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MYB transcription factors orchestrating the developmental program of xylem vessels in Arabidopsis roots

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Abstract Xylem vessel elements play an important role in conducting water and nutrient in land plants. The in vitro culture system, in which subcultured Arabidopsis cells are induced to transdifferentiate into xylem vessel elements, was used to uncover the comprehensive gene expression profile by microarray analysis, which resulted in the identification of many genes encoding transcription factors including two transcriptional key regulators, VASCUAR-RELATED NAC DOMAIN6 (VND6) and VND7. Here we analyze the detailed expression pattern and function of ten genes encoding MYB transcription factors, MYB20, 43, 46, 52, 63, 83, 85, 99, 103, and 118, with the significantly up-regulated expression during the in vitro vessel element differentiation. Of these, six exhibited the specific expression in differentiating xylem vessels in Arabidopsis roots. In addition, *MYB46* and *MYB83* were shown to upregulate five of these *MYB* genes, *MYB43*, *52*, *63*, *85*, and *103*. Our results suggest that several MYB genes comprise a transcriptional network during xylem vessel element differentiation in roots.

Key words: Arabidopsis, MYB, secondary cell wall, transcription factor, xylem vessel element differentiation.

Xylem vessel elements play an important role in land plants as a component to conduct water and nutrients, and to support the plant. Plants provide two types of vessels, protoxylem vessels with annular and spiral secondary cell walls (SCWs) and metaxylem vessels with reticulate and pitted SCWs. In Arabidopsis roots, it is well illustrated that two protoxylem vessels develop at the outermost position of the vascular system, between which up to four metaxylem vessels are located. However, the molecular mechanisms controlling vessel formation is poorly understood. We established the Arabidopsis *in vitro* xylem vessel element inducible system, which was used for microarray analysis with the Arabidopsis full-genome GeneChip array ATH1 (Affymetrix) to uncover the comprehensive gene expression profile during xylem vessel element differentiation. Through the analysis, two transcriptional key regulators, *VASUCULAR-RELATED NAC DOMAIN6* (*VND6*) and *VND7*, were found to activate transdifferentiation of various cells into metaxylem vessels and protoxylem vessels, respectively (Kubo et al. 2005). In addition to *VND6* and *VND7*, a number of genes encoding various types of transcription factors including MYB transcription factors are expressed during the in vitro vessel element differentiation (Kubo et al. 2005). In this study, we characterize the detailed expression pattern and transcriptional regulatory network of ten *MYB* genes with the up-regulated expression during in vitro vessel element differentiation.

Materials and methods

Plant materials and growth condition

Arabidopsis thaliana, ecotype Columbia (Col-0), was used in both the wild-type and transgenic experiments. Arabidopsis seeds were surface-sterilized by immersion in a solution of 1% (v/v) sodium hypochlorite and 0.1% Triton X-100 for 10 min, and rinsed five times with sterile deionized water prior to plating. Seeds were plated on half-strength MS medium containing with 0.5% sucrose and 0.4% phytagel (Sigma-Aldorich), and incubated for 3 days at 4ºC, and then incubated in a growth chamber (16 h light/8 h dark) at 22ºC.

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Table 1. List of PCR primers

