region containing the SNBE/TERE-like motif. (A) Nucleotide sequences of the 33 bp *VND7* promoter fragments used for EMSA. The gray box and red line indicate the SNBE/TERE-like motif and mutated nucleotide sequence, respectively. (B) The 33 bp *VND7* 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 as the competitor where indicated (×20 or ×200). The yellow arrowhead indicates free probe.

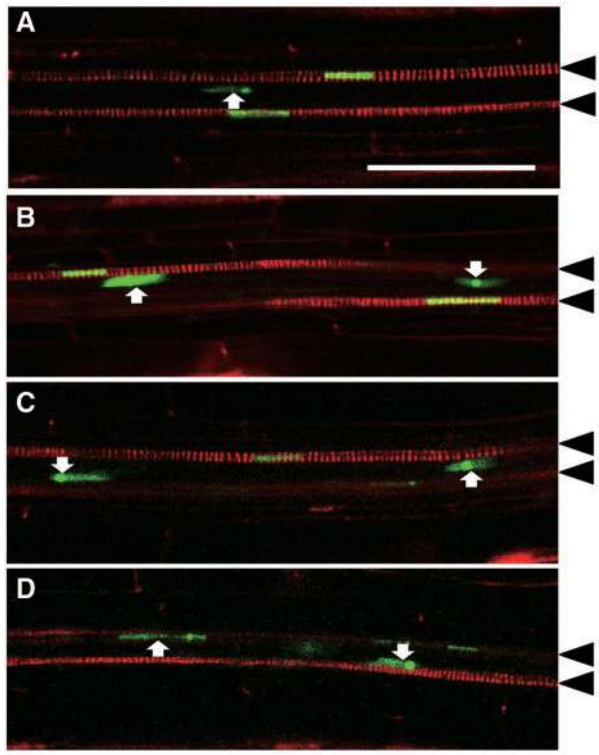

Fig. 4 Expression patterns of the transcription factors shown to induce *VND7* promoter activity in vitro. Confocal microscopy images of (A) *VND7pro::YFP-NLS*, (B) *GATA5pro::YFP-NLS*, (C) *GATA12pro::YFP-NLS* and (D) *ANAC075pro::YFP-NLS* in the roots of (A and D) 9-day-old and (B and C) 10-day-old plants. The roots were counterstained with propidium iodide. Black arrowheads indicate protoxylem vessel strands, and white arrows indicate fluorescent signals in putative metaxylem precursor cells. Scale bar = 100 μm.

up-regulated 24 h after induction of the *VND* genes, whereas only *MYB46* was up-regulated by *ANAC075* induction and none of the target genes was up-regulated by *GATA12* (**Fig. 6**), presumably reflecting the frequency of ectopic xylem vessel element-like cell formation. In contrast, surprisingly, endogenous *VND7* mRNA levels were not markedly changed by induction of any of the transcription factors (**Fig. 6A–F, H, I**). These data suggest that *VND1–VND5* genes can also regulate the downstream genes independently of *VND7*.

Discussion

A regulatory system for the induction of *VND7* expression within the VND family

The *VND* gene family consists of seven genes in Arabidopsis (*VND1–VND7*) (Kubo et al. 2005). All *VND* genes are up-regulated at the early stages of xylem vessel element formation in the Arabidopsis in vitro xylem vessel element formation system (Kubo et al. 2005), whereas *VND* genes are differently expressed in vascular tissues in planta (Kubo et al. 2005, Yamaguchi et al. 2008). In this study, we showed that all VND proteins can activate the *VND7* promoter, at least partially through binding to the SNBE/TERE-like motif located in the *VND7* promoter (**Table 1**; **Figs. 1–3**; **Supplementary Fig. S3**, **Supplementary Table S1**). However, the transactivation activity of VND7 for other *VND* genes was not strong (**Fig. 1**). These results suggest that *VND7* might be a terminus of a transcriptional network within the VND family. Based on the different expression patterns of *VND* family genes in vascular cells and the formation of heterodimers between VND7 and other members of this family, it was thought that each *VND* family gene had a distinct role in vascular development (Yamaguchi et al. 2008). Here we revealed an additional function of *VND* family genes, namely the activation of *VND7* transcription. Moreover, the EMSA results suggested that the *VND7* promoter contains multiple recognition sites of VND proteins, including the SNBE/TERE-like motif and other novel motif(s) (**Figs. 2**, **3**). However, the exact mechanism by which VND proteins regulate VND7 transcription remains to be elucidated.

We found that inducible expression of *VND1–VND5* triggered ectopic secondary cell wall deposition and the up-regulation of several direct target genes of VND6 and VND7 (**Figs. 5**, **6A–G**). Together with our EMSA results (**Figs. 2**, **3**), these findings show that VND1–VND5 proteins possess the potential to regulate directly the expression of genes involved in secondary cell wall formation through binding to the SNBE/TERE motifs, in a similar fashion to VND6 and VND7 (Ohashi-Ito et al. 2010, Zhong et al. 2010, Yamaguchi et al. 2011). Likely, overexpression of members of the Arabidopsis NAC SECONDARY WALL THICKENING PROMOTING FACTOR (NST)/SND and SOMBRERO (SMB) family, which are closely related to members of the VND family and are involved in fiber cell differentiation and root cap maturation, respectively (Mitsuda et al. 2007, Zhong et al. 2006, Willemsen et al. 2008, Yamaguchi and Demura 2010), causes ectopic secondary cell wall formation (Mitsuda et al. 2007, Zhong et al. 2006, Ko et al. 2007, Bennett et al. 2010). Moreover, recent studies revealed that heterologous overexpression of homologs of VND, NST/SND and SMB (VNS) family proteins isolated from *Populus trichocarpa* and *Physcomitrella patens*, induced ectopic secondary cell wall deposition in Arabidopsis (Ohtani et al. 2011, Xu et al. 2014). These findings support the idea that the transactivation activity of VNS family proteins to activate the downstream target genes evolved in the last common ancestor of land plants, and is widely shared among current land plant species (Ohtani et al. 2011, Xu et al. 2014).

VND7 expression is positively regulated by multiple classes of transcription factors

We isolated 14 transcription factors that positively regulate the expression of *VND7* (**Table 1**; **Supplementary Fig. S2**). Among them, *LBD15/ASL11*, *LBD30/ASL19*, *SND2* and *REV* have been reported to be involved in xylem tissue formation (Soyano et al. 2008, Zhong et al. 2008, Ohashi-Ito et al. 2010, Zhong et al. 2010, Carlsbecker et al. 2010, Yamaguchi et al. 2011). *LBD30/ASL19* and *LBD18/ASL20* were previously shown to maintain *VND7*