Gene	Forward	Reverse
MYB20 pro	CACCTAGGGTGTACTTCAAATGGGAGCCACCGG	TCTCCCCATTTCTCTCTCTCTCTCTCG
MYB43 pro	CACCAATCTTTTAATTTTATCGTATGTGACAATC	CCTCCCCATCTCTCTCCCTCTCTCGCTAAA
MYB46 pro	CACCTACACTTCTACAGTTGTTAACCTCACACTA	CTTCCTCATATTTTTGGTTGAGTTAATTGT
MYB52 pro	CACCAGTTTGGCTTTGTTCCTACACACGTG	ACACATCATTTTTTAATACCTCTCTCC
MYB63 pro	CACCTTTGGCGCATGAGTTGTTTTCTAAAT	CTTCCCCATTCTCTCTCTCTCACTCCCTCT
MYB83 pro	CACCTGATCTTCTACCATCTGAGAATGGCAAATC	CATCATCATTTTGGACTGGTGGAGCCTT
MYB85 pro	CACCGAATCAGGTTACATCCCTTATTTCGGTACG	TCTCCCCATTAAATACTATATAGAAATG
MYB99 pro	CACCACGTATCTTCCACTCACTTTCGTTCCAAG	ACCACCCATTATTTATATTATATGGTTTAA
MYB103 pro	CACCACGTAAGCTTGGTGCCCTGGTCTACAGTTC	ATGACCCATGATTAGTAGCTCCTCAAAG
MYB118 pro	CACCGTGACTCCACCAAACCACTCAACACAC	GAACTCCATATGATTATGATGGCAAA
MYB20 RT-PCR	TTCGAGTGGTCCGACTATGGTAATA	CCAAAAGTGGAAGAACTCTCATCAT
MYB43 RT-PCR	ATACGATGAACTTGTGGGACATCAA	GCTAGAGGAGATCAAATGTCCATGA
MYB46 RT-PCR	GAATGTGAAGAAGGTGATTGGTACA	CGAAGGAACCTCAGTGTTCATCA
MYB52 RT-PCR	TAAACACGGATTCGAAGATACACGA	ATAAACCCTGAGAGGCAGAGTTTCC
MYB63 RT-PCR	GAAGAAGAGTCTGATGAGGATGAGG	CATGAGCTCGTAGTTCTTCAAGAGTG
MYB83 RT-PCR	CTTTGTCATTCAGAAAGCTTCAAGG	ATCGACTTGGAAATCAAGGAAGGGAAA
MYB85 RT-PCR	GGTGTTGGTGGAACATTATATGACC	GGTGAACAGTCAAAACCCAAAATC
MYB99 RT-PCR	CTTACGATGCTATTGTCCGGTGAC	CAACCTACGGCCTAATTAAACATAC
MYB103 RT-PCR	GATCAATGGGACGATTCACAACAAT	AAACGAAGAAGGGAAAGAAGAAGATAA
MYB118 RT-PCR	AAGTGGTGTGACCATGGAGATTGAT	TCGACTTGTTTATATTGCACGGTCT
MYB46 ORF	CACCATGAGGAAGCCAGAGGTAGCCATTG	TGTGTGTGGCTAAGAATCTTGAGTA
MYB83 ORF	CACCATGATGATGAGGAAACCGGACATTA	AAGATCGATCCACTTCATGTTAAAAA
Act2 RT-PCR	GTCCAGGAATCGTTCACAGAAAAT	GGGAAATGAAACAAACAAATGGAG

CACC was added for directional cloning in pENTR/D/TOPO cloning system (Invitrogen).

Vector construction

The approximately 2.0 kb of promoter sequences upstream of the ATG translation start site of each gene were amplified from *Arabidopsis* Col-0 genomic DNA by PCR with gene-specific primer sets (Table 1), and then subcloned into the pENTR/D/TOPO vector (Life Technologies). For promoter analysis, these promoter sequences were integrated into the pBGGUS and pBGYN binary vectors (Kubo et al. 2005). For transactivation assay, these promoter sequences were fused with firefly luciferase to generate reporter plasmids. The effector constructs contained *MYB46* or *MYB83* between the *CaMV35S* promoter and *NOS* terminator.

Transformation of Arabidopsis

Agrobacterium tumefaciens, strain GV3101::pMP90 was used to transform Arabidopsis plants. Four-week-old Arabidopsis plants were infected with the *Agrobacterium* strain carrying the binary vextors, according to the floral dip method (Clough and Bent 1998). Transgenic plants were selected by half-strength MS medium containing with 0.5% sucrose, 10 mg mL^{-1}, and 0.4% phytagel.

Gene expression analyses

The 8-day-old transgenic Arabidopsis plants carrying gene-specific promoter fused with YFP-NLS or GUS were analyzed. GUS staining method was previously described (Pyo et al. 2007). Images of YFP fluorescence and differential interference contrast were captured with a microscope (BX51, Olympus) and photographed with a digital camera (DP70, Olympus).

RT-PCR analysis

Total RNA was purified from 10-day-old seedlings of *MYB46*ox, *MYB83*ox, and wild type, using RNeasy plant Mini Kit (Qiagen) with DNA-free (Life Technologies). The purified total RNA (2 μg each) of RNA was used as the template for first-strand cDNA synthesis using SuperScript III RT (Life Technologies) with oligo $(dT)_{12–18}$ primers in a reaction volume of 20 μl. A 1 μl aliquot of the reaction mixture was amplified by PCR using ExTaq DNA polymerase (Takara Bio) in a reaction volume of 20 μl with 6 μM of each primer (Table 1). The number of cycles varied depending on the gene to avoid saturation of the amplification as follows: *MYB46*, *52*, *63*, *83*, and *85*, 30 cycles; *MYB99*, 38 cycles; *MYB103*, *Act2*, 23 cycles. After PCR, the reaction products were separated on 1.5% (w/v) agarose gels and stained with ethidium bromide. *Act2* was used as an extraction and loading control.