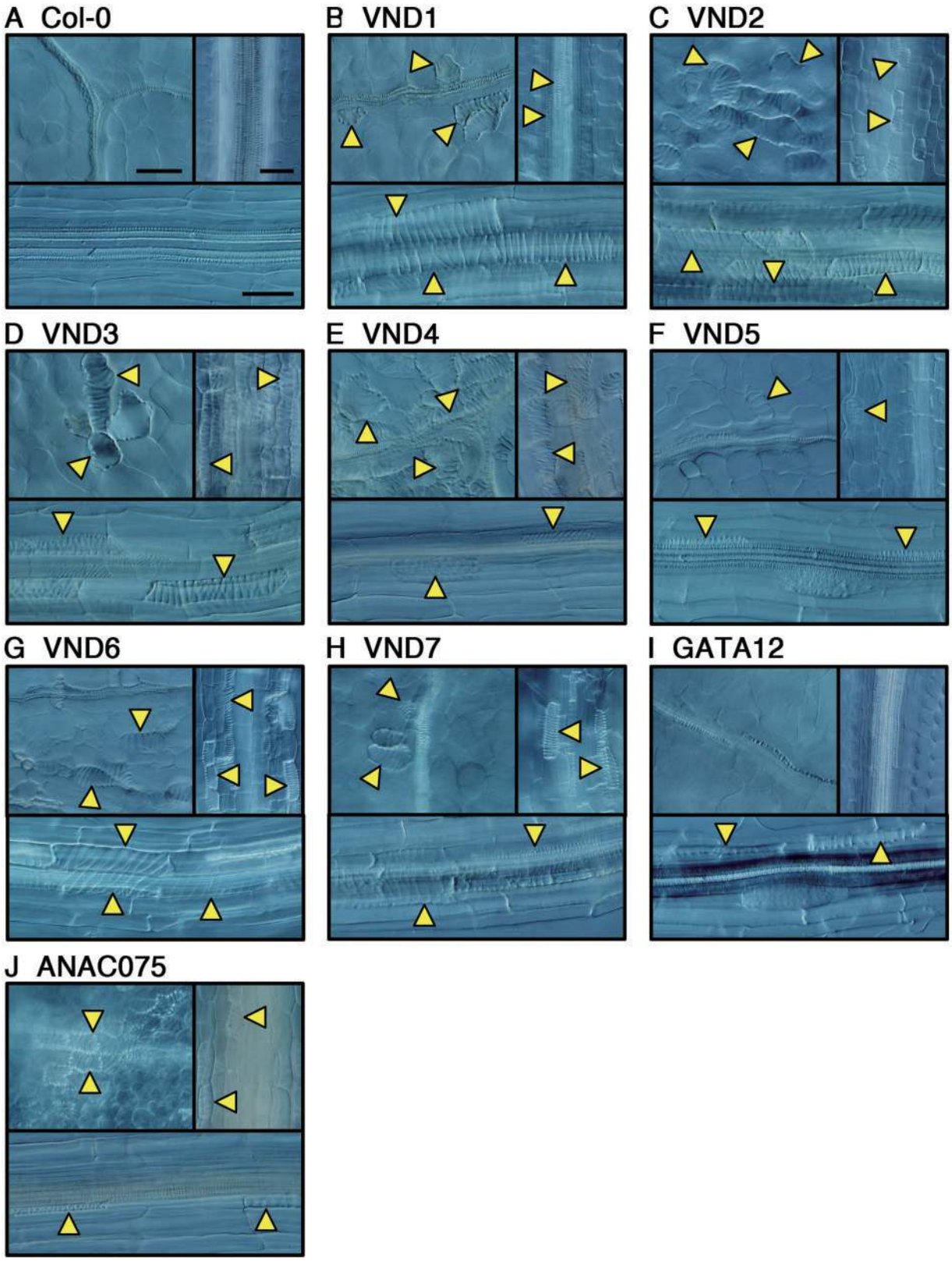

Fig. 5 Morphology of transgenic plant organs overexpressing transcription factors shown to up-regulate *VND7* expression. DIC images of leaves (top left), hypocotyls (top right) and roots (bottom) of 7-day-old (A) Col-0 and transgenic plants expressing (B–H) *VND1–VND7*, (I) *GATA12* and (J) *ANAC075* under the control of an estrogen-inducible system. Plants were grown on medium containing 10 μM β-estradiol for 7 d. Yellow arrowheads indicate the induced ectopic xylem vessel-like elements. Scale bar = 50 μm.

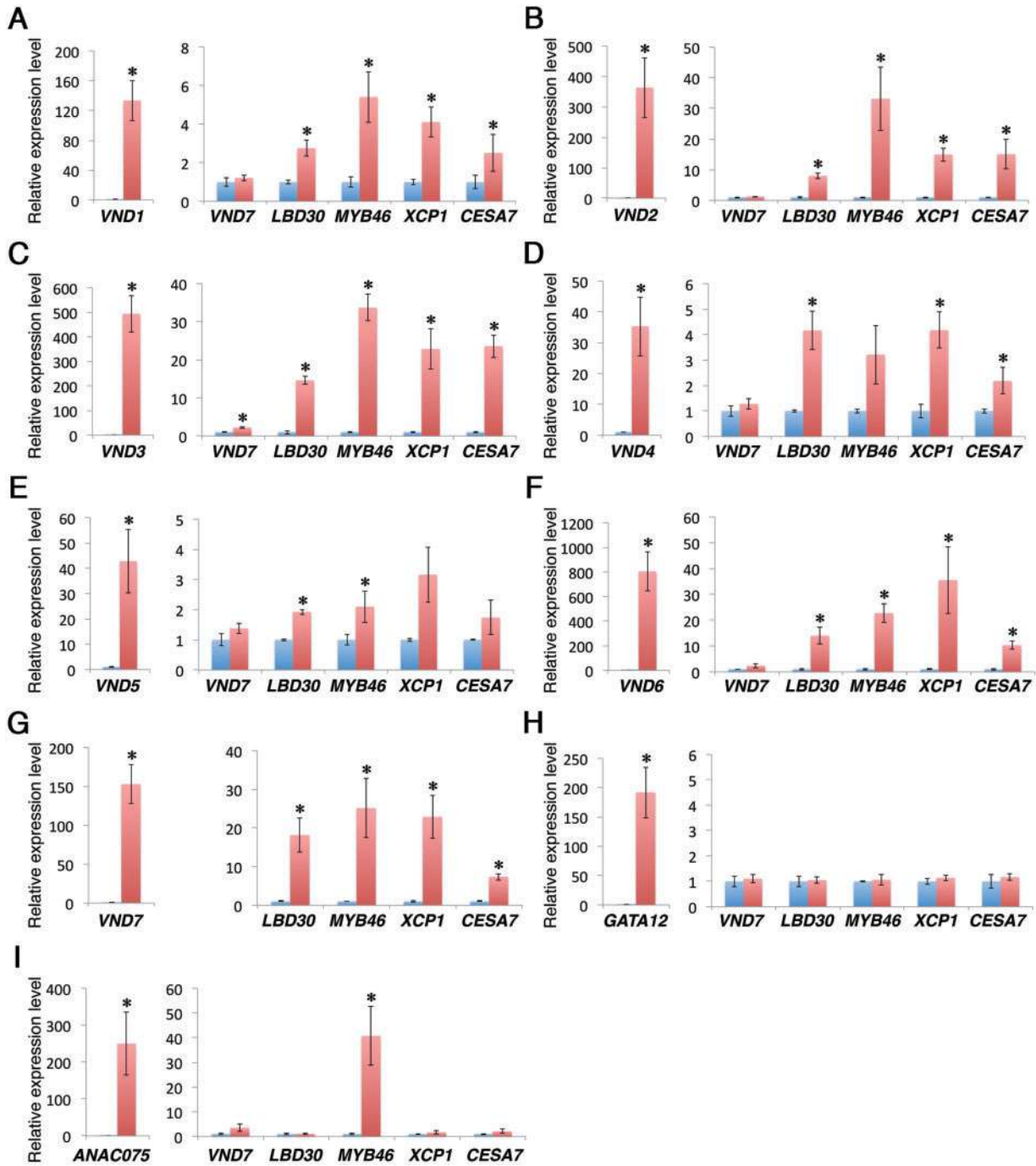

Fig. 6 Expression analysis of *VND7* and other genes involved in xylem vessel formation. The expression of the indicated genes was monitored by quantitative real-time PCR analysis. Five-day-old transgenic seedlings harboring (A–G) *VND1–VND7*, (H) *GATA12* and (I) *ANAC075* were treated with (red) or without (mock, blue) 10 μM β-estradiol for 24 h. The mRNA levels of each gene were normalized to those of *UBQ10*. The blue and red bars indicate mock and β-estradiol treatment, respectively. Error bars indicate the SD ($n = 3$). Asterisks indicate statistically significant differences (Welch's *t*-test; $^*P < 0.05$) compared with values of the mock control.

expression during xylem vessel element differentiation via a feedback pathway (Soyano et al. 2008), and *LBD15/ASL11* and *LBD30/ASL19* were shown to be direct targets of VND7 (Zhong et al. 2010, Yamaguchi et al. 2011). Our data suggested that LBD15/ASL11 could also function in a positive feedback loop that regulates *VND7* expression during xylem vessel differentiation, as do LBD30/ASL19 and LBD18/ASL20 (Soyano et al. 2008). SND2 is a NAC transcription factor expressed in both fiber cells and xylem vessels, and is transcriptionally regulated by NST3/SND1, a master regulator of fiber cell differentiation (Zhong et al. 2006, Mitsuda et al. 2007, Mitsuda et al. 2008, Zhong et al. 2008). *REV* is a member of the Class III HD-Zip transcription factor family and functions to initiate vascular formation and differentiation of xylem vessels (Carlsbecker

et al. 2010, Miyashima et al. 2011). Thus, our screening strategy effectively identifies the regulators of *VND7* expression during xylem vessel formation.