Dual luciferase transient transactivation assay

The effector, reporter, and reference plasmid containing *Renilla reniformis* luciferase were delivered to rosette leaves of 4-week-old Arabidopsis by particle bombardment (GE Healthcare) and luciferase activity was assayed with the Dual-Luciferase Reporter Assay System (Promega) using Mithras LB940 (Berthold) as described previously (Yamaguchi et al. 2008, 2010).

Accession numbers

The Arabidopsis Genome Initiative locus identifiers or Genbank database accession numbers for the genes investigated in this study are *MYB20* (At1g66230), *MYB43* (At5g16600), *MYB46* (At5g12870), *MYB52* (At1g17950), *MYB63* (At1g79180), *MYB83* (At3g08500), *MYB85* (At4g22680), *MYB99* (At5g62320), *MYB103* (At1g63910), *MYB118* (At3g27780), *VND6* (At5g62380), *VND7* (At1g71930), *Act2* (At3g18780), *EgMYB2* (AJ576023), *PtMYB1* (AY356372), *PtMYB4* (AY356271), and *PtMYB8* (DQ399057).

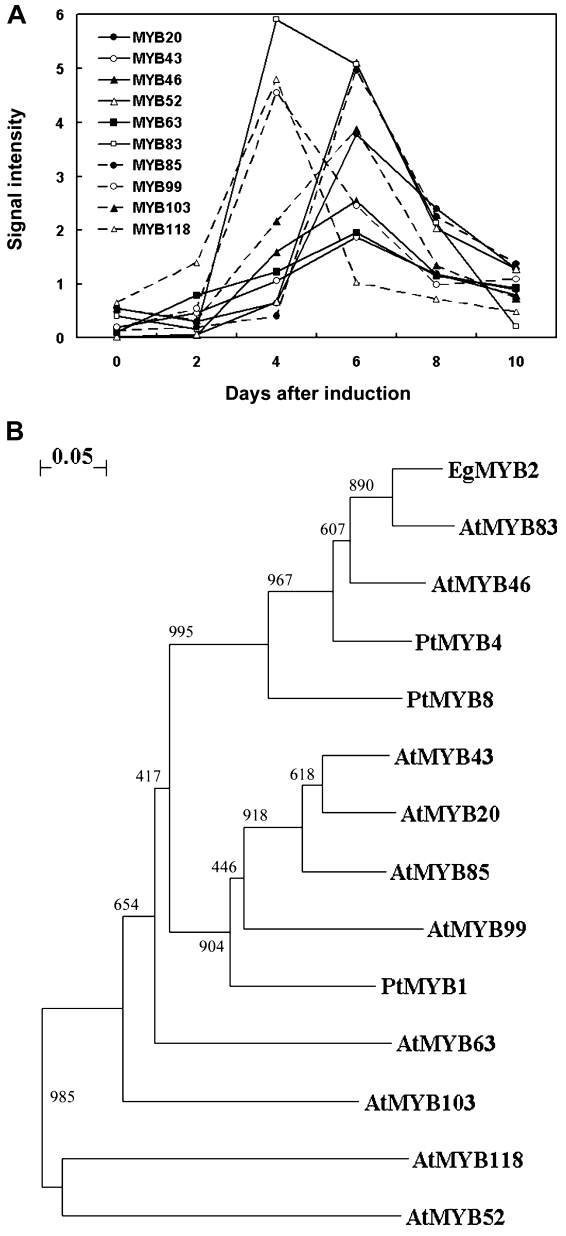

Figure 1. Selected ten *MYB* genes. (A) Expression patterns of ten *MYB* genes during the *in vitro* xylem vessel element differentiation. Signal intensities of each gene were captured from the microarray data described by Kubo et al. (2005). (B) Phylogenetic tree of MYB proteins. Amino acid sequences of ten MYB proteins analyzed in this study and four other MYB proteins previously shown to be involved in regulation of secondary cell wall biosynthesis were aligned by using the ClustalX 2.0 (Larkin et al. 2007) and the tree was generated with NJPlot (Perrière and Gouy 1996). Bootstrap values of 1,000 replicates are indicated on the tree.