In this study, we identified *GATA5*, *GATA12* and *ANAC075*, which had not previously been reported to be involved in xylem vessel formation, as putative positive regulators of *VND7* expression (**Table 1**; **Supplementary Table S1**). The Arabidopsis genome contains 29 GATA domain-containing transcription factors, some of which are known to function in a wide range of processes, including embryogenesis, morphogenesis, the circadian clock, light responses, seed dormancy and hormone cross-talk (Liu et al. 2005, Manfield et al. 2007, Luo et al. 2010, Nawy et al. 2010, Kanei et al. 2012, Zhang et al. 2013). In silico analysis suggested that ANAC075, one of the closest homologs of SND2, which controls secondary cell wall formation, is a novel candidate gene for control of secondary cell wall formation (Zhong et al. 2008, Shen et al. 2009, Hussey et al. 2011). The transcriptome analysis of the Arabidopsis in vitro xylem vessel element formation system showed that the expression of *GATA5*, *GATA12* and *ANAC075* peaks before that of *VND7* (**Supplementary Fig. S7**). Expression analysis showed that these genes are expressed in vascular tissue and that their expression overlapped with that of *VND7* in the xylem vessels in the root maturation zone, where two strands of protoxylem vessels start to appear (**Fig. 4**), suggesting that *GATA5*, *GATA12* and *ANAC075* are involved in xylem vessel formation, probably by regulating the expression of *VND7*.

These results indicate that multiple classes of transcription factors, such as members of the NAC, LBD/ASL, HD-Zip and GATA transcription families, regulate *VND7* transcription. This finding provides insight into the complex and co-ordinated transcriptional regulatory network underlying vascular development and concomitant secondary cell wall formation.

VND7 expression is tightly regulated in vivo

Our data showed that VND1–VND7, GATA12 and ANAC075 could induce *VND7* promoter activity in a transient expression assay using Arabidopsis leaves (**Table 1**; **Supplementary Fig. S3**; **Supplementary Table S1**) and that VND1–VND7 could directly bind to the SNBE/TERE-like motif on the *VND7* promoter region (**Figs. 2**, **3**; **Supplementary Fig. S4**). However, none of the *VND1–VND6* overexpression lines showed significant accumulation of endogenous *VND7* mRNA, despite these lines showing up-regulated mRNA levels of the other putative direct target genes, such as *LBD30/ASL19* and *MYB46* (**Fig. 4A–F**).

It was previously reported that expression of *VND7pro::GUS* was restricted to cells adjacent to the endogenous xylem vessels of hypocotyl explants cultured in the presence of phytohormones that play pivotal roles in xylem vessel formation (Kubo et al. 2005, Ohashi-Ito and Fukuda 2010). Similarly, *CONSTANS* (*CO*), which is a critical factor for daylength measurement in photoperiodic flowering, is mainly expressed in vascular tissue in a tissue/cell type-specific manner (Ito et al. 2011). Ectopic overexpression of the upstream basic helix–loop–helix (bHLH) transcription factor genes, *FLOWERING BHLH 1* (*FBH1*) and *FBH2*, highly up-regulates the expression of *CO* without changing its spatio-temporal expression patterns (Ito et al. 2011). Thus, endogenous *VND7* expression appears to be tightly regulated in a tissue/cell type-specific manner in vivo, and this tight regulation might be accomplished through unknown motifs located outside of the *VND7* promoter sequence used in our transient expression assays. Therefore, it would be interesting to investigate the mechanism that regulates *VND7* expression in specific cell types.

Based on all of these findings, we propose a model in which the transcription of *VND7* is regulated by multiple transcription factors (**Fig. 7**). Vascular development is a critical aspect of plant development, and multiple developmental and hormonal signals regulate when and where vascular tissues are formed (Caño-Delgado et al. 2010). Recent advances in our understanding of vascular development show that multiple types of signaling molecules regulate vascular development and vascular specification, often by controlling the expression of certain transcription factors (Kubo et al. 2005, Demura and Fukuda 2007, Ohhashi-Ito and Fukuda 2010, Kondo et al. 2011, Miyashima et al. 2012, Kondo et al. 2014). Therefore, in the process of vascular development, multiple upstream transcription factors mediate developmental cues that precisely control VND7 function to achieve appropriate xylem formation (**Fig. 7**). Moreover, the fact that endogenous *VND7* expression was not significantly induced by ectopic overexpression of the upstream transcription factor candidates suggests that unknown inhibitory factors are involved in the transcriptional regulation of *VND7* in non-xylem vessel cells (**Figs. 6**, **7**). To understand fully the transcriptional regulation of *VND7* during xylem vessel differentiation, the upstream transcription factors need to be characterized in detail, and the inhibitory factors need to be identified.

Materials and Methods

Selection of candidate transcription factors

Candidate transcription factors were selected from the transcriptome analysis data of the Arabidopsis in vitro xylem vessel transdifferentiation system generated by Kubo et al. (2005). Expression data were recorded at six time points, namely at 0, 2, 4, 6, 8 and 10 d after induction of xylem vessel transdifferentiation (Kubo et al. 2005). The transcription factors were chosen based on the criteria that they had more than twice the normalized signal intensity at 2–6 d after induction when compared with the expression data at 0 d after induction, or that at 4–6 d after induction when compared with the expression data at 2 d after induction, since the normalized signal intensity of *VND7* peaked at 6 d after induction (**Table 1**; **Supplementary Fig. S2**; Kubo et al. 2005).

Plasmid construction

To generate the Gateway destination vector for the dual LUC transient transfection assay, the pA35SG effector plasmid (Yamaguchi et al. 2008) was digested with *Sma*I and ligated into the *Eco*RV-digested GATEWAY Reading Frame Cassette (GWRFC) B (Invitrogen, http://www.invitrogen.com/). Likewise, the GAL4UAS:TATA:LUC reporter plasmid containing *fLUC* (Ohta et al. 2000) was digested with *Hin*dIII and *Sma*I to remove the GAL4UAS and TATA-box sequences, blunted using the BKL Kit (TAKARA BIO INC., http://www.takara-bio.com/) and ligated into *Eco*RV-digested GWRFC B. The resultant effector and

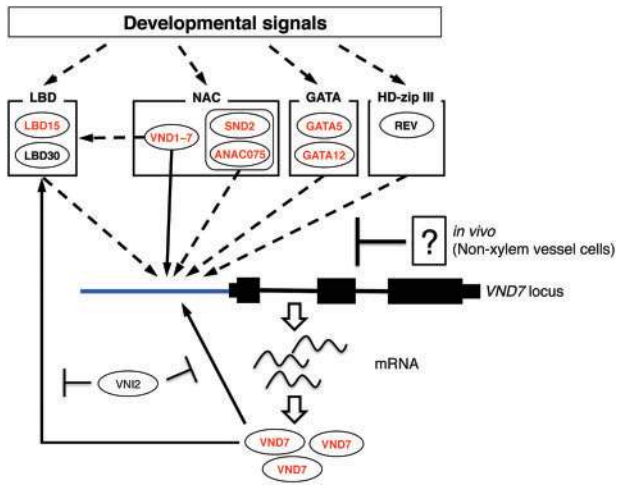

Fig. 7 Schematic model of the local transcriptional network that controls *VND7* expression. The expression of *VND7* is positively regulated by several transcription factor families. Access to the *VND7* promoter by the transcription factors is somehow tightly controlled and limited to differentiating xylem vessel cells. VNI2 negatively regulates the transcriptional activation activity of VND7 (Yamaguchi et al. 2010b). Transcription factors in red font were suggested in this study to regulate *VND7* expression positively. Arrows with solid lines indicate that direct transcriptional regulation has been confirmed. The blue line, black lines, large black boxes and small black boxes of the *VND7* locus indicate the promoter, intron, exon and 5′ or 3′ untranslated regions, respectively.