Results and discussion

We found that ten *MYB* genes, *MYB20*, *43*, *46*, *52*, *63*, *83*, *85*, *99*, *103*, and *118*, showed significantly up-regulated expression just when the xylem vessel elements were actively forming (4 to 6 day after induction) (Figure 1). The detailed expression patterns of these *MYB* genes in Arabidopsis roots were obtained by the promoter analysis with the yellow fluorescence protein (*YFP*) gene fused to the SV40 nuclear localization signal (NLS) or the *GUS* reporter gene. 8-Day-old transformants harboring the transgenes were observed.

It was revealed that six of these ten *MYB* genes, *MYB46*, *52*, *83*, *85*, *99*, and *103*, have specific expression in differentiating xylem vessels, which was shown by the clear YFP signals on elongated nuclei characteristic of differentiating vessels (Figure 2) and by restricted GUS staining in differentiating vessels (Figure 3). *MYB46*, *83*, and *99* showed similar pattern of expression: specific expression both in immature protoxylem and metaxylem vessels indicated by obscure thickening of SCWs, suggesting fundamental function of *MYB46*, *83*, and *99* at earlier stages of differentiation of both types of vessels. *MYB52* and *85* exhibited almost identical expression pattern: the strong expression was observed in protoxylem and metaxylem vessels with clear SCW thickening, accompanied with the weak expression in pericycle and/or endodermis around the region where clear SCWs of metaxylem vessels were observed (Figure 3). The expression of *MYB103* was detected only in metaxylem vessels with relatively clear SCW thichening but not in protoxylem vessels, suggesting specific function of MYB103 in metaxylem vessels.

The expression of *MYB20*, *43*, and *63* was not specific for vessel elements, but basically predominant in vascular stele (Figure 2 and 3) The YFP signal of *MYB20* was detected in vascular cells at the cell maturation zone but not the meristematic and elongation zones while the GUS signal of *MYB20* was obscure. Both the YFP and GUS signals of *MYB43* were observed in vascular cells (Figure 2 and 3) as well as in cortical cells of meristematic and cell elongation regions (data not shown). The signals of *MYB63* were observed in vascular cells including developing metaxylem vessels (Figure 2, yellow arrowheads; Figure 3) as well as in cortical cells of cell elongation region (data not shown). We could not detect any YFP and GUS signals of *MYB118* in roots of Arabidopsis transformants (data not shown). It was reported that *MYB118* is predominantly expressed in silique and is involved in embryogenesis (Zhang et al. 2009), suggesting the function of MYB118 independent of vessel differentiation. Taken together, our promoter analysis in roots indicated that these MYB genes showed diverse expression patterns: *MYB46*, *83*, and *99* are expressed from earlier stages of vessel formation while *MYB52*, *85*, and *103* are expressed at later stages, and the vessel expression of *MYB63* and *103* is restricted to metaxylem vessels.

It was recently found that *MYB46* and *83* are key regulators for the SCW synthesis in Arabidopsis roots: transgenic Arabidopsis plants overexpressing *MYB46* or *MYB83* under the control of CaMV35S promoter (*MYB46*ox and *MYB83*ox) showed an ectopic vessel

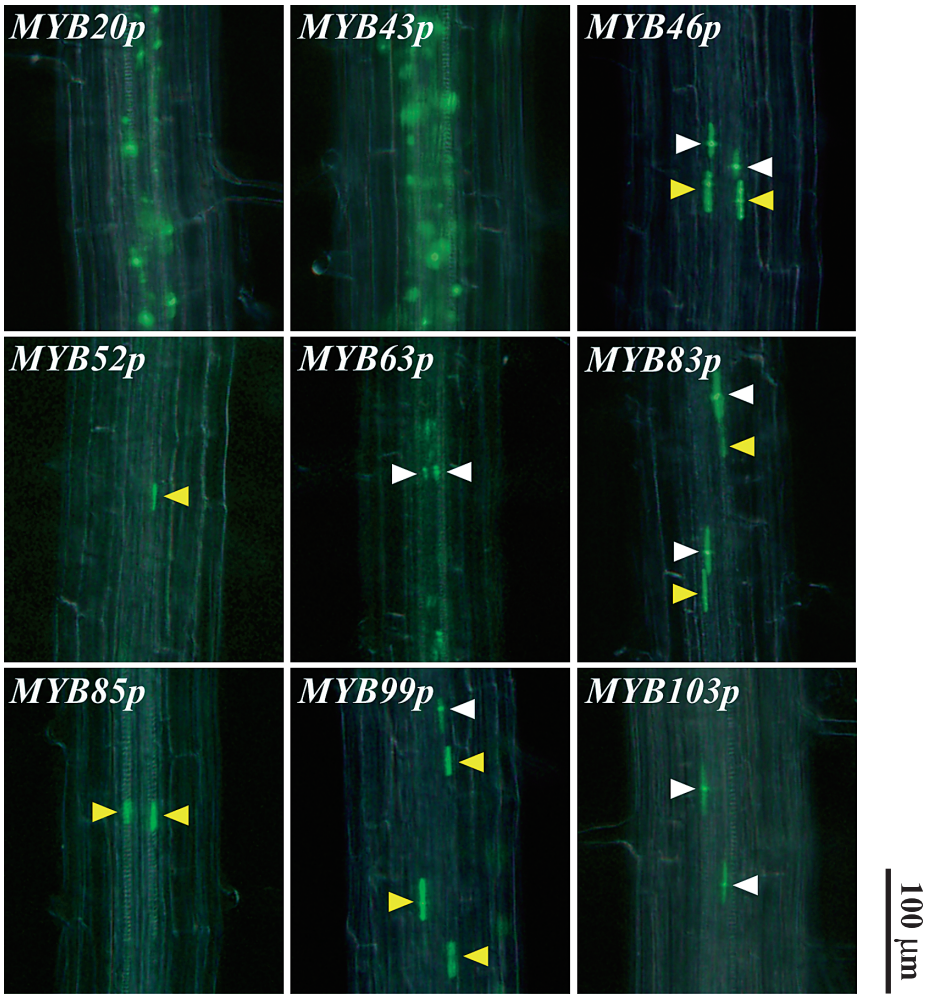

Figure 2. Expression patterns of vessel-related *MYB* genes in Arabidopsis roots. Middle part of roots from 8-day-old transgenic Arabidopsis plants carrying gene-specific promoter fused with *YFP-NLS* were observed under the fluorescent microscope. Images of differential interference contrast and YFP fluorescence were merged. YFP signals observed in protoxylem and metaxylem vessels were indicated by yellow and white arrowheads, respectively. Bar=100 μm.

element formation in several types of cells with up-regulated expression of several SCW-related genes (McCarthy et al. 2009; N. Nishikubo, Y. Nakano, and T. Demura, unpublished data; Zhong et al. 2007, 2008), suggesting that *MYB46* and *MYB83* are upstream of other vessel-related *MYB* genes. Thus, we further investigated whether *MYB46* and *MYB83* induce the expression of vessel-related *MYB* genes by RT-PCR. Strong upregulation of *MYB52* and *63* and slight but significant upregulation of *MYB43*, *85*, and *103* was observed both in *MYB46*ox and *MYB83*ox while no significant change in expression of MYB20, 99, and 118 was detected (Figure 4). These results indicate that the expression of at least half of *MYB* genes that are expressed preferentially during vessel formation is regulated by *MYB46* and *MYB83*.

To confirm the idea that *MYB46* and *MYB83* upregulate the expression of the vessel-related *MYB* genes, we employed the particle bombardment-based transactivation assay. Arabidopsis rosette leaf cells were cotransfected with the firefly luciferase reporter gene linked to the promoter sequences of all the vessel-related *MYB* genes (except for *MYB20* and *MYB118*) which were used for the promoter analysis and CaMV 35S promoter-driven *MYB46* or *MYB83* gene together with a reference plasmid containing the *Renilla reniformis* luciferase gene, and then luciferase activity was assayed. It was found that *MYB46* and *MYB83* genes highly induced luciferase reporter activity driven by the promoter of *MYB43*, *52*, *63*, *85*, and *103* but not that of *MYB46*, *83*, and *99* (Figure 5). These findings suggest that *MYB46* and *MYB83* activate the expression of at least 5 *MYB* genes, *MYB43*, *52*, *63*, *85* and *103*, during vessel element formation.