reporter plasmids were designated pA35G and pAGL, respectively. The amplified CDSs of candidate transcription factors and the promoter regions of *VND1–VND7* were cloned into the pENTR/D/TOPO vector (Invitrogen, http://www.lifetechnologies.com) and then integrated into the Gateway destination vectors, pA35G and pAGL, using LR Clonase (Invitrogen), respectively. The primer information is described in **Supplementary Table S3**. The nucleotide sequence of the multicloning site (MCS) 5′-CACCTAGTGGATCCCCCGGG CTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGTGATG-3′, which includes a stop codon at the 5′ end and a start codon at the 3′ end, was used as an effector control (Yamaguchi et al. 2008). The RIKEN Arabidopsis full-length (RAFL) clones (Seki et al. 2002) containing full-length cDNAs of the genes listed in **Table 1** and labeled with B were digested with *Sfi*I, and the released cDNA fragments were subcloned into *Sfi*I-digested pBIG2113SF binary vector (Ichikawa et al. 2006). The empty vector pBIG2113SF was used as an effector control (Ichikawa et al. 2006). A reference plasmid containing rLUC was prepared as described in Ohta et al. (2000). For the promoter analysis, the promoter fragments of *GATA5*, *GATA12* and *ANAC075* (**Supplementary Table S3**) 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–VND7*, *GATA5*, *GATA12* and *ANAC075* were subcloned into the *Pac*I/*Asc*I sites of the pER8 vector, an estrogen receptor-based chemically inducible system for use in transgenic plants (Zuo et al. 2000). For the EMSA, the NAC domain regions of *VND1–VND7* (**Supplementary Table S2**) 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 and growth conditions

Arabidopsis thaliana (ecotype Col-0) was used as the wild type. Plants were germinated on sterile Murashige and Skoog medium (0.6% agar) at 22°C under continuous light conditions after cold treatment (at 4°C in the dark for 2–3 d). Two to three weeks after germination, plants grown on plates were transferred to soil and grown further in a growth chamber at 22°C with a photoperiod of 16 h light and 8 h darkness. For transformation, the plasmids generated were electroporated into *Agrobacterium* strain *GV3101::pMP90*, and 3- to 4-week-old Arabidopsis plants were transformed by the floral dip method (Clough and Bent 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 a Biolistic PDS-1000/He Particle Delivery System (BIO-RAD, http://www.bio-rad.com; Mitsuda et al. 2009), and LUC activity was assayed with the Dual-Luciferase Reporter Assay System (Promega, http://www.promega.com) using the Mithras LB940 or LB941 (Berthold, http://www.bertholdtech.com).

Transient expression assay using *VND7pro:GUS* leaves

The effector plasmids was introduced into the rosette leaves of 18-day-old transgenic plants expressing the *GUS* reporter gene 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 d 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 the reaction solution (1 mM 5-bromo-4-chloro-3-indolyl glucuronide, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 100 mM sodium phosphate pH 7.0) at 37°C for 10 h. After bleaching by ethanol, the samples were mounted in the clearing solution (8 g of chloral hydrate, 1 ml of glycerol and 2 ml of water). Observation was made 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 regions of VND1–VND7, containing whole NAC domains (**Supplementary Table S2**), were expressed in *Escherichia coli* strain BL21*trxB* (DE3) and purified as previously described (Yamaguchi et al. 2010b). Biotinylated promoter fragments (approximately 20 fmol) were incubated in reaction buffer (LightShift EMSA Optimization and Control Kit; Thermo Scientific) for 30 min at 4°C with 5 pmol purified recombinant protein and/or an excess of unlabeled fragments as competitors. The protein-bound biotinylated DNA fragments were separated by PAGE. The DNA was electroblotted onto a positively charged nylon membrane (Hybond-N+; GE Health Care, http://www.gelifesciences.com) and detected using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific), 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 the oligo(dT) 20 primer and Transcriptor Reverse Transcriptase (Roche; http://www.roche.com). Real-time PCR was performed with the 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 analysis are presented in **Supplementary Table S3**. *UBQ10* cDNA served as an internal control for all experiments.

Microscopy analysis

To observe the overexpression lines, plant samples were fixed with 90% acetone for >1 week at −30°C. Samples were mounted in clearing solution (8 g of chloral hydrate, 1 ml of glycerol and 2 ml of water) before observation. Nomarski (differential contrast; DIC) images were captured using a polarizing microscope (BX51; Olympus) equipped with a digital camera (DP70; Olympus). The roots of overexpression lines stained by safranin-O (Kitin et al. 2000) were detected using a confocal laser scanning microscope (Zeiss AX10 observer Z1) equipped with a digital camera (Zeiss LSM 710). The roots of *VND7pro::YFP-NLS*

(Kubo et al. 2005, Yamaguchi et al. 2008), *GATA5pro::YFP-NLS*, *GATA12pro::YFP-NLS* and *ANAC075pro::YFP-NLS* plants were counterstained with propidium iodide and observed with a laser scanning confocal microscope.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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