Function of a number of transcription factors including NACs and MYBs during differentiation of vessel elements and fibers has been described so far (Ko et al. 2009; Kubo et al. 2005; McCarthy et al. 2009;

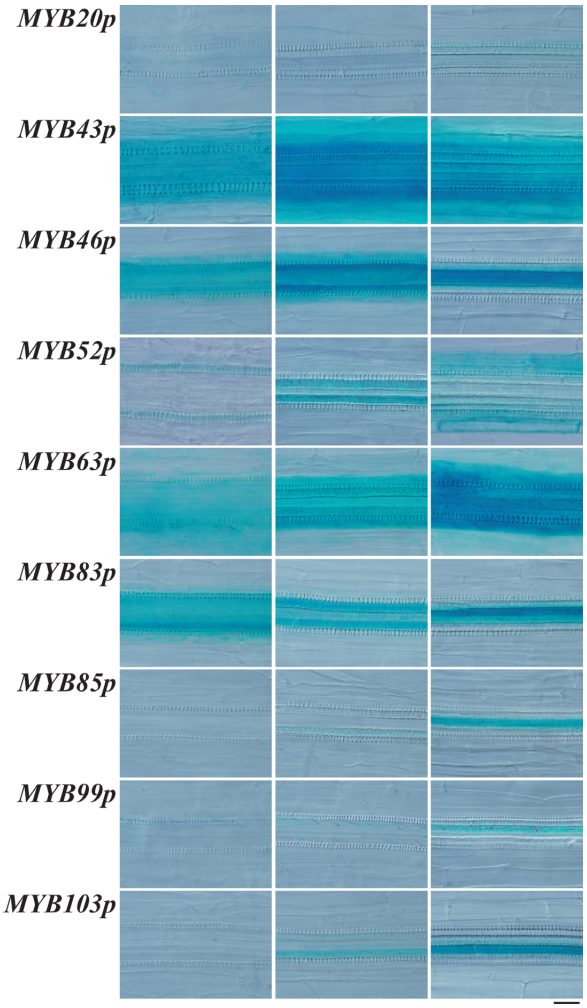

Figure 3. Expression patterns of *MYBp::GUS* in 8-day-old Arabidopsis roots. GUS staining method was described previously (Pyo et al. 2007). GUS stainings observed in differentiating protoxylem vessels (left panels), in differentiating outermost metaxylem vessels (middle panels), and in differentiating inner metaxylem vessels (right panels). Bar=20 μm.

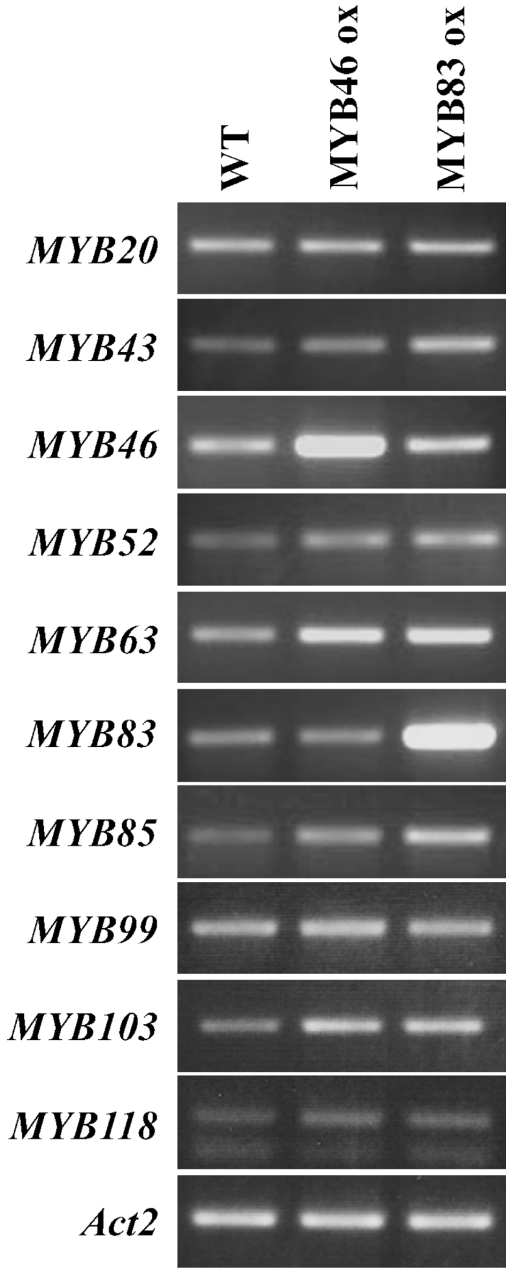

Figure 4. Gene expression of vessel-related *MYB* genes in *MYB46* and *83* overexpressors. Expression levels of the vessel-related *MYB* genes in 10-day-old transgenic Arabidopsis seedlings were analyzed by RT-PCR. The expression level of *Act2* was used as a control.

Mitsuda et al. 2005, 2007; Zhong et al. 2006, 2007, 2008, 2010; Zhou et al. 2009), suggesting the transcriptional network comprised of these transcription factors (Yamaguchi and Demura 2010 in this issue). Our data shown in this paper is basically consistent with the previous reports, in terms of the function of *MYB46* and *MYB83* genes which activate several downstream *MYB* genes. However, our findings added the new findings that these downstream *MYB* genes were not always spatially and temporally expressed in the same manner as the *MYB46* and *MYB83* genes during vascular development especially during vessel element formation in roots. For instance, promoter activity of *MYB103* was detected only in metaxylem vessels while that of *MYB46* and *MYB83* both in proxylem and metaxylem vessels (Figure 2 and 3). Another new finding is that *MYB99* uncharacterized previously shows vessel-specific expression pattern in roots similar to that of *MYB46* and *MYB83* (Figure 2 and 3). The transactivation assay suggests that the expression of *MYB99* is not under the control of *MYB46* and *MYB83* (Figure 5). In addition, our recent preliminary results of transactivation assay suggested that *MYB99* does not have any activity to induce *MYB46* nor *MYB83*. Further functional analysis of MYB99 may shed light on the transcriptional regulatory network during vessel element formation.

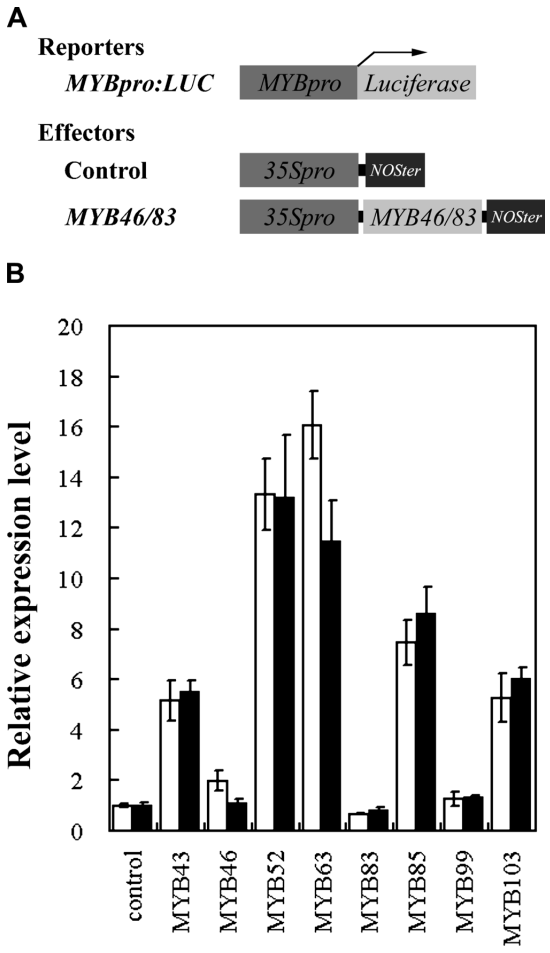

Figure 5. *MYB46* and *MYB83* Activate Expression of Several *MYB* genes. (A) Schematic diagrams of the effector and reporter constructs. The reporter construct contains the promoter of *MYB43, 46, 52, 63, 83, 85, 99*, or *103* with a minimal *CaMV35S* promoter (min pro) upstream from the firefly luciferase reporter gene. The effector constructs contained *MYB46* or *MYB83* between the *CaMV35S* promoter and the *NOS* terminator. (B) Relative luciferase activities after bombardment of one combination of the reporter constructs and the effector constructs. The reporter gene activity was normalized to the activity of *Renilla luciferase*, the gene for which was cobombarded as a reference. White bars, 35S-MYB46; Black bars, 35S-MYB83; Error bars represent S.E. (n=4).

